"How I Do It" Session: Pancreatitis—Molecular Biology Update

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INTRODUCTION

The Board of Directors and Advisory Board of the Pancreas Club, Inc., decided this year to somewhat redirect the focus of the "How I Do It" session for this year's program. Recent past sessions have focused on intraductal papillary mucinous neoplasms, modern imaging of the pancreas, and resection strategies for pancreatic adenocarcinoma. This year the topic turned to pancreatitis, with attention being directed to recent progress in the molecular understanding of both acute and chronic pancreatitis.

The topic of acute pancreatitis was discussed by Dr. David C. Whitcomb, from the University of Pittsburgh. Dr. Whitcomb and his laboratory have led the initiative toward a more thorough understanding of a pivotal molecule in acute pancreatitis: cationic trypsinogen. His lecture beautifully highlighted the three-dimensional conformational changes in trypsinogen brought about by the common mutations of hereditary pancreatitis and the interaction of calcium.

The topic of chronic pancreatitis was nicely reviewed by Dr. Helmut Friess, from the University of Heidelberg. Drs. Friess and Büchler have been leaders in the advancement of our knowledge about chronic pancreatitis. Dr. Friess' discussion focused on the molecular events observed in conjunction with neural prominence and inflammatory cells, as well as recent findings from DNA array technology. Short synopses of these two lectures follow.

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Acute Pancreatitis: Molecular Biology Update

David C. Whitcomb, M.D., Ph.D.

Acute pancreatitis represents a clinical syndrome that is defined by the sudden onset of "typical" pain associated with elevated digestive enzymes (amylase and lipase) in the blood stream. My task is to provide a molecular biology update of this syndrome. This assignment reminded me of an urban legend told to me by a surgical resident at Duke University during my house staff training. He told me that a bank robber accidentally tripped an alarm during a bank holdup and, in the heat of the moment, took three physicians hostage as he jumped into his getaway car. Realizing that he could only manage one, he determined to kill two and keep the physician who was most valuable to him. The first physician was in family practice. Because the bandit already had his influenza vaccination, he shot the doctor and threw him out the window. The second physician was a molecular biologist. "What does a molecular biologist do?" he asked. "STOP, shoot me now!" cried the trauma surgeon. "I would rather die than hear another lecture about molecular biology!"

To be sure, there are innumerable traumatic, metabolic, toxicologic, pathologic, inflammatory, and other redundant pathways that crisscross and backstep in multiple isotypes and forms described somewhere in excruciating detail. However, today I want to focus on a molecule of singular importance: trypsin. If one understands trypsin, then one understands the origins of acute pancreatitis.

TRYPSIN IS CENTRAL TO PANCREATIC FUNCTION

There are three forms of trypsin that are expressed in the human pancreas.[1] The most abundant is cationic trypsinogen, also known as "PROTEASE, SERINE, 1; PRSS1." Anionic trypsinogen (PRSS2) and mesotrypsinogen (PRSS3) are similar to PRSS1 in that they all attack peptide chains at arginine or lysine residues. Trypsin is a highly active and efficient enzyme that rapidly digests dietary proteins within the intestinal lumen. It is synthesized in the pancreatic acinar cells as inactive trypsinogen, and normally remains inactive until it comes in contact with enterokinase within the intestinal brush border.

Trypsin serves two additional functions. First, trypsin is the activator of nearly all of the other pancreatic digestive enzymes. It is the match that lights the gunpowder. Without trypsin, the other digestive enzymes remain inactive and maldigestion develops. Second, trypsin regulates pancreatic secretion. The duodenum has a trypsin "sensor" that measures luminal trypsin activity. When free trypsin activity falls, as occurs during a protein-rich meal or with ingestion of a protein inhibitor, then luminal trypsin-sensitive factors (such as CCK-releasing factors) avoid trypsin-mediated destruction. As CCK-releasing factors increase in concentration, they stimulate CCK release,[2] which in turn causes pancreatic secretion of more pancreatic enzymes until the protein and CCK-releasing factors are digested and baseline trypsin activity is restored. Thus trypsin plays a key regulatory role in pancreatic digestive enzyme physiology by activating digestive enzymes and regulating duodenal enzyme activity through a feedback system.

PATHOLOGY OF TRYPSIN

Premature trypsinogen activation to trypsin within the pancreas causes an activation cascade with additional trypsinogen and other digestive proenzymes actively converted to active enzymes, leading to pancreatic digestion and inflammation. This is recognized clinically as acute pancreatitis. There are now multiple lines of evidence demonstrating that trypsinogen activation is among the earliest events in acute pancreatitis.[3] In essence, trypsin activation initiates acute pancreatitis,[4] whereas trypsin inhibition reduces or prevents pancreatitis.[5] Furthermore, gain-of-function genetic trypsin mutations or loss-of-function mutations in trypsin inhibitors increase the risk of acute pancreatitis.[6,7] Moreover, products

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of trypsin activation (trypsinogen activation peptide; TAP) are identified early in the course of acute pancreatitis.[8]

REGULATING TRYPSIN

A key to preventing the development of acute pancreatitis is the regulation of trypsin activity within the pancreas. Fortunately, the body uses a number of strategies to keep trypsin under control. These can be divided into two main categories: intra-acinar cell mechanisms and ductal mechanisms. In addition, there are trypsin inhibitors and other protective mechanisms in the interstitial space, which will not be considered here.

Trypsinogen is synthesized within the acinar cells. As noted earlier, the first protective mechanism is the synthesis of trypsinogen in an inactive form (trypsinogen) with an activation site located in the target organ, which is the duodenal lumen. Trypsinogen activation occurs when the exposed TAP is cleaved off the native molecule by enterokinase or trypsin. Cleavage of this peptide causes a conformational change in the core peptide, with opening of the specificity pocket and active site. Several mutations in cationic trypsinogen enhance trypsin activation,[7] and these mutations increase susceptibility to acute pancreatitis.

If trypsinogen is activated within the acinar cells, it can be inhibited by pancreatic secretory trypsin inhibitor (PSTI, also known as serine protease inhibitor, Kazal type 1; SPINK1). This peptide is a specific trypsin inhibitor and an acute-phase protein. Mutations in SPINK1, which likely reduce its ability to inhibit trypsin, are associated with pancreatitis in children, some familial pancreatitis, and various forms of tropical pancreatitis. These mutations are common and are seen in 2% of most studied populations.[9] Although intracellular SPINK1 is an effective trypsin inhibitor, the amount of trypsinogen greatly outnumbers the number of SPINK1 molecules, so that the inhibitory capacity is limited. If more trypsinogen is activated than SPINK1 can inhibit, then other protective mechanisms must be employed.

Our studies in hereditary pancreatitis drew attention to the important trypsin self-destruct mechanism.[6] The trypsin molecule has two globular domains held together by a single side chain. This side chain is critical to trypsin regulation. In the middle of the side chain is an arginine, the target amino acid of trypsin. Biochemical studies demonstrate that this site is sensitive to trypsin hydrolysis, and cleavage of this site is the first step in trypsin autolysis. The importance of this site and the autolysis mechanism is demonstrated by patients with hereditary pancreatitis who have mutations at R122 and recurrent acute pancreatitis when trypsin cannot be destroyed within the acinar cells.

The R122 autolysis site is regulated by calcium. If trypsinogen is activated in a calcium-free buffer, it quickly undergoes autolysis.[10] In the presence of calcium, trypsin remains active indefinitely. Structural studies suggest that the trypsin molecule has a calcium binding site near the autolysis loop (i.e., connecting side chain).[11] Calcium binds to the calcium pocket and the R122 site, preventing a second trypsin from attacking, thereby stabilizing active trypsin. This makes physiologic sense because trypsin is stabilized *after* being secreted from the acinar cell into the duct, and remains stable in the duodenum and into the jejunum, where calcium is normally absorbed. Trypsin activity then disappears within the ileum. Elevated calcium levels within the acinar cell are pathologic because they eliminate the trypsin autolysis mechanism and lead to acute pancreatitis[12,13] just as is seen in hereditary pancreatitis. Of note, acinar cells are very sensitive to bile salts, which cause marked increases in intracellular calcium levels.[14] This observation may link bile reflux to acute pancreatitis.

Once trypsinogen is secreted into the pancreatic ducts, which have a high calcium concentration, there is danger of trypsinogen activation because the autolysis mechanism is eliminated. Fortunately, some SPINK1 is also secreted with trypsin. However, if too much trypsinogen is activated, then other mechanisms must be employed to protect the pancreas. One key mechanism is rapid flushing of the duct system. This is mediated by the duct cells and is dependent on cystic fibrosis transmembrane conductor regulator (CFTR) function. Mutations in CFTR reduce the fluid-secreting capacity of the pancreas, causing active trypsin to remain within the pancreas for prolonged periods of time. This may also lead to acute pancreatitis and chronic pancreatitis, and is seen in patients with cystic fibrosis or atypical cystic fibrosis with pancreatitis.[15,16]

CONCLUSION

Molecular genetic studies provide critical information that points to trypsin as the central molecule in initiating acute pancreatitis. Susceptibility to acute pancreatitis appears to be related to the body's ability to protect itself from trypsin activation. Once acute pancreatitis is initiated, the extent, severity, and complications are largely controlled by other factors, such as the immune response. Knowledge of these concepts will likely become more and more important

as risk and pathways are better defined and early interventions developed.

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Molecular Pathophysiology of Chronic Pancreatitis—An Update

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Chronic pancreatitis is an inflammatory disease of the exocrine pancreas characterized by the progressive destruction of the whole organ resulting in severe exocrine and endocrine insufficiency, as well as the leading clinical symptom, severe abdominal pain.[1,2] Heavy alcohol consumption is the main etiologic factor leading to chronic pancreatitis in Western countries; however, other causes of chronic pancreatitis have been identified including various gene mutations. Mutations include those of the cationic trypsinogen gene,[3] the serine protease inhibitor Kazal type 1 (SPINK1) gene,[4] and the cystic fibrosis transmembrane conductor regulator (CFTR) gene.[5] These gene mutations, especially the cationic trypsinogen mutation, have been the focus of chronic pancreatitis research in the past several years, and they explain, at least in part, the cause of chronic pancreatitis in a subset of patients. In addition, other causes of chronic pancreatitis have been identified such as gallstone disease, certain metabolic disorders, and some tropical variants (for whom the etiologic agent has not been identified so far). Irrespective of the underlying cause, chronic pancreatitis is characterized histologically by acinar cell atrophy, dedifferentiation of acinar cells into tubular complexes, immune cell infiltration, alteration of pancreatic nerves, and parenchymal fibrosis.

NEUROIMMUNE INTERACTION

In recent years, modern molecular biological techniques have provided important clues to clarify the morphologic changes and pathophysiologic aspects of chronic pancreatitis.[2] One aspect that has been extensively investigated in recent years is the neuroimmune interaction in chronic pancreatitis (i.e., the alteration of nerves and the infiltration of inflammatory cells). In one of the first studies on nerve alterations in chronic pancreatitis by Bockman et al.,[6] it was noted that chronic pancreatitis tissues exhibit an increase in both the number and the diameter of pancreatic nerve fibers. In addition, it was observed that inflammatory cells often surround these pancreatic nerves, exhibiting a damaged perineurium and invasion by inflammatory cells. It has also been observed that these enlarged nerve fibers exhibit an intensification of immunostaining for calcitonin gene-related peptide and substance P, which are regarded as pain transmitters.[7] These findings together indicate that changes in pancreatic nerves themselves might be responsible for the pain syndrome in chronic pancreatitis. In addition to the changes in nerves, there is growing evidence that inflammatory cells infiltrating the pancreas actively participate in the disease process of chronic pancreatitis. For example, Hunger et al.[8] have recently demonstrated that perforin, a marker for activated cytotoxic cells, is significantly elevated in the pancreas of patients with alcoholic chronic pancreatitis. Because activated cytotoxic cells are preferentially located in the vicinity of residual intact parenchyma, the cells might be directly involved in pain generation in chronic pancreatitis. In addition, inflammatory cells also seem to be involved in neural changes in chronic pancreatitis, because infiltration of pancreatic nerves by immune cells and destruction of the perineurium have been correlated with pain intensity in patients with chronic pancreatitis.[9]

The recruitment of inflammatory cells into the inflamed pancreatic tissue depends on the production of chemotactic factors. The pancreatic parenchyma is actively involved in the induction of inflammation in chronic pancreatitis through the production of different kinds of chemokines. For example, interleukin-8, ENA 78, and MCP 1 have been localized at high levels in certain tissue compartments in chronic pancreatitis.[10] In addition, increased levels of RANTES, Mip-1 alpha, and their receptor CCR 5 (which is mainly expressed on infiltrating macrophages) have been observed in chronic pancreatitis.[11] Other factors have also been identified, which have the potential

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to participate in the abnormal neural milieu and therefore indirectly in pain generation in chronic pancreatitis. For example, nerve growth factor and its receptor TrkA are expressed at increased levels in chronic pancreatitis. Nerve growth factor was observed in tubular complexes and in dedifferentiating acinar cells in chronic pancreatitis, whereas TrkA was mainly present in the perineurium in chronic pancreatitis.[12] Interestingly, there appears to be a strong correlation between nerve growth factor levels and pancreatic fibrosis and acinar cell damage, as well as between TrkA levels and pain intensity.[12] It has therefore been suggested that the NGF/TrkA pathway might influence neural morphologic changes in the pain syndrome of chronic pancreatitis.

DNA TECHNOLOGY

Despite this progress in the research of chronic pancreatitis, much of the pathophysiology and the underlying mechanisms remain to be fully elucidated. With the almost complete sequencing of the human genome[13] in 2001 and the development of novel technologies such as DNA array technology, it is now possible to analyze large numbers of genes simultaneously and to identify disease-specific genes. The first study employing DNA array technology in chronic pancreatitis was conducted by Friess et al.[14] in 2001. Screening 5600 human genes, six chronic pancreatitis-specific genes were identified that showed a significantly increased expression in chronic pancreatitis compared to the normal pancreas and to pancreatic cancer. These six genes included the cartilage oligomeric matrix protein (COMP), the cysteine-rich secretory protein-3 (CRISP3), and tryptase.[14]

Tryptase is a serine protease stored in mast cells, which is known to induce the synthesis of type 1 collagen by human fibroblasts, and to stimulate fibroblast proliferation and chemotaxis. Mast cells are well-known effector cells of immediate-type allergic reactions. A cross-linking of IgE receptor–bound IgE on mast cell membranes by specific allergens leads to the release of preformed mediators and to the synthesis of new mediators. Through these and other mechanisms, mast cells exert their role in allergic reactions as well as in acute and chronic inflammatory settings. In human chronic pancreatitis, there is a significant increase in the total number of mast cells compared to the normal pancreas.[15] Interestingly, there is also a correlation between the number of mast cells and the extent of fibrosis and intensity of inflammation.[15] Furthermore, IgE-dependent mast cell activation is higher in chronic pancreatitis than in the normal pancreas; however, there is no difference in the number of mast cells or in IgE-positive mast cells between chronic pancreatitis of different etiologies. In chronic pancreatitis, mast cells are mostly located in the fibrous tissue and around regenerating ducts, which are positive for the c-kit receptor, the receptor for the mast cell growth factor stem cell factor.[15] It is thought that mast cells are a relevant component of the inflammatory infiltrate in chronic pancreatitis, which might play a role in tissue destruction and remodeling.

Cysteine-rich secretory protein 3 (CRISP3) is a member of the cysteine-rich secretory protein family that has been detected in several human tissues, with predominance in the salivary gland, pancreas, and the prostate. In addition, CRISP3 is considered a defense-associated molecule in mammals. In chronic pancreatitis there is increased expression of this molecule, which is mainly localized in the cytoplasm of the dedifferentiating acinar cells and in tubular complexes.[16] Interestingly, CRISP3 expression was weak to absent in pancreatic cancer cells, as well as in normal acini and ductal cells distant from the pancreatic cancer, and in the normal pancreas.[16] The predominant localization of CRISP3 in acinar cells dedifferentiating into tubular complexes, as well as in these tubular structures itself, suggests that this molecule has some role in the pathophysiology of chronic pancreatitis.

A third gene that is selectively upregulated in chronic pancreatitis is COMP. COMP is a member of the thrombospondin family of extracellular matrix proteins. In human chronic pancreatitis, COMP expression was evident in the cytoplasm of the degenerating acinar cells but not in tubular complexes or in normal acini or ductal cells.[17] In addition, COMP is also present in the fibrotic tissue in chronic pancreatitis. The preferential expression of COMP in dedifferentiating acinar cells in chronic pancreatitis suggests a potential role for this gene in the cause of acinar cell degeneration and dedifferentiation.[17] COMP might thus serve as a marker for tissue destruction and disease activity in chronic pancreatitis.

CONCLUSION

The pathogenesis of chronic pancreatitis is still not completely understood. However, recent advances in cellular and molecular biology have revealed complex interactions between inflammatory cells and pancreatic parenchyma cells, as well as alterations in nerves. Furthermore, as a result of novel technologies, it has become possible to identify key genes in the disease process of chronic pancreatitis. Tryptase,[15] CRISP3,[16] and COMP[17] are three of the candidate

genes that may serve as disease markers and therapeutic targets in the future. Further molecular and cell biology studies will increase our knowledge of the pathogenesis and pathophysiology of this disease, hopefully resulting in better diagnostic and therapeutic modalities in the future.

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Is Extended Resection for Adenocarcinoma of the Body or Tail of the Pancreas Justified?

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Patients with body or tail tumors of the pancreas often have contiguous organ involvement or portal-splenic confluence adherence requiring extensive resection in order to obtain grossly negative margins. The aim of this study was to determine whether long-term survival is possible after contiguous organ or portal vein resection in patients with adenocarcinoma of the body or tail of the pancreas. Between 1983 and 2000, a total of 513 patients with adenocarcinoma of the body or tail of the pancreas were identified from a prospective database. Distal pancreatectomy with or without splenectomy was performed in 57 patients (11%). Extended resection was necessary in 22 patients (39%): 14 (64%) for contiguous organ involvement and eight (36%) for portal vein resection Estimated blood loss, blood transfused, and length of hospital stay were significantly greater in patients requiring extended resection compared to standard resection ($P = 0.02$, $P = 0.01$, and $P = 0.02$, respectively). Median follow-up for patients still alive was 84 months (range 40 to 189 months). Median survival following resection was 15.9 months compared to 5.8 months in patients who were not resected ($P < 0.0001$). Actual 5- and 10-year survival rates were 22% and 18%, respectively, following extended resection, 8% and 8% following standard resection, and 0% and 0% if no resection was attempted because of locally unresectable disease. Patients undergoing extended resection for adenocarcinoma of the pancreatic body or tail have long-term survival rates similar to those for patients undergoing standard resection; they also have markedly improved long-term survival compared to those who are not considered resectable because of locally advanced disease. Extended distal pancreatectomy is justified in this group of patients. (J GASTROINTEST SURG 2003;7:946–952)

KEY WORDS: Distal pancreatectomy, pancreatic adenocarcinoma, pancreatic resection

Adenocarcinoma of the body and tail of the pancreas has historically been considered a disease with a dismal prognosis. In the past decade, few patients have been reported to survive long term.[1–3] Because of the late appearance of symptoms, most patients with adenocarcinoma of the pancreatic body and tail present with advanced disease, including metastasis, precluding surgical treatment.[3–5]

The only viable chance for cure of this aggressive cancer is surgical resection.[6] However, pancreatic body and tail tumors often infiltrate surrounding regional blood vessels and adjacent organs. Efforts to increase resectability with en bloc resections of the pancreas including the superior mesenteric vein (SMV) and portal vein (PV) confluence have been attempted with no compromise in oncologic outcome for adenocarcinoma of the pancreatic head.[7–9] Data regarding outcome after extended resection for distal adenocarcinoma, including the SMV/PV junction and surrounding organs, remain scarce. The purpose of the study was to review our experience with adenocarcinoma of the body and tail of the pancreas, and to determine whether long-term survival is possible following contiguous organ or portal vein resection.

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METHODS

Between 1983 and 2000, a total of 513 consecutive patients with adenocarcinoma of the body or tail of the pancreas were admitted to our institution and were identified from a prospective database maintained by the Department of Surgery at Memorial Sloan-Kettering Cancer Center. Following routine imaging, patients were considered unresectable either because of metastatic disease (M1) or locally advanced disease (T4). Surgical exploration was performed in select patients at the surgeon's discretion either by laparoscopy or open laparotomy to confirm the extent of the disease. Distal pancreatectomy with or without splenectomy was performed in all resected patients.

Patients who underwent distal pancreatectomy with portal vein resection and/or contiguous organ resection including gastrectomy, adrenalectomy, or colectomy were considered to have extended resection. Those undergoing only pancreatectomy with or without splenectomy were considered to have standard resection. The extent of portal vein invasion was not consistently noted, but the degree of portal vein resection performed was what was thought necessary by the surgeon to obtain negative margins. Patients undergoing other surgical procedures not related to the pancreatic resection, such as cholecystectomy or herniorraphy, were analyzed with the standard resection group. Clinical, operative, and pathologic details were noted in all patients. Inpatient and outpatient records were thoroughly reviewed to confirm clinical course. Anesthesia records were reviewed to verify operative time, blood loss, and amount of blood transfused. Pathologic findings were reconfirmed in all 5-year survivors.

For the majority of patients, death was due to pancreatic cancer. Disease-specific survival was calculated from the time of operation to the time of death from disease. Death rate was estimated by means of the Kaplan-Meier method. Follow-up of patients is through September 2002. Actual 5- and 10-year survival were calculated from patients undergoing surgery prior to September 1997 (N = 42) and 1992 (N = 23), respectively. Univariate associations of prognostic factors were analyzed using the log-rank test. Multivariate analysis was based on the Cox regression model. Only factors identified to be significant in the univariate analysis were evaluated in the multivariate analysis. The Mann-Whitney test was applied to compare continuous data (e.g., hospital stay, blood loss, tumor size, duration of operation) between extended resection and standard resection groups.

RESULTS

Of the 513 patients admitted to our hospital with a diagnosis of adenocarcinoma of the body or tail of the pancreas, 57 (11%) underwent resection with curative intent. Fig. 1 outlines the course of selection

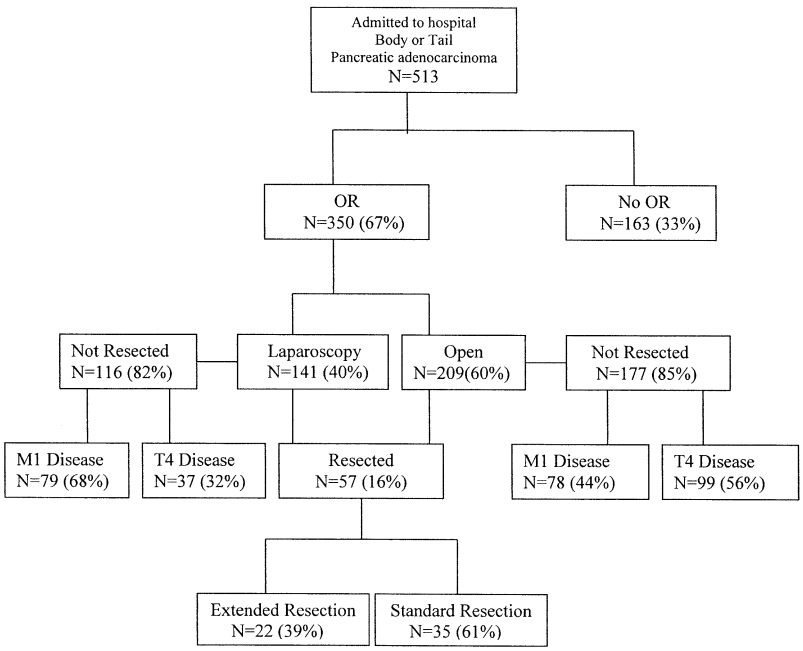

Fig. 1. Selection for surgical exploration and resection among patients with adenocarcinoma of the pancreatic body or tail.

for surgical exploration and resection among the entire group. Of the 350 patients (67%) who underwent surgical exploration, 57 (16%) were resected. Extended resection was performed in 22 patients (39%), and standard resection in 35 (61%). Extended resection included portal vein resection in eight patients and contiguous organ resection in 14. Patients who underwent surgical exploration without resection had either laparoscopy (N = 116, 40%) or open laparotomy (N = 177, 60%).

Among the 57 patients who underwent distal pancreatectomy, 30 (53%) were female and 27 (47%) were male with a median age of 66 years (range 38 to 81 years). Median follow-up was 15 months for the entire resected group and 84 months for those still alive (N = 6) at last contact (range 40 to 189 months). Median disease-specific survival for the entire group after resection was 15.9 months (range 1 to 189 months), and for the 456 patients not undergoing resection median disease-specific survival was 5.8 months ($P < 0.0001$). Predictors of survival following resection are shown in Table 1. Lymph node–positive and poorly differentiated tumors were associated with poorer survival. Fig. 2 demonstrates that there was no difference in actuarial disease-specific survival when extended resection and standard resection were compared ($P = 0.8$). After extended resection, positive lymph nodes and poorly differentiated tumors were found in 10 (45%) and 9 (41%) patients, compared to 18 (51%) and 13 (37%) patients after standard resection ($P = 0.79$ and 0.78, respectively).

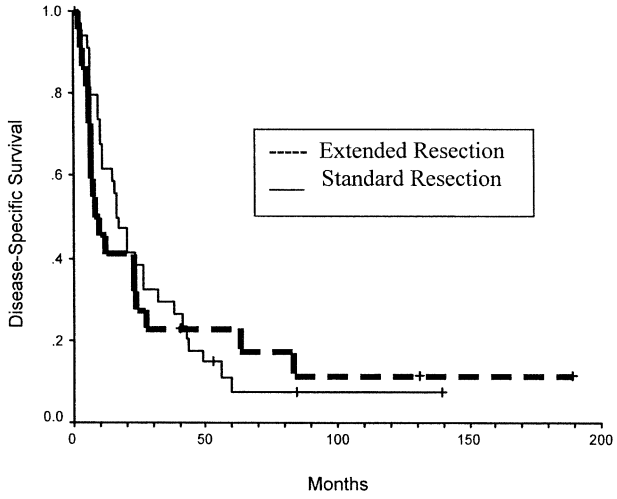

Fig. 2. Actuarial disease-specific survival following resection for distal pancreatic adenocarcinoma.

Table 1. Prognostic indicators for survival following distal pancreatectomy for adenocarcinoma

	N	Median DSS (mg)	DSS (*P* value)	MV (*P* value)	RR	CI
Lymph nodes						
Positive	28	11	0.05	0.02	0.50	0.3–0.9
Negative	29	16				
Size						
⩽2 cm	6	59	0.07			
>2 cm	51	15				
Differentiation						
Poor	21	6	0.003	0.008	0.42	0.23–0.80
Moderate well	36	23				
Margins						
Positive	16	10	0.63			
Negative	41	17				
Splenectomy						
Yes	51	15	0.29			
No	6	12				
Extended resection						
Yes	22	9	0.80			
No	35	16				

CI = 95% confidence interval; DSS = disease-specific survival; MV = multivariate analysis; RR = relative risk.

Perioperative variables comparing extended resection to standard resection are shown in Table 2. There were no postoperative deaths in either group. Tumor size was not significantly different between groups. Length of hospital stay, estimated blood loss, and amount of blood transfused were all increased after extended resection. The duration of the operation tended to be longer after extended resection, as did the incidence of reoperation, but neither reached significance.

Patients who underwent surgical exploration without resection (N = 293) had a median survival of 4.7 months. Those undergoing laparoscopic exploration (N = 116) had a median survival of 7.2 months, and those underoing open surgical exploration (N = 177) had a median survival of 4.3 months ($P = 0.04$). Among all of the patients undergoing exploration but not resection, 157 (54%) were found to have histologically proved M1 disease to either the peritoneum or liver, and they had a median survival of 4.0 months. The 136 patients (46%) who were surgically explored and thought to have locally advanced, unresectable disease (T4), but no metastatic disease, had a median survival of 7.8 months ($P = 0.0004$ compared to M1 disease).

Actual 5- and 10-year disease-specific survival was 22% and 18%, respectively, after extended resection, 8% and 8% after standard resection, and 0% and 0% if no resection was attempted because of surgically

Table 2. Perioperative variables following distal pancreatectomy for adenocarcinoma

Characteristic	Extended resection (N = 22)	Standard resection (N = 35)	Univariate *P* value	Multivariate *P* value
Tumor size (cm)*	5.0 (1.6–10)	4.5 (1–13)	0.37	
Length of stay (days)*	14 (7–88)	9 (5–51)	0.004	0.02
Length of surgery (min)*	270 (125–625)	205 (1.5–460)	0.06	
Estimated blood loss (ml)*	1500 (300–4700)	600 (200–5500)	0.002	0.02
Blood transfused (ml)*	500 (0–1750)	0 (0–2250)	0.004	0.01
Reoperation	2 (9%)	(0%)	0.08	

*Expressed as median (range).

documented T4 disease. There were six 5-year survivors, three of whom were 10-year survivors (5.3%). Of the 5-year survivors, four had extended resection (two each had portal vein resection and other organ resection) and two had standard resection. Among the 10-year survivors, one had standard resection and two had extended resection. One of the 10-year survivors, who was still alive at 11 years, had a tumor less than 2 cm with 12 positive lymph nodes, and the other two had tumors larger than 2 cm in size but negative lymph nodes.

DISCUSSION

Most patients with adenocarcinoma of the body or tail of the pancreas have metastatic disease.[3–5] Up to 32% of patients with potentially resectable pancreatic cancer have involvement of surrounding organs or major vessels.[7,10] Extended resection has been shown to result in similar survival rates as standard resection for patients with tumors of the pancreatic head,[7–9] but the role of extended resection has not been clearly defined for adenocarcinoma of the pancreatic body and tail.

In the current series 11% of patients admitted to our institution with adenocarcinoma of the body or tail of the pancreas were resected, and this is similar to earlier reports from our group[1] as well as others.[2,3,11] This is a dismal resectability rate, but it is probably elevated because it excludes those patients who were clearly not resectable and therefore not admitted. Approximately 85% of the patients who had exploratory procedures, either with laparoscopy or with open surgery, were found to have unresectable disease. Those who were not resected but explored with laparoscopy had a longer median survival than those who had open exploratory surgery. The longer survival is likely a result of a less invasive surgery and shorter recovery rather than the extent of disease.

Among those who underwent resection, only nodal status and histologic differentiation had a significant impact on survival. Earlier reports from our institution did not identify node-positive tumors as predictors of poorer outcome.[1,12] Although the improvement was of minor biological significance, with more patients, longer follow-up, and rereviewed pathologic findings to confirm adenocarcinoma, our current data confirm that lymph node status is significant and is similar to that in other reports of cancer of the pancreatic head.[13,14]

Reports of 5- and 10-year survivors following resection for distal pancreatic adenocarcinoma are rare.[1–3] In our series, with a median follow-up of 80 months for patients still alive, we had six 5-year survivors, three of whom were also 10-year survivors. These data demonstrate that long-term survival is possible; however, in our series half of the patients who survived 5 years died of disease within the next 5 years. Therefore it is inappropriate to associate 5-year survival with cure.

Of the 57 patients who underwent distal pancreatectomy, 39% required extended resection. Patients undergoing extended resection had a higher estimated blood loss, required more blood transfusions, and had a longer hospital stay. However, there were no perioperative deaths, and disease-specific survival rates did not differ in comparison to the standard resection group. The median disease-specific survival was 9 months for extended resection and 16 months for standard resection, and the Kaplan-Meier survival curves overlap at 5 and 10 years. The survival data are not significantly different, but these data underscore the fact that long-term survival in patients with distal pancreatic adenocarcinoma most likely reflects the biology of the tumor rather than the surgical procedure. Regardless, with no long-term survivors among nonresected patients, we believe that if extended surgery is necessary to achieve complete gross resection, it should be performed.

Our experience with survival and extended resection is similar to findings in earlier studies. A recent report by Sasson et al.[10] noted that extended resection for pancreatic adenocarcinoma was associated with longer operative time but no difference in blood loss,

hospital stay, or overall survival. The difference is that their series of 116 patients included 101 pancreatic head tumors and 15 body and tail tumors. Harrison et al.[7] found that extended resection, including portal vein resection, for tumors of the head of the pancreas was associated with similar survival rates as standard resection.

Our study is similar to other reports[15,16] of multiorgan resection for abdominal malignancies. Martin et al.[15] found that aggressive resection of gastric cancer, including removal of up to three other organs, was associated with increased morbidity but acceptable 5-year survival. Studies suggest that for gastric cancer the strongest predictors for long-term survival are tumor characteristics rather than extent of resection.[15,16] Our data support similar conclusions for distal pancreatic adenocarcinoma.

We have demonstrated that 5- and 10-year survival is possible after distal pancreatectomy for adenocarcinoma of the body or tail of the pancreas. In this series the strongest predictors of long-term survival were lymph node status and tumor differentiation. Extended resection requiring SMV/PV or contiguous organ resection is associated with increased blood requirements and length of hospital stay but long-term survival rates similar to those for standard resection. At the time of surgery, if extended resection is thought to be necessary to achieve complete resection of the tumor, this aggressive approach is justified in patients with distal pancreatic adenocarcinoma.

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Discussion

Dr. H. Reber (Los Angeles, CA): In my practice, for patients with carcinoma of the body or tail of the pancreas, we too would go ahead and do a resection even if the tumor appeared to be locally invading the stomach or colon, as you have suggested. But with some frequency we find, after the specimen has been resected and the pathologists have examined it, that what we thought was tumor invasion was just inflammatory adherence. I wonder if you could tell us a little bit about whether each of the patients in whom you did an extended resection really did have cancer invading these associated organs?

Dr. Shoup: Unfortunately it was difficult to go back 20 years and look at some of our earlier patients to see whether that was really the case. In a paper presented yesterday on that same topic, it was noted that frequently patients undergo extended resections that we think might have been unnecessary. But the bottom line is that it was at the surgeon's discretion at the time of surgery that the only way to be assured of achieving a

complete gross resection was to perform an extensive resection.

Dr. C. Fernandez-del Castillo (Boston, MA): I wonder if you could tell us about the adjuvant treatment for these patients. Surely many of them must have received radiation or chemotherapy. Did this have any effect on their survival?

Dr. Shoup: That is an excellent question. We have very thorough follow-up, in terms of outcome, but no good details on the type or extent of radiation/chemotherapy. Patients who do not have follow-up treatment are reviewed constantly until they are dead of disease; because that happens soon, and for most, there is excellent follow-up until death.

As you know, many patients come to Memorial Sloan-Kettering Cancer Center to undergo surgery, and then they go elsewhere or back home, which could be miles and miles away. We do not necessarily hear back from them until we get a letter saying that they have died of their disease. So we do not really have good details on chemoradiation therapy and because of that we did not do an analysis of that.

Dr. D. Rattner (Boston, MA): I was going to follow up on the same theme. It seems odd that according to your data a positive margin did not affect length of survival. Really this study was not done on an intent-to-treat basis; there was no randomization to either extend the resection or not extend it. It was sort of what you found at surgery, I presume, as to what was removed. Given the retrospective nature of the study and the fact that the groups being compared may not have been equivalent, I wonder if you would comment on whether you still can claim that extended resection fails to improve survival?

Dr. Shoup: We think that extended resection can be associated with improved survival because two of our three long-term survivors had undergone extended resection; if the surgeon thought that carrying out the operation to that extent was not worth it, then I am sure the patient would not be alive for long, because we have no long-term survivors among the patients who did not undergo resection at all.

Dr. S. Pedrazzoli (Padova, Italy): Congratulations for this large experience. In the past, left pancreatic cancer was considered unresectable. But now, in our experience, we also have long-term survivors. I have two questions for you.

First, how many of the extended operations were amenable to a standard operation? I believe you performed an extended operation when a standard operation was not possible, so that you resected the portal vein and so forth. The two groups are not at all comparable.

Second, did you perform the Appleby operation on these patients?

Dr. Shoup: To answer your second question first, we did not perform any arterial resections in these patients. The only vessel resections we did were the portal vein resections when we thought this was necessary.

As to the first question, it is difficult to say whether the extended resection was really necessary, and if we could have performed just a standard resection. It is really left to the surgeon's discretion at the time of the surgery. All we are trying to say, based on the data we presented here, is that if you think you need to do an extended resection in order to remove this tumor, then go ahead and do it because you really may be helping the patient.

Dr. R. Prinz (Chicago, IL): This is a very nice review, but it encompasses a substantial number of years. Certainly our ability to assess unresectability preoperatively and our attitudes about resection have changed quite dramatically over that period of time. I wonder if you were able to glean from your data whether some of the patients who were considered unresectable during the study would now be resectable with an extended resection?

Also, I am interested in the role of laparoscopy and how it has affected your approach to these patients. It seems, and maybe this is a presumption on my part, that you are now using laparoscopy in these patients before they undergo exploratory operations. That is our approach. Has that made you more likely to do an extended resection or less likely to do so?

Dr. Shoup: If you look at the earlier patients compared to the ones now, the only thing I can say is that the majority of the surgeons were the same, and it is hard for me to tell you exactly whether our selection criteria have changed over that time. I can certainly go back and look at that, though, and analyze two different time periods and see if it looks like there has been a difference.

As far as laparoscopy is concerned, this database goes back to 1983. We did not really start doing laparoscopy until 1993. So none of the earlier patients had laparoscopy, and that is why there were so many patients who had open surgery in the schema.

Of the ones who have been operated on since 1993, 75% to 80% underwent laparoscopy, and some were at the surgeon's discretion, but it is hard for me to really glean exactly from the notes why some had this procedure and some did not. Certainly the standard approach at Memorial Sloan-Kettering is to examine all patients who have pancreatic cancer laparoscopically, especially those with cancer of the body and tail. I doubt whether the laparoscopy itself affected our willingness to perform an extended resection, but it did, as you know, prevent an unnecessary open operation in a number of patients.

Dr. A. Warshaw (Boston, MA): This comment is precipitated by the observation that positive margins do not seem to make a difference in the outcome after resection of pancreatic cancer in the body or tail. It is becoming clear that pancreatic cancer has a spectrum of behaviors depending on which biological factors affecting cell growth and survival are operating in a given tumor. How many times has a patient asked you, "Doctor, how long have I had this tumor," and you cannot answer knowledgeably. Although we think of pancreatic cancer as fast growing and fulminant, some cancers may be relatively slow growing and already present for years longer than we suspect.

That may explain why it may be more than 5 years or even 10 years before there is sufficient new growth for the cancer to reappear, whereas others may recur lethally within months. The implication is that the outcome after resection may be more beholden to the biology of a given cancer than to what the surgeon does or whether adjuvant treatments are used.

Dr. Shoup: I definitely agree. Even in those studies that show a difference in margin status, this difference is a few percentage points at best. With our numbers and the poor prognosis overall, this difference is not detected. We still know little about the biology of the disease from this retrospective study.

Invited Discussion—Expert Commentator

Sean J. Mulvihill, M.D. (Salt Lake City, UT): In this paper Shoup and colleagues, from Memorial Sloan-Kettering Cancer Center, address surgical treatment of patients with adenocarcinoma of the body and tail of the pancreas, a group with very poor outcome in historical series. The authors subjected 350 such patients over an 18-year period to open or laparoscopic exploratory operations, but resection was possible in only 57. Today, improved preoperative imaging has reduced this proportion of nontherapeutic procedures. Among these 57 resected patients, extended resection including portal vein resection, adrenalectomy, gastrectomy, or colectomy was performed in approximately one third with two thirds undergoing standard distal pancreatectomy and splenectomy. The patients undergoing extended resection had higher blood loss, transfusion rates, and length of hospital stay, but there were no operative deaths. Extended resection appears warranted because survival was equal to or better than when standard resection sufficed (19% vs. 9% at 5 years). Interestingly, one of the 10-year survivors had 12 positive lymph nodes. One wonders about this patient's tumor metabolic activity. Could this good outcome with a very advanced tumor be predicted by preoperative position emission tomography? This is clearly a very difficult group of patients, but as with the Whipple resection for cancers of the pancreatic head, it appears that previous nihilism is misplaced and we should consider resection in fit patients, even in the face of adjacent organ or vascular involvement.

18-Fluorodeoxyglucose Positron Emission Tomography in Predicting Survival of Patients With Pancreatic Carcinoma

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The prediction of survival of patients with pancreatic cancer is usually based on tumor staging and grading and on the level of tumor markers. However, accurate tumor staging can be obtained only after resection, and still there is a great difference in survival rates among patients with the same clinicopathologic parameters. Recently the uptake of 18-fluorodeoxyglucose (FDG) by positron emission tomography (PET) has been found to be correlated with survival in patients with pancreatic cancer. This study evaluated the role of ^{18}FDG PET as a prognostic factor for patients with pancreatic cancer. From June 1996 to July 2002, a total of 118 patients underwent PET for pancreatic cancer. The standardized uptake value (SUV) of ^{18}FDG was calculated in 60 of them, and these patients were divided into high (>4) and low ($\leqslant 4$) SUV groups. They were also evaluated according to the tumor node metastasis (TNM) classification system of the International Union Against Cancer, and by tumor grade, medical or surgical treatment, diabetes, age, sex, and CA 19-9 serum levels. Twenty-nine cancers showed high and 31 showed low SUVs. Survival was significantly influenced by tumor stage ($P = 0.0001$), tumor grade ($P = 0.01$), and SUV ($P = 0.005$). Multivariate analysis showed that only stage ($P = 0.001$) and SUV ($P = 0.0002$) were independent predictors of survival. When patients who were analyzed for SUV were stratified according to the other variables, FDG uptake was related to survival also after stratification for the following: stage III to IVa ($P = 0.002$), stage IVb ($P = 0.01$), tumor resection ($P = 0.006$), moderately differentiated tumors ($P = 0.01$), age less than 65 years ($P = 0.006$), CA 19-9 levels greater than 300 kU/L ($P = 0.002$), and absence of diabetes ($P = 0.0001$). The SUV calculated with ^{18}FDG PET is an important prognostic factor for patients with pancreatic cancer and may be useful in selecting patients for therapeutic management. (J GASTROINTEST SURG 2003;7:953–960)

KEY WORDS: Fluorodeoxyglucose, positron emission tomography, standardized uptake value, pancreatic cancer, prognosis

With an age-standardized incidence and a mortality rate of 11.4 and 11.3 per 100,000 for males and 7.0 and 7.4 per 100,000 for females, respectively, pancreatic cancer accounts for 4.1% of cancer deaths in men and 4.8% in women in Europe.[1] The correlation between incidence and mortality underscores the grim prognosis for patients with pancreatic cancer. However, some of these patients have a comparatively good prognosis. To predict length of survival for patients with pancreatic cancer, many clinicopathologic factors have been well studied, especially tumor stage and grade,[2,3] R0 resection,[4] postoperative normalization of tumor markers,[5] and demonstration of disseminated tumor cells.[6] However, there are conflicting results, and differing survival rates among patients within the same stage grouping are not infrequent.[7]

18-Fluorodeoxyglucose (FDG) positron emission tomography (PET) is a relatively recent, noninvasive imaging technique that is based on the principle of

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specific tissue metabolism because of selective ^{18}FDG uptake and retention by malignant cells.[8,9] PET has been proposed for diagnosing and staging different malignancies including pancreatic carcinoma.[10,11] Furthermore, there is evidence that FDG uptake in malignant tumors is related to tumor aggressiveness.[12] A correlation between FDG uptake and survival in patients with gliomas was reported by Patronas et al.[13] in 1985. More recently some investigators[7,14–16] have reported their findings regarding prognostic information obtained with FDG PET in patients with pancreatic cancer.

The aim of the present study was to determine in a large series of patients whether glucose metabolism as assessed by means of ^{18}FDG PET provides prognostic information independent of established prognostic factors in patients with pancreatic cancer.

MATERIAL AND METHODS

From June 1996 to July 2002, 60 of 118 patients who had a PET scan as part of a preoperative workup for pancreatic cancer underwent additional abdominal PET with semiquantitative analysis of the uptake. This subset of 60 patients with pancreatic cancer who had a semiquantitative analysis of the uptake of the tracer was enrolled in a retrospective study. Informed consent was obtained from each patient.

The mean age of these patients was 66.3 years (range 48 to 82 years), and the male:female ratio was 34:26. In all patients the presence of pancreatic ductal adenocarcinoma was histologically proved with specimens obtained by surgery or percutaneous fine-needle biopsy. Pathologic diagnosis and classification of the tumors were made according to the International Union Against Cancer (UICC) staging system.[17] The clinical and pathologic records of each patient were reviewed, and the following features were analyzed: age, sex, diabetes, type of treatment (resection surgery, palliative surgery, or medical treatment), preoperative CA 19-9 serum levels (RIA; Centocor Inc., Malvern, PA, serum reference <37 kU/L), tumor stage, and grade.

^{18}FDG PET images were obtained using a dedicated tomograph (Siemens, ECAT EXACT 47) with a field of view of 16.2 cm. After an overnight fast, 444 MBq (12 mCi) of ^{18}FDG was injected intravenously into each patient. To avoid interference due to hyperglycemia, blood glucose levels were checked just before the procedure and lowered to less than 120 mg/dl with insulin administration whenever necessary. Two transmission scans of the abdomen, 15 minutes each, were obtained by 68 Ge/68 Ga rod sources before the administration of FDG to obtain cross sections for attenuation correction of the emission images. Then two emission scans, 15 minutes each, were acquired starting 60 minutes after the administration of FDG. The reconstruction was performed in a 128 × 128 matrix with a Hanning filter 0.3 cutoff. Transaxial, coronal, and sagittal sections were obtained for visual analysis. To perform a quantitative analysis, the standardized uptake value (SUV) was calculated in the suspected neoplastic foci (SUV = tissue tracer concentration/injected dose/body weight). For the SUV analysis, a circular region of interest was placed over the area of maximal focal FDG uptake suspected to be a tumorous focus, and the mean radioactivity values were obtained.

For univariate analysis the patients were divided into two groups, with the median SUV as the cutoff value. Differences in patient characteristics between groups were tested for significance using the Mann-Whitney U test, chi-square test, or Fisher's exact test. In addition, the effect on survival of the variables age, sex, tumor stage and grade, type of treatment, diabetes, and CA 19-9 levels was investigated using univariate and multivariate analysis. The survival data were estimated by the Kaplan-Meier method and examined by the log-rank test. Multivariate analysis of survival was performed using the Cox proportional hazards model. Significance was considered as $P < 0.05$.

RESULTS

Twenty-seven patients had distant metastases (stage IVb), 14 had lymph node metastases (stage III), 17 had a locally advanced tumor (stage IVa), and only two patients had a stage I tumor (Table 1). Fourteen tumors were found to be well differentiated, 24 were moderately differentiated, and 22 were poorly differentiated. Nine patients underwent pancreaticoduodenectomy, one total pancreatectomy, six distal pancreatectomy, and 22 biliary and/or digestive bypass; 22 patients had only medical treatment.

The median SUV for the 60 patients was 4.0 with values ranging from 2.1 to 13.0 (31 patients had SUVs ⩽4.0 and 29 had SUVs >4.0). The two groups did not differ statistically with regard to sex, age, number of patients with diabetes, tumor stage, Ca 19-9 serum levels, and type of treatment or tumor grade (see Table 1). The median CA 19-9 level for the 60 patients was 300 kU/L, ranging from 3.0 to 27,000 kU/L. Follow-up was available for all patients and ranged from 1 to 35 months.

The overall median survival was 221 days. The median survival for patients with SUVs ⩽4.0 was 265 days vs. 178 days for patients with SUVs >4.0

Table 1. Standardized uptake value and clinicopathologic factors

	Low SUV (≤4.0)	High SUV (>4.0)
SUV (mean ± SD)	3.18 ± 0.53	5.82 ± 2.04
Median survival (days)	265	178
Age (yr) (mean ± SD)	66.71 ± 10.55	65.07 ± 10.24
Sex		
Male	15	16
Female	16	13
UICC		
I	1	1
II	0	0
III	7	7
IVa	10	7
IVb	13	14
Grading		
Well	9	5
Moderate	12	12
Poor	10	12
Site		
Head	16	16
Body-tail	15	13
CA 19-9 (kU/L) (means ± SD)	2767.47 ± 6831.50	3461.46 ± 7439.52
Diabetes		
No	21	19
Yes	10	10
Treatment		
Resective	8	8
Palliative	11	11
Medical	12	10

SD = standard deviation; SUV = standardized uptake value; UICC = International Union Against Cancer.

($P = 0.005$) (Fig. 1). Ten (32%) of 31 patients with low SUVs survived longer than 12 months, whereas only 2 (7%) of 29 in the high SUV group did so ($P < 0.01$). Univariate analysis showed that survival was significantly influenced also by tumor stage ($P = 0.0001$) and tumor grade ($P = 0.01$). Type of treatment, diabetes, age, sex, and preoperative CA 19-9 levels (≤ or >300 kU/L) did not significantly influence survival. Detailed results are presented in Table 2. A stepwise multivariate analysis showed that tumor stage and SUV were independent predictors of survival (Table 3). When the patients who were analyzed for SUVs were stratified according to the other variables, FDG uptake was related to survival also after stratification for the following: stage III to IVa ($P = 0.002$), stage IVb ($P = 0.01$), tumor resection ($P = 0.006$) (Table 4; Fig. 2), bypass operation ($P = 0.04$) (see Table 4), moderately differentiated tumors ($P = 0.01$), age less than 65 years ($P = 0.006$),

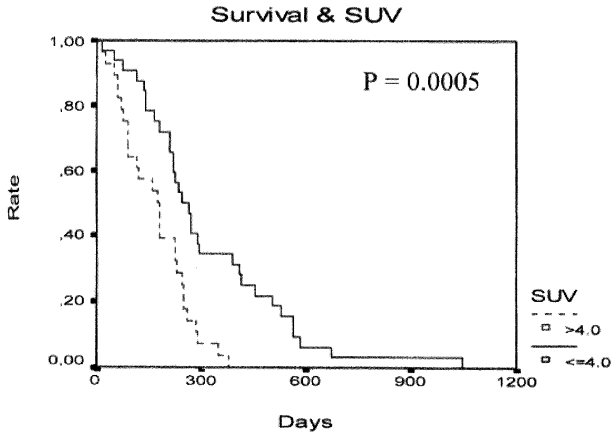

Fig. 1. Survival curves of patients with standardized uptake values (*SUV*) >4 (29 patients; *broken line*) or ≤4 (31 patients; *unbroken line*).

CA 19-9 levels greater than 300 kU/L ($P = 0.002$), and absence of diabetes ($P = 0.0001$).

Analyzing the subset of 16 patients who underwent pancreatic resection, the median survival of patients

Table 2. Univariate analysis of clinicopathologic factors and survival

Variable	No. of patients	Median survival ± SE (days)	*P* value
Treatment			
Resective	16	284.00 ± 22.00	0.11
Palliative	22	222.00 ± 50.31	
Medical	22	180.00 ± 22.12	
SUV			
≤4.0	31	265.00 ± 28.38	0.005
>4.0	29	178.00 ± 14.80	
Stage			
III and IVa	31	265.00 ± 21.82	0.0001
IVb	27	162.00 ± 37.00	
Grade			
Well	14	220.00 ± 31.80	0.01
Moderate	24	232.00 ± 13.47	
Poor	22	139.00 ± 35.76	
CA 19-9			
≤300 kU/L	31	261.00 ± 30.59	0.36
>300 kU/L	29	224.00 ± 15.93	
Diabetes			
No	40	222.00 ± 19.26	0.55
Yes	20	217.00 ± 15.65	
Sex			
M	31	207.00 ± 26.90	0.3932
F	29	249.00 ± 26.01	
Age			
≤65 yr	31	217.00 ± 21.80	0.7745
>65 yr	29	222.00 ± 22.42	

SE = standard error; SUV = standardized uptake value.

Table 3. Multivariate analysis using Cox regression model

Variable	Hazard ratio	95% Confidence interval	*P* value
SUV	3.96	1.92–8.17	0.0002
Tumor stage	4.76	1.87–12.05	0.001
Age	1.94	0.96–3.90	0.06
Tumor grade	1.55	0.95–2.53	0.07
Treatment	1.43	0.89–2.29	0.13
Diabetes	0.63	0.32–1.22	0.17
CA 19-9	1.29	0.69–2.40	0.41
Sex	1.26	0.58–2.71	0.55

with low SUVs was 386 days vs. 224 days for patients with SUVs greater than 4 (see Table 4). None of the patients with a high SUV survived for more than 12 months after surgery, whereas only two of eight patients with a low SUV died within 12 months; two subjects in this subgroup are still alive 10 and 17 months after operation, respectively. The two subgroups of resected patients, divided according to SUVs, basically differ for more left-sided and poorly differentiated cancers in the subgroup with high SUVs and shorter survival; six of them received chemo- or chemoradiotherapy after surgery, compared to one patient in the low-SUV group who had chemotherapy after operation (Table 5).

DISCUSSION

An accelerated rate of glucose transport and an increased rate of glycolysis are among the most characteristic biochemical markers of malignant transformation. Overexpression of glucose transporter 1 (Glut-1)[18,19] and glycolytic enzymes[20] has been shown in human pancreatic adenocarcinomas. FDG is a glucose analogue that is actively taken up by Glut-1 into the cell and phosphorylated by hexokinase during the first step of the glycolytic pathway. However, unlike normal glucose, phosphorylated FDG cannot continue glycolysis and becomes trapped within the cell. FDG accumulation correlates with the tumor proliferation rate in patients with malignant head and neck tumors[21] and in vitro with the number of viable tumor cells.[22] FDG PET is therefore useful in distinguishing benign from malignant tumors in the diagnosis of tumor recurrence and in the evaluation of tumors after neoadjuvant chemoradiation.[10] A close relationship between tumor aggressiveness and metabolic activity was initially reported by Patronas et al.[13] for glioma, and more recently a correlation between FDG uptake and prognosis was reported for a variety of cancers.[8,9] Nakata et al.[14] introduced FDG PET and SUV, a semiquantitative parameter of glucose consumption, as new metabolic predictors of prognosis in patients with pancreatic carcinoma. In a small series of 14 patients, they found that survival was significantly shorter in the high-SUV group (>3.0) as compared with the low-SUV group (<3.0) ($P < 0.05$). These results were partially confirmed by these same investigators 4 years later.[7] In a series of 37 patients with histologically proved pancreatic cancer, the SUV was not able to predict survival in the subgroup of patients with resectable tumors. However, in the subgroup with unresectable tumors, patients with a low SUV survived significantly longer than those with a high SUV ($P = 0.03$). Moreover, multivariate analysis showed that tumor SUV was an independent prognostic indicator for patients with unresectable tumors.

In the current study we analyzed FDG uptake in a large cohort of patients (n = 60) with histologically proved pancreatic cancer. Patients were divided into high (⩾4)– and low (<4)–SUV groups. The two groups did not differ statistically with regard to age, sex, tumor stage and grade, CA 19-9 level, diabetes, and treatment. Survival was significantly influenced by SUV. The median survival time for patients with low FDG uptake was 265 days compared to 178 days for patients with high FDG uptake in the tumor ($P = 0.005$) (Fig. 1). Among the clinicopathologic parameters tested, tumor stage and tumor grade were significantly related to survival after univariate analysis ($P = 0.001$ and $P = 0.01$, respectively). Multivariate analysis showed that only SUV ($P = 0.0002$) and

Table 4. Survival according to treatment and SUV

Treatment Total number; cases with low/high SUV	Low SUV (⩽4.0) Survival median ± SE (days)	High SUV (>4.0) Survival median ± SE (days)	*P* value
Resection (n = 16; 8/8)	386.00 ± 114.55	224.00 ± 60.81	0.006
Bypass (n = 22; 11/11)	244.00 ± 59.45	121.00 ± 54.56	0.043
Medical (n = 22; 12/10)	210.00 ± 8.66	172.00 ± 166.96	0.082

SE standard error.

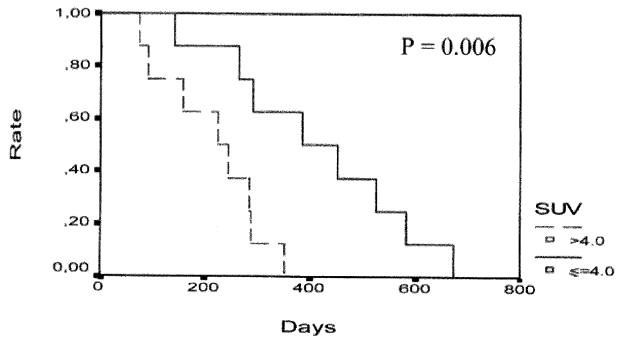

Fig. 2. Survival curves of resected patients with standardized uptake values >4 (8 patients; *broken line*) or ≤4 (8 patients; *unbroken line*).

tumor stage ($P = 0.001$) were independent predictors of survival.

Interestingly, even when patients who were analyzed for SUV were stratified according to the other variables considered, ^{18}FDG uptake significantly influenced survival, independent of tumor stage, in the following groups: patients who underwent resection ($P = 0.006$) (Fig. 2), patients 65 years of age or younger ($P = 0.006$), patients with CA 19-9 levels greater than 300 kU/L ($P = 0.002$), those with moderately differentiated tumors ($P = 0.01$), and those without diabetes ($P = 0.001$). The different biological aggressiveness of the tumor, detected by the SUV, may explain the difference in survival after a potentially curative resection with otherwise similar prognostic variables. It is well known that ^{18}FDG PET is less accurate in patients with diabetes, and therefore it can be unable to adequately predict survival. On the other hand, it is difficult to explain the differences that were verified within the other groups.

Table 5. Clinicopathologic features and SUV in resected patients (n = 16)

Features	Low SUV (≤4.0); n = 8 Number of cases	High SUV (>4.0); n = 8 Number of cases
Operation		
PD	6	3
TP	1	0
LP	1	5
Resection margins		
Positive	2	3
Negative	6	5
Lymph nodes		
Positive	6	6
Negative	2	2
Grading		
Well	2	1
Moderate	3	2
Poor	3	5
Extended resection*		
Yes	4	5
No	4	3
Postoperative therapy		
No	7	2
Chemotherapy	1	2
Radiochemotherapy	0	4

PD = pancreaticoduodenectomy; TP = total pancreatectomy; LP = left pancreatectomy.
*Large vessels or adjacent organs.

In a group of 52 patients with pancreatic cancer, Zimny et al.[16] reported that survival was significantly influenced by SUV (cutoff 6.1) and CA 19-9 serum levels, using both univariate and multivariate analysis. The different cutoff values chosen by Nakata et al.,[7] by Zimny et al.,[16] and by us in the present study are related to the different median values recorded in the three studies. Because the cutoff values vary greatly, ranging from 3.0[7] to 6.1,[16] it is unwise to suggest a cutoff value for further studies; rather it should be determined on the basis of the personal (possibly large) data.

We were unable to find a statistically significant difference in survival between resected and nonresected patients (see Table 2). This may be due to the small number of resected patients (16 of 60) and to the small number of early-stage cancers (stage I/II 2 of 60). However, high and low SUVs were able to separate patients with a significantly different survival after a potentially curative tumor resection (see Fig. 2).

Most previous studies demonstrated that tumor-associated histologic characteristics are important in defining the prognosis of patients with pancreatic cancer, especially after resection of the tumor.[2,3,5,6,23,24] However, most of them are available only after a resection procedure has been performed. The great advantage of SUVs calculated by ^{18}FDG PET is that this parameter can be obtained before any treatment has been performed. Whether the availability of a pretreatment factor related to prognosis may influence the clinical management of patients with pancreatic cancer was not investigated in the present study because it was retrospective. Post-surgical chemo- or radiotherapy in our patients was given as part of randomized international trials, and most of the treated patients belonged to the high-SUV subgroup of patients who had a shorter survival. Because the prognostic value of the SUV is at least equivalent to that of tumor staging, stratification of patients on the basis of the extent of the disease evaluated by multidetector CT scan and on the SUV may improve our understanding of the actual effect of different

treatments. In as much as the number of PET/CT scans will increase rapidly in the near future, a single procedure can give both diagnostic and prognostic information.[25,26]

There is evidence that increased glycolytic activity as determined by FDG uptake represents tumor growth and resembles the biological behavior of the tumor. Therefore the effects of chemo- and/or radiotherapy can be evaluated on the basis of SUV variations. Rose et al.[10] performed pre- and post-treatment FDG PET scans to assess the response to neoadjuvant therapy in patients with potentially resectable pancreatic cancer. All patients who demonstrated a 50% or greater reduction in SUV after chemoradiation had histologic evidence of tumor necrosis. Maisey et al.[15] demonstrated that the absence of FDG uptake at 1 month after chemotherapy for pancreatic cancer is an indicator of improved survival, and FDG PET may be a useful tool for assessing early response to treatment of pancreatic cancer.

CONCLUSION

The ^{18}FDG PET SUV calculation provides relevant prognostic information in patients with pancreatic cancer before any surgical or medical treatment, and may therefore be considered for stratifying patients for prospective studies when different therapeutic options are to be compared.

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Discussion

Dr. L. McHenry (Indianapolis, IN): The standard uptake value (SUV) was 4.0. In your previous paper in the *Annals of Surgery* you had an SUV of 2.6 as a cutoff to detect pancreatic cancer in cystic neoplasms. Could you explain why that would be different in this situation?

Dr. S. Pedrazzoli: The cutoff SUV of 4 was the mean value for patients with pancreatic cancer, whereas 2.5 was the maximum or upper limit for benign disease. This number has changed a little since then because of the increased number of patients studied. We published the paper when we had 56 cystic tumors. Now we have more than 85 cystic tumors, and the sensitivity, specificity, and accuracy are better. This is why we considered the upper limit of normal values to be 2.5 instead of 2.6. It depends on the number of patients studied, the procedure performed, and the machine.

Dr. McHenry: I have one last question. Have you seen a change in your SUV now with CT/PET as compared to PET alone? Or is the SUV uptake the same? Is the PET scan with the CAT scan giving you the same PET information?

Dr. Pedrazzoli: We do not yet have the PET/CT. We will have the PET/CT within a few months. But I introduced the combination of PET/CT imaging because I believe it is important for the future. The CT scanner is very useable, and the PET images with increased uptake can be correlated with the CT images.

Dr. R. Prinz (Chicago, IL): It is interesting that you have not shown any correlation with CA 19-9. A number of reports find that CA 19-9 relates to prognosis. At the present time ^{18}FDG PET is not approved for use in pancreatic cancer in the United States and probably one of the reasons why is that there is no reason to believe that it will change how we currently manage patients with pancreatic cancer. Do you do anything different on the basis of your PET findings?

Dr. Pedrazzoli: I believe there will be a big difference now that we have reviewed all of our experiences. Unfortunately only 60 out of 118 of our patients had their SUVs calculated. Why? Because calculation of SUVs in the initial period required a great deal of time, more time than for a simple total-body PET. So the nuclear medicine personnel decided not to perform SUV calculations in all patients. Our first patients were without SUVs. But now that we have enough patients—and I believe 60 patients are enough—there is an inverse correlation of SUV with survival despite the same stage as UICC.

What about CA 19-9 and prediction of prognosis? I believe it does predict prognosis. I believe that it corresponds to the tumor burden but not to tumor aggressiveness, two different perspectives that relate to prognosis.

Dr. J. Howard (Toledo, OH): Relative to your CA 19-9 data, were your measurements all at the time of admission?

Dr. Pedrazzoli: Yes, before any treatment was begun.

Dr. Howard: I think the literature and our data indicate there is a definite prognostic relationship to the CA 19-9 level after resection.

Dr. Pedrazzoli: I agree with you. We published a paper 10 years ago about possible normalization of CA 19-9 values after resection, and a CA 19-9 level of less than 300 before resection was a predictor of improved survival.

Dr. J. Moser (Pittsburgh, PA): I have a brief comment. We have been using SMART (PET/CT) for a couple of years in an effort to distinguish cancer from inflammatory masses caused by chronic pancreatitis. In our selected cases, the SUV calculated from CT/PET had only about 65% sensitivity for pancreatic cancer. False negative findings were not infrequent. In each case we confirmed the final diagnosis by histologic examination of the resected pancreatic mass. I am not sure that PET/CT is going to be a complete panacea for the problems of PET as it applies to pancreatic lesions.

Dr. Pedrazzoli: I agree with you about the unsatisfactory value of PET in differentiating between chronic pancreatitis and pancreatic cancer, but this paper reports on the very useful prognostic value of the SUV in all patients who have had pancreatic cancer.

Invited Discussion—Expert Commentary

Sean J. Mulvihill (St. Louis, MO): It is clear that our current TNM staging system for pancreatic cancer is inadequate by some standards. First, in comparison to other cancers, patients with node-negative disease have a relatively poor outcome, implying that either our pathologists are missing lymphatic metastases or other

features are more important in predicting systemic disease. Second, preoperative clinical and radiographic staging is incomplete, making it difficult to stratify patients who might benefit from neoadjuvant therapy. Sperti and colleagues address these issues by using ^{18}FDG PET as a preoperative staging tool, dividing patients into groups with high or low glucose uptake, representing greater or lesser tumor metabolic activity. With this functional assay of tumor behavior, they demonstrated a statistically significant difference in survival, with patients exhibiting low tumor metabolic activity having improved outcome independent of tumor stage. Interestingly, they also show significant discrimination in survival even after resection. This implies that ^{18}FDG PET may be useful clinically not only in identifying occult metastases that are not apparent on clinical evaluation and cross-sectional imaging but also as a staging and prognostic tool. We are entering an exciting time of functional imaging. This, or similar technology, may help us to decide preoperatively whether or not a given patient might benefit from neoadjuvant therapy or even how aggressive we should be with surgical resection. I believe ^{18}FDG PET will become a useful tool in the management of patients with pancreatic cancer, but a number of issues related to imaging technique and analysis require refinement.

Troponin I Peptide (Glu94-Leu123), a Cartilage-Derived Angiogenesis Inhibitor: In Vitro and In Vivo Effects on Human Endothelial Cells and on Pancreatic Cancer

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Several inhibitors of angiogenesis have been identified in bovine and shark cartilage. One of them is troponin I, which is the molecule responsible for the inhibition of the actomyosin ATPase during muscle contraction. In this study we sought to investigate if the active site of troponin I (peptide Glu94-Leu123; pTnI) is also the one responsible for the antiangiogenic properties of this protein. The effects of pTnI on endothelial cell tube formation and endothelial cell division were investigated using human umbilical vein endothelial cells, Matrigel, light microscopy, carboxyfluorescein diacetate, succinimidyl esterlabeling, and flow cytometry. Its effects on induction of ICAM-1 and production of vascular endothelial growth factor by pancreatic cancer cells (CAPAN-1) were also investigated, as was its efficacy in a mouse model of pancreatic cancer metastases. Our results show that concentrations as low as 1 pg/ml of pTnI significantly inhibit endothelial cell tube formation, and that endothelial cell division was inhibited at 96 hours by 3 μg/ml pTnI ($P = 0.0001$). No effects were seen using troponin peptide 124-181 as a control. pTnI-treated supernatant from the pancreatic cancer cell line CAPAN-1 downregulated ICAM-1 expression on human umbilical vein endothelial cells up to 10 ng/ml pTnI, and a significant reduction in vascular endothelial growth factor production was seen by treating CAPAN-1 cells with up to 1 μg/ml pTnI. After intrasplenic injection of CAPAN-1 cells, mice treated with pTnI had fewer liver metastases compared to control mice (liver/body weight 5.5 vs. 11.1; $P = 0.03$). The active region of troponin I is the one responsible for its antiangiogenic effect. The mechanism of action of this peptide is probably multifactorial. (J GASTROINTEST SURG 2003;7:961–969)

KEY WORDS: Angiogenesis, troponin I, pancreatic cancer

Angiogenesis, the formation of new blood vessels, is recognized as a fundamental process in and an essential component of tumor growth and metastasis, and has become a target for the treatment of cancer.[1,2] The process of neovascularization may be induced by a number of mediators including growth factors, cytokines, and cell adhesion molecules, and their inhibition or antagonism has been the focus of extensive basic and clinical research.[3,4]

Bovine and shark cartilage have been investigated as treatments for cancer, and the findings that several substances in cartilage have antiangiogenic activity provides a theoretical rational for possible effective use.[5–7] One of these substances is troponin I, first described by Moses et al.[8] in 1999. Troponin is a globular molecule consisting of three subunits (troponin C, troponin T, and troponin I), each of which possesses a specific function. The principal known function of troponin I is the inhibition of the actomyosin ATPase during muscle contraction.[9] It is a 21 to 23 kDa protein that consists of 181 amino acid residues. Several isoforms have been described and are

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coded by three different genes.[10] The active site of troponin I is located in the amino acid residues 96 to 116. This peptide inhibits 85% of the ATPase activity in a concentration-dependent manner.[11]

Very little is known about the mechanism of troponin I as an angiogenesis inhibitor.[8,12] In this study we investigated whether the active site of troponin I (peptide Glu94-Leu123; pTnI) is also responsible for the antiangiogenic effect of the molecule by studying its effects on the following: (1) endothelial cell tube formation; (2) endothelial cell division; (3) induction of intercellular adhesion molecule 1 (ICAM-1) by pancreatic cancer cells, and (4) production of vascular endothelial growth factor (VEGF) by pancreatic cancer cells. We also investigated the effects of this peptide in limiting the establishment and progression of metastases in a mouse model of metastatic pancreatic cancer.

MATERIAL AND METHODS

Troponin I Peptide

The troponin I peptide 94-123 (Glu-Asp-Met-Asn-Gln-Lys-Leu-Phe-Asp-Leu-Arg-Gly-Lys-Phe-Lys-Arg-Pro-Pro-Leu-Arg-Arg-Val-Arg-Met-Ser-Ala-Asp-Ala-Met-Leu), as well as the fluorescein isothiocyanate– and AlexaFluor ®594–labeled peptide 94-123 and the peptides 124-181 were synthesized and given to us by Dr. Paul Leavis (Boston Biomedical Research Institute, Watertown, MA).

pTnI was dissolved in Hanks' balanced salt solution (HBSS) (BioWhittaker, Inc., Walkersville, MD) at a stock concentration of 10 mg/ml (2.76 mmol/L) and frozen at −20C. For in vitro experiments pTnI was dissolved in the respective cell culture medium; for in vivo experiments pTnI was suspended in physiologic saline solution.

Cell Lines, Cell Culture, and Conditioned Cell Supernatant

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics® Human Cell Systems (BioWhittaker, Inc.) and subcultured up to passage number n+15. CAPAN-1 cells, derived from a hepatic metastases of a human pancreatic adenocarcinoma, were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Culture media for HUVEC was EGM-2® (BioWhittaker, Inc.) supplemented with human recombinant epidermal growth factor, hydrocortisone, human fibroblast growth factor basic with heparin, VEGF, human recombinant insulin-like growth factor, ascorbic acid, gentamicin, amphotericin-B, heparin, and fetal bovine serum (FBS). CAPAN-1 cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) supplemented with 10% FBS.

CAPAN-1 cells used for in vivo experiments were grown to almost 100% confluence, harvested with Trypsin-EDTA (BioWhittaker, Inc.), washed, centrifuged at 1750 rpm for 4 minutes at room temperature, and then resuspended in HBSS to a final concentration of 5×10^6 cells/100 µl. Cell viability was confirmed by trypan-blue exclusion.

For conditioned CAPAN-1 cell supernatant, cells were seeded into a six-well plate and allowed to grow to 75% confluence. pTnI was added in the desired final concentrations in fresh RPMI medium, and the cells were incubated for 48 hours. Then the supernatant was removed, centrifuged, and frozen at −20C.

Effects of pTnI on Endothelial Cell Tube Formation

For endothelial cell tube formation, 50 µl/well of Matrigel® basement membrane matrix (Becton Dickinson, Bedford, MA) was added to a 96-well plate and allowed to gel for 60 minutes at 37C. Then 1.5×10^4 HUVEC were seeded onto the surface of the gel in the presence or absence of troponin I peptides or conditioned CAPAN-1 cell supernatant, and incubated for 14 hours at 37C in a CO_2 incubator. The serine protease inhibitor gabestate mesilate (FOY®, Ono Pharmaceutical Co., Ltd, Osaka, Japan) was used as a positive control for inhibition of tube formation.[13] The cultures on the gel were fixed for 10 minutes in 25% glutaraldehyde, washed, and stained with Mayer's hematoxylin. Tube formation was inspected under a light microscope at 40× magnification and analyzed using MetaMorph 4.6 for Windows. For all cultures the center section of the well was selected and the quantity of tube formation measured. The results for tube formation of HUVEC without troponin I was defined as 100%.

Effects of pTnI on Endothelial Cell Division

Carboxyfluorescein diacetate, succinimidyl ester (CFDA,SE) was used as a cell division marker. Then 1×10^5 HUVEC were seeded on six-well plates and allowed to attach for 24 hours. Cells were washed once and incubated for 15 minutes at 37C with 3 µm CFDA,SE (Molecular Probes Inc., Eugene, OR) in Cellgro-free® medium (Mediatech, Inc., Herndon, VA). Cells were then washed once with prewarmed Cellgro-free medium and once with EGM-2 for 15 minutes at 37C. Finally, 2 ml of EGM-2 medium containing pTnI in different concentrations was added. CFDA,SE-labeled HUVEC grown in pTnI-free medium were collected after 0, 24, 48, 72, and 96 hours. All other cells were cultured for 4 days,

with changing of the medium on day 3. Cells were then trypsinized, washed once in HBSS containing 1% bovine serum albumin and 1% NaN_3 (fluorescence-activated cell sorter [FACS] buffer), and fixed in 3.7% paraformaldehyde. After one more wash, cells were resuspended in 50 µl FACS buffer and analyzed by flow cytometry (FACScan, Becton Dickinson) using excitation at 488 nm and the FL1 detection channel. Data were analyzed and converted to histograms by WinMDI (J.Trotter; Scripps Research Institute, La Jolla, CA).

Effects of pTnI on Intracellular Cell Adhesion Molecule 1 (ICAM-1) Expression

One $\times 10^5$ HUVEC were seeded on six-well plates and allowed to grow to almost 100% confluence, and then stimulated with 2 ml of conditioned CAPAN-1 supernatant for 6 hours at 37C with different concentrations of pTnI. Stimulation with TNF-α (10 ng/ml) was used as control. After washing once with HBSS, cells were harvested by trypsinization and washed again with ice-cold HBSS containing 1% bovine serum albumin and 1% NaN_3 (FACS buffer) before incubating with normal mouse serum for 20 minutes. After two more washes, cells were incubated with a fluorescein-conjugated mouse monoclonal antihuman ICAM-1 antibody (R&D Systems, Inc.) (7.5 µg/ml) for 30 minutes at 4C. Fluorescein-conjugated mouse IgG_1 was used as a negative control. After two washes in FACS buffer, cells were fixed in ice-cold 3.7% paraformaldehyde, washed again, and analyzed by flow cytometry. The mean fluorescence intensity expressed by the pTnI-free cells was determined as 100%.

Effects of pTnI on Vascular Endothelial Growth Factor Production by CAPAN-1 cells

To measure VEGF in CAPAN-1 supernatant, 2.5×10^4 cells per well were seeded in a 96-well plate without pTnI or with 0.1 µg/ml pTnI and the supernatant was collected from 0 to 72 hours.

VEGF in CAPAN-1 cell supernatant was measured using the ChemiKine™ VEGF Sandwich enzyme-linked immunosorbent assay kit (Chemicon International, Inc., Temecula, CA) according to the manufacturer's instructions. Briefly, 100 µl of CAPAN-1cell supernatant was added to the plate precoated with mouse antihuman VEGF monoclonal antibody, then biotinylated rabbit antihuman VEGF antibody was added, and the plate was incubated for 3 hours. After washing, streptavidin–alkaline phosphatase was added and incubated for 45 minutes. The plate was washed again, color reagent was added, and the optical density was read at 490 nm.

Animals and Liver Metastases Model

Six-week-old male athymic nude mice, weighing 20 to 25 g, were obtained from Charles River Laboratories (Wilmington, MA) and acclimatized for 1 week. Animals were caged in groups of five in a barrier care facility with free access to food and water. All procedures were performed in accordance with federal, local, and institutional guidelines and were approved by the subcommittee on animal research at our institution.

The hematogenous liver metastases model of pancreatic carcinoma was used as previously described by our laboratory.[14] Briefly, animals were anesthetized with ketamine, 100 mg/kg (Monarch Pharmaceuticals, Rochester, MI), and xylazine, 5 mg/kg (Lloyd Laboratories, Shenandoah, IA), followed by a small left subcostal incision and exteriorization of the spleen. Using a 30-gauge needle and a 1 ml syringe, 5×10^6 CAPAN-1 cells in 100 µl HBSS were injected under the capsule of the spleen. Hemostasis was performed by gentle pressure on the injection site with a cotton applicator for 30 seconds. The peritoneum and skin were closed in a single layer using 5-0 Dexon sutures.

Mice were divided into three groups. Group 1 ($n = 5$) received 3.5 mg/kg pTnI intraperitoneally daily for 7 days before tumor cell injection and for 14 days after tumor cell injection. Group 2 ($n = 8$) received the same volume of vehicle alone (0.9% NaCl) according to the same schedule. Group 3 ($n = 6$) consisted of mice without tumor induction who served as control group. Animals were killed at 6 weeks. Body weight was recorded, and the abdominal and thoracic cavities were inspected. The liver was excised and weighed.

Tissue was collected for histologic evaluation. Representative sections of liver were fixed for 72 hours in 10% formalin, embedded in paraffin, and stained with hematoxylin-eosin for light microscopy.

Statistical Analysis

Data are presented as mean ± standard error of the mean (SEM). Continuous normally distributed variables were analyzed by Student's t test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of pTnI on Endothelial Cell Tube Formation

HUVEC on Matrigel-coated wells form a capillary-like network within 14 hours. A significant failure to form continuous networks between cells

was observed in the presence of pTnI with concentrations as low as 1 pg/ml pTnI ($P < 0.0001$). No inhibitory effect was seen with the use of the same concentrations of troponin I peptide 124-153.

CAPAN-1 cell supernatant stimulated the cell tube formation in a volume-dependent fashion. The capillary network increased by 62% with 5 µl supernatant and by 88% with 50 µl supernatant. Addition of

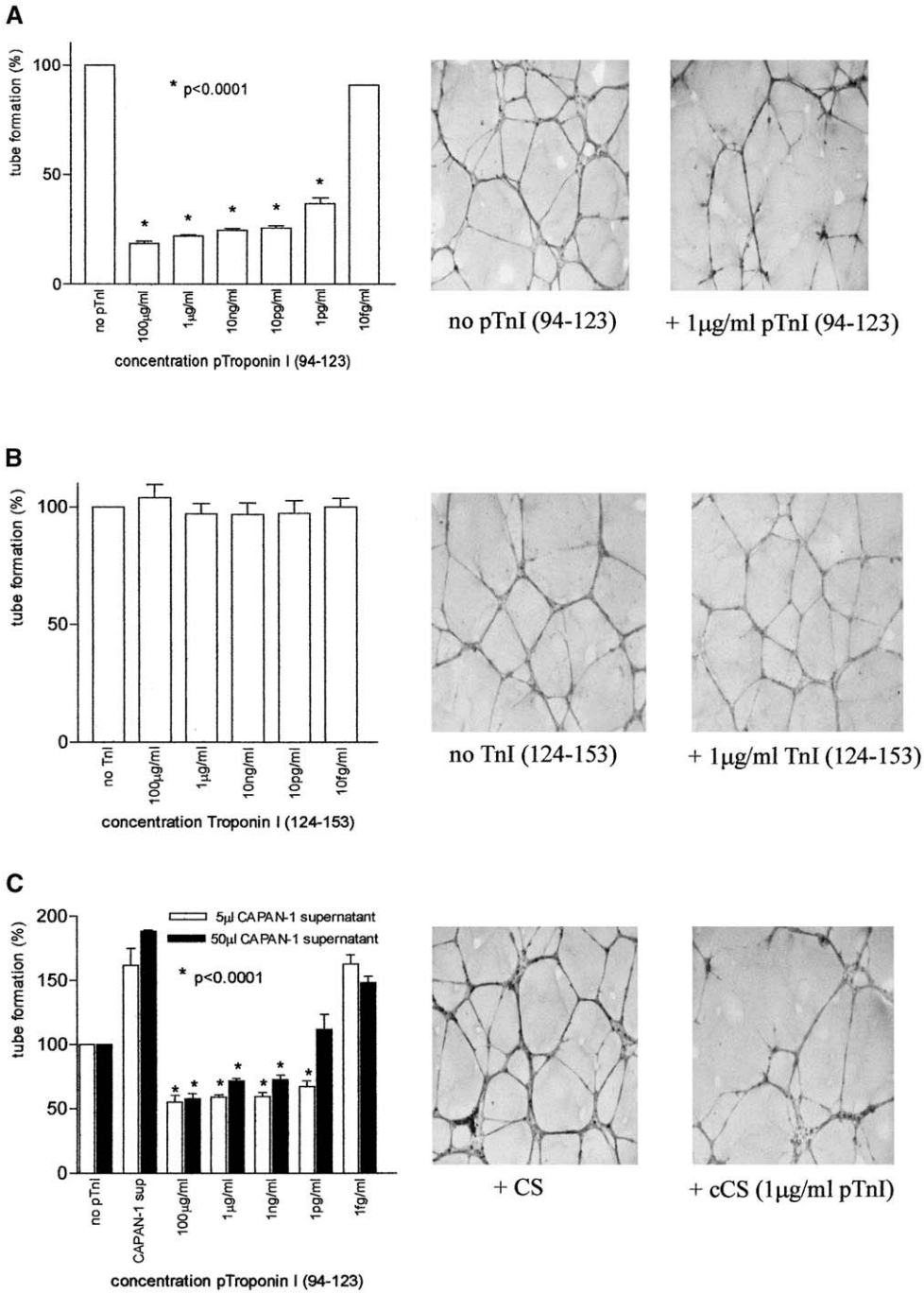

Fig. 1. Effect of troponin I on tubelike structures formed by HUVEC cultured on Matrigel. The stained cell cultures were viewed by microscope and the tube network of HUVEC was analyzed by computer. Troponin I peptide 94-123 inhibited tube formation up to a concentration of 1 pg/ml (**A**). No inhibition was observed with troponin I peptide 124-153 (**B**). Supernatant of pancreatic cancer cells CAPAN-1 (*CS*) accelerates the tubelike network in volume-dependent fashion. Supernatant of CAPAN-1 coincubated with troponin I peptide 94-123 (*cCS*) inhibits the network significantly up to 1pg/ml pTnI with 5 µl CAPAN-1 supernatant and up to 1 ng/ml pTnI with 50 µl supernatant (**C**).

CAPAN-1 cell supernatant conditioned with pTnI inhibited the tube formation in a volume- and concentration-dependent manner (Fig. 1).

Endothelial Cell Division

CFDA,SE labeling was used for the first time on endothelial cells. Labeled HUVEC showed a decrease in fluorescence intensity, which is inversely proportional to cell division and cell generations (Fig. 2).

HUVEC cultured in medium containing 3 μg/ml pTnI showed fewer cell divisions after 96 hours, resulting in a significantly higher mean fluorescence intensity compared to cells grown in pTnI-free medium ($P = 0.0001$) and shifting the histogram to the right (Fig. 3). On the other hand, CAPAN-1 supernatant added to HUVEC medium accelerated cell division and shifted the histogram to the left (data not shown).

ICAM-1 Expression on Endothelial Cells

CAPAN-1 supernatant stimulated endothelial cells to express ICAM-1. Stimulation of HUVEC with conditioned, pTnI-treated CAPAN-1 cell supernatant resulted in a downregulation of ICAM-1 expression. The downregulation was significant up to a concentration of 10 ng/ml pTnI ($P < 0.05$) (Fig. 4). Stimulation with CAPAN-1 cell supernatant was less effective than with TNF-α (data not shown).

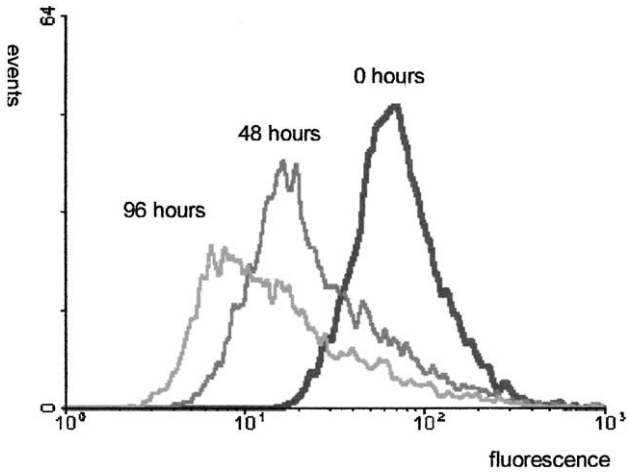

Fig. 2. Detection of cell division of HUVEC labeled with carboxyfluorescein diacetate, succimidyl ester. Peaks from right to left represent successive generations of cells 0, 48, and 96 hours after labeling.

pTnI and VEGF Production by CAPAN-1 Cells

Incubation of CAPAN-1 cells with troponin I peptide for 48 hours resulted in a significant decrease of VEGF production. The reduction was significant ($P < 0.05$) up to a concentration of 1 μg/ml pTnI (Fig. 5).

Effects of pTnI on Liver Metastases

Splenic injection of CAPAN-1 cells resulted in growth of multiple metastatic nodules in the livers

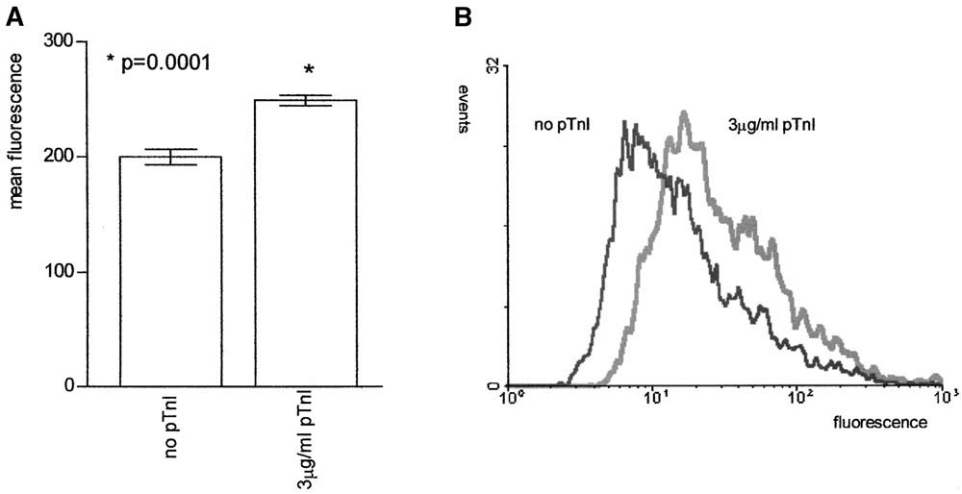

Fig. 3. Effect of troponin I peptide 94-123 on cell division of HUVEC. Cells cocultured for 96 hours with 3 μg/ml pTnI show fewer cell divisions compared to untreated cells. Flow cytometry analysis shows a higher mean fluorescence, representing fewer cell divisions, in the cocultured cells (mean ± SEM) (**A**). The histogram of cocultured cells is shifting to the right (**B**).

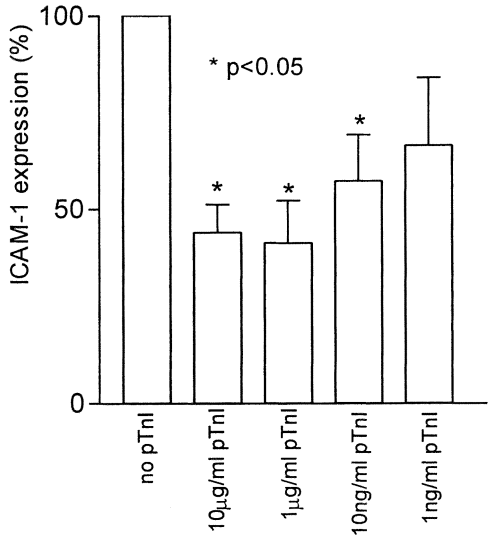

Fig. 4. Inhibition of ICAM-1 expression on stimulated HUVEC by troponin I peptide 94-123 (*pTnI*). HUVEC were stimulated with CAPAN-1 supernatant conditioned with 0, 10 µg/ml, 1 µg/ml, 10 ng/ml, and 1 ng/ml pTnI. Flow cytometry analysis shows a significant downregulation of ICAM-1 up to 10 ng/ml pTnI (mean ± SEM).

of nude mice. Histologically, metastases consisted of well-differentiated neoplastic glands. Ascites was observed in none of the treated animals but in three of the animals in the nontreated group. One mouse in this group also had a splenic tumor.

Macroscopically, tumor burden in the liver of mice treated with troponin I peptide 94-123 was minimal compared to the control group (Fig. 6). Hepatic tumor loads were calculated from the liver-to-body weight ratio. The mean liver-to-body weight ratio in the treated group was 5.54 (range 4.88 to 6.56), which was comparable to the ratio in normal mice (5.25). A significantly greater ratio of 11.08 (range 5.18 to 16.41) was seen in the nontreated group (Fig. 7).

DISCUSSION

Bovine and shark cartilage have been investigated as treatments for cancer for years, and several substances that have antitumor activity have been identified in cartilage.[15–18] One of these substances is troponin I, a protein shown to have an antiangiogenic effect.[8] Our study shows that the central part of the protein responsible for the ATPase inhibitory action of troponin I in muscle is also the one responsible for the antiangiogenetic effect. The synthetic peptide Glu94-Leu123 inhibited capillary tube formation by endothelial cells in concentrations as low as 1 pg/ml. Additionally, pTnI inhibited endothelial cell division. Both effects were not seen using another part of the troponin I protein.

The effects of pTnI were not limited to endothelial cells. Our studies show that pTnI decreases VEGF expression by CAPAN-1 cells and downregulates

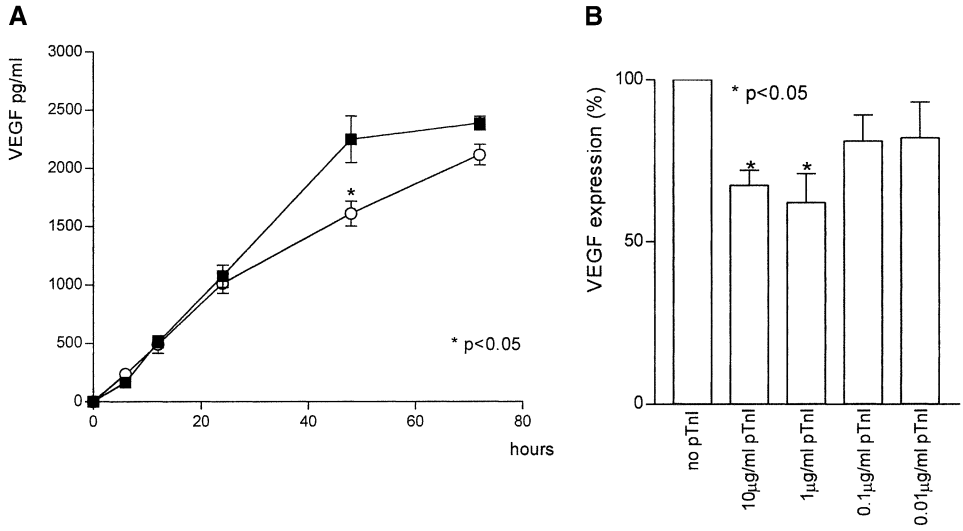

Fig. 5. VEGF production by CAPAN-1 cells and inhibition by troponin I peptide 94-123 (*pTnI*). **A**, Time course without (■) or with (○) 1 µg/ml pTnI. Forty-eight hours after coincubating the cells with pTnI, a significant reduction of VEGF production was observed. **B**, CAPAN-1 cells were incubated for 48 hours with different concentrations of pTnI. A significant reduction in VEGF production is seen up to a concentration of 1 µg/ml pTnI (mean ± SEM).

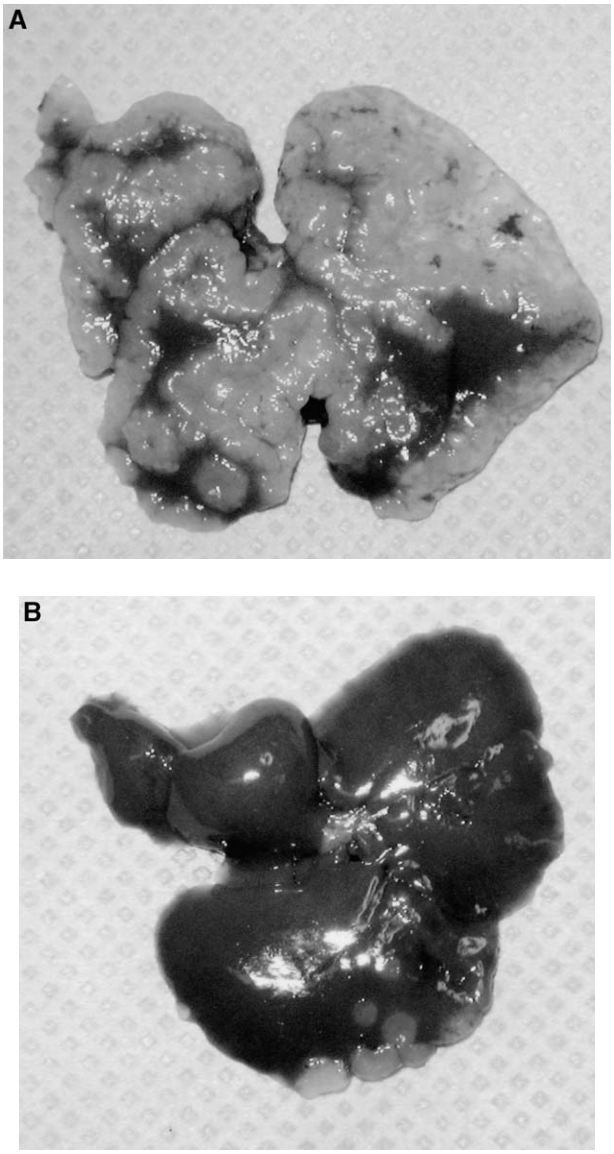

Fig. 6. Effects of troponin I peptide 94-123 on hepatic metastases from pancreatic cancer. **A**, Six weeks after intrasplenic injection of CAPAN-1 cells, the liver of an untreated animal is almost replaced by cancer. **B**, Cancer growth is minimal in the treated animal.

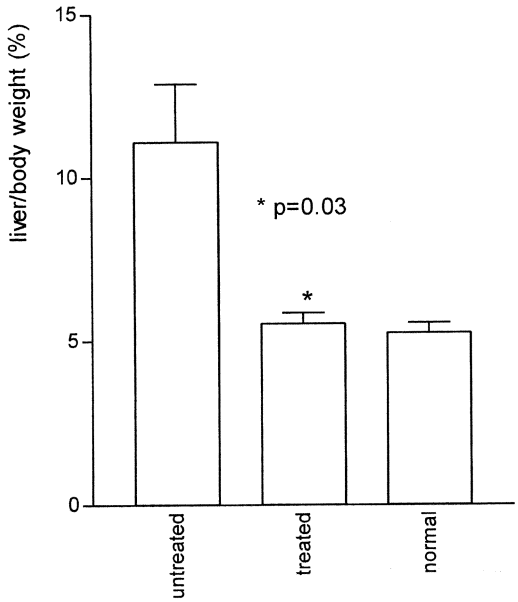

Fig. 7. Reduction of tumor burden in the liver by troponin I peptide 94-123. Reduction between untreated and treated group is significant. Liver/body weight in the treated group is similar to that in normal animals.

ICAM-1 expression on HUVEC stimulated by CAPAN-1 supernatant. VEGF, a potent angiogenic polypeptide, plays a key role in the formation of new blood vessels,[19] and is overexpressed in cancer and associated with disease progression.[20,21] Another function of VEGF is the upregulation of ICAM-1 on endothelial cells,[22] and therefore the downregulation of ICAM-1 by pTnI seen in this study could be an indirect effect of the peptide on VEGF production by cancer cells. The mechanism whereby pTnI inhibits VEGF expression is unknown. Feldman and Rouleau[12] suggested that troponin I may interact with the basic fibroblast growth factor receptor on endothelial and nonendothelial cells, and perhaps pTnI competes with VEGF and basic fibroblast growth factor on the cell surface for their receptors.

In vivo, pTnI inhibited pancreatic cancer metastases in the liver metastases model. The tumor burden of the treated animals was significantly less than that of the control group, and the intraperitoneal application of pTnI was well tolerated. As animals were treated before tumor inoculation, no conclusions can be drawn about the effect against established liver metastases, but the magnitude of the effect does suggest that this component alone or in combination with other drugs may be useful in cancer treatment.

In summary, our results indicate that the active region of troponin I, which is the one that inhibits ATPase during muscle contraction, is also the part of the protein responsible for the antiangiogenic effect. pTnI has an inhibitory effect on endothelial cells as well as on tumor cells in vitro, and in vivo reduced metastases from pancreatic cancer to the liver. The mechanism of action on endothelial and tumor cells is unclear but may be associated with growth factors and their receptors.

Although the findings in this study cannot be generalized to other pancreatic cancer cell lines or to other in vivo pancreatic cancer models, they do add to the increasing evidence that inhibition of angiogenesis can affect cancer progression. Whether or not troponin I can become a useful agent for this

purpose requires further laboratory study including comparison to other antiangiogenic therapies.

We thank Dr. Paul Leavis for the synthesis of troponin I and Dr. Gerald Waneck for assistance with flow cytometry.

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Discussion

Dr. M. Callery (Boston, MA): Have you tested whether you can potentiate chemotherapeutic agents with your troponin I compound?

Dr. B. Kern: No, we did not. We only did it with troponin I, not in combination with other chemotherapies.

Dr. J. Moser (Pittsburgh, PA): I was just wondering if you have any speculation about the cell surface receptor or effector mechanism for pTnI that would mediate these effects?

Dr. Kern: There are some hints that troponin I competes in some way with growth factor receptor, VEGF receptor, or fibroblast growth factor receptor. The latter was suggested as a likely target in a paper published last year.

Invited Discussion—Expert Commentator

Dr. Sean J. Mulvihill, M.D. (Salt Lake City, UT): This is an important paper describing possible antineoplastic effects of troponin I, the protein responsible for inhibition of actomyosin ATPase during muscle contraction. Previous studies have suggested an angiogenesis inhibitory effect of this protein. The work of Folkman and others increasingly shows that inhibition of angiogenesis is an important potential therapeutic target in cancer therapy, and several compounds are now in clinical trials. The main findings in this study include the following effects of troponin I: (1) dose-dependent inhibition of endothelial cell tube formation; (2) inhibition of endothelial cell division; (3) downregulation of ICAM-1 expression in endothelial cells; (4) minor inhibition of VEGF production by endothelial cells; and (5) inhibition of development of liver metastases in a nude mouse model.

Although the mechanism of action of troponin I is not clear from this study, the marked inhibitory effect of pretreatment in vivo in the nude mouse model suggests a possible therapeutic application. This represents cutting-edge work. It is gratifying to see a paper presented at the SSAT meeting citing very recent references from *Science* and the *Journal of Biological Chemistry*. I congratulate the authors for being on top of a rapidly evolving field and bringing us this report of their work.

Cystic Lesions of the Pancreas: Selection Criteria for Operative and Nonoperative Management in 209 Patients

Peter J. Allen, M.D., David P. Jaques, M.D., Michael D'Angelica, M.D., Wilbur B. Bowne, M.D., Kevin C. Conlon, M.D., Murray F. Brennan, M.D.

Because of the inability to determine benign from malignant, many have recommended that all cystic lesions of the pancreas be resected. Patients evaluated between January 1995 and December 2000 for the ICD-9 diagnosis of pancreatic cyst (577.2) or benign neoplasm of the pancreas (211.6) were reviewed. Patient, cyst, and treatment characteristics were recorded. Comparisons were made between patients who underwent operative and nonoperative management. Over the 5-year period, 209 patients were evaluated. Nonoperative treatment was initially chosen for 144 patients (69%). In this group the average cyst diameter was 2.5 cm (range 0.5 cm to 13.0 cm), and the median change in diameter during follow-up was zero cm (range 1.5 cm to 4.0 cm). In six patients (4%) changes occurred within the cyst that resulted in resection. None of these patients had a malignant diagnosis. Operative treatment was initially chosen for 65 (31%) of the 209 patients. Malignancy was found in six of the operative patients (6 [9%] of 65). Differences in patient and cyst characteristics between groups included the cyst size, septations, a solid component, and the presence of symptoms. Selected patients with cystic lesions of the pancreas may be safely followed radiographically. Selection criteria identified in this study (symptoms, cyst size, solid component, and septations) and the utilization of new imaging techniques allow the creation of treatment plans for these patients that can be prospectively tested. (J GASTROINTEST SURG 2003;7:970–977)

KEY WORDS: Pancreas, cyst, neoplasm

A "cystic lesion of the pancreas" is a radiographic finding that has a broad histologic differential. The histologic entities included within this radiographic grouping include pseudocysts, benign neoplastic cysts, premalignant neoplastic cysts, malignant neoplastic cysts, and non-neoplastic cysts.[1] Over the past two decades, a variety of laboratory, radiographic, and endoscopic tests have been evaluated for their ability to distinguish between benign and malignant cysts of the pancreas. Despite this effort, the present ability to determine nonoperatively the precise histologic identity of a cystic lesion of the pancreas is limited.[2]

Because of this inability to determine benign from malignant cysts, many have recommended that all cystic lesions of the pancreas be resected.[3–5] Given the increasing use of abdominal CT scanning and the associated increase in the number of incidental pancreatic cystic lesions, this treatment strategy is currently impractical.[6,7] Studies that have recommended this approach demonstrate that when this treatment strategy is followed, 30% to 40% of the patients exposed to the risks of pancreatic resection are those undergoing resection of a benign serous cystadenoma.[4,5] The benefits of resection for a patient with an asymptomatic serous cystadenoma are unclear.

This study was designed to identify all patients evaluated at our institution for a cystic lesion of the pancreas over a 5-year time period. Patients managed both operatively and nonoperatively were included in an attempt to better define the selection factors associated with either management strategy and to address the past recommendation that all cysts of the pancreas need to be resected.

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METHODS

Institutional physician billing data (from January 1995 to December 2000) were obtained, and all patients who were evaluated at our institution and billed for the ICD-9 diagnosis of pancreatic cyst (577.2) or benign neoplasm of the pancreas (211.6) were identified. The electronic records of these patients (n = 840) were then reviewed. All patients, who upon review, were found to have been evaluated for the radiographic diagnosis of a cystic lesion of the pancreas were included (n = 209). Patients who were given the ICD-9 diagnosis of pancreatic cyst but who were thought, after radiographic evaluation, by the attending surgeon to have a specific pathologic entity were excluded. These patients included those presumed to have a pseudocyst, cystic neuroendocrine tumor, or intraductal papillary mucinous neoplasm (IPMN). No patient thought to have adenocarcinoma with cystic degeneration was given the ICD-9 diagnosis of pancreatic cyst.

Patient-, cyst-, diagnosis-, and treatment-related variables were recorded. Diagnostic studies performed at our institution during this time period included ultrasound, CT, magnetic resonance cholangiopancreatography (MRCP), endoscopy with or without cholangiography and endoscopic ultrasound, and percutaneous or endoscopic biopsy. Cyst fluid analysis consisted only of cytologic examination. Cyst characteristics recorded included the cyst diameter, the presence of septations, the presence of calcium, and the presence of a solid component. When multiple cysts were present within the pancreas, the cyst diameter of the largest cyst was recorded. When the recorded cyst diameter was discrepant between radiographic studies, the average diameter was recorded. A cyst was considered to be symptomatic if it was identified on an imaging study performed specifically for the evaluation of upper abdominal symptoms. For patients managed nonoperatively, radiographic follow-up was not standardized; however, in general, most patients underwent serial imaging with high-quality CT or MRCP every 3 to 6 months for 2 years, and annually thereafter.

The analyses in this study were performed on an intention-to-treat basis by whether the initial decision was to operatively or nonoperatively manage the patient. The association between patient-, cyst-, and diagnosis-related variables with the selection of operative or nonoperative treatment was assessed using Fisher's exact test. Means of continuous variables were compared using a two-sample *t* test. All statistical tests were two sided, with type I error controlled at 5%.

RESULTS

Over the 5-year time period, 209 patients were evaluated at our institution for a cystic lesion of the pancreas. The average age of the patients was 65 years (range 30 to 89 years), and there were 89 males (43%) and 120 females (57%) in the study (Table 1). A current or prior history of malignancy (nonpancreatic) was present in 88 patients (42%), and 24 of these patients (11%) had documented metastatic disease at the time of cyst diagnosis. Cystic lesions were discovered incidentally in 75% of the patients. Patients with a history of malignancy were more likely to have the cystic lesion discovered incidentally (86 [98%] of 88) as compared to patients without a history of malignancy (72 [60%] of 121). The majority of patients were evaluated and managed by the surgical service (n = 186, 89%); however, 23 patients (11%) were evaluated and managed by the gastroenterology service.

The radiographic study most commonly used in the original diagnosis of the cystic lesions was computed tomography (CT), with 99% of patients undergoing a CT scan at the time of diagnosis. Magnetic

Table 1. Patient and cyst characteristics of 209 patients evaluated for cystic lesion of the pancreas

	No. of patients	%
Sex		
Male	89	43
Female	120	57
Other malignancy		
Yes	88	42
Reason for diagnosis		
Incidental	158	75
Symptomatic	51	25
Location		
Head	81	39
Body	63	30
Tail	65	31
Septated		
Yes	88	42
Calcium with cyst		
Yes	24	12
Solid component		
Yes	43	21
Management		
Operative	65	31
Nonoperative	144	69
Factor	Mean	SD
Age (yr)	65	13
Cyst diameter (cm)	3.5	3.0
Serum amylase (U/l) (n = 47)	5	7
Serum CA 19-9 (U/ml) (n = 32)	23	19

resonance imaging (MRI) or MRCP was subsequently performed in 50% of patients, and 29 patients (14%) underwent endoscopy with or without cholangiography or ultrasound. Serum amylase levels were obtained in 47 patients (22%). Amylase levels were less than 50 U/L in all patients except one. This patient was incidentally diagnosed with a 1.9 cm cystic lesion and an amylase level of 3,500 U/L, and was followed for a presumed pseudocyst. Serum CA19-9 levels were obtained in 32 patients (15%). CA19.9 levels were less than 80 U/ml in all patients except one. This single patient had a CA19.9 level of 10,200 U/ml, a 10 cm cystic lesion with septations, a solid component, and metastatic disease in the liver at the time of diagnosis. A biopsy was performed either percutaneously or endoscopically in 23 patients (11%). In two of the 23 patients who underwent biopsy, the cytologic analysis was either suspicious for or consistent with malignancy; however, evaluation of the resected specimen was consistent with serous cystadenoma (false positive, 9%).

The cyst characteristics at the time of diagnosis for all 209 patients evaluated in this study are listed in Table 1. The average initial cyst diameter was 3.5 cm (range 0.4 to 20.0 cm). Cyst location within the pancreas was evenly distributed (head 39%, body 30%, and tail 31%). Multiple cysts within the pancreas were documented in 33 patients (16%), and extrapancreatic cysts were noted in 115 patients (55%). The most common locations for extrapancreatic cysts were the kidney (n = 71) and the liver (n = 53). In this study 92 patients (44%) presented with a cyst that was less than 3 cm in diameter, asymptomatic, without a solid component, and without septations.

Initial management was operative in 65 patients (31%) and nonoperative in 144 patients (69%). The associations between patient and cyst characteristics and the selection of operative or nonoperative management are depicted in Table 2. Patient characteristics associated with the selection of operative management included a younger age and the lack of a history of malignancy. Cyst characteristics associated with the selection of operative management included location within the body or tail of the pancreas, larger diameter, septations, the presence of a solid component, and the presence of symptoms. All patients (n = 23) who presented with persistent or ongoing symptoms underwent operative resection.

For patients initially undergoing nonoperative management, the median cyst radiographic follow-up was 31 months. The median change in cyst diameter

Table 2. Patient and cyst variables, and their association with the selection of operative and nonoperative management

	Operative (n = 65)	Nonoperative (n = 144)	*P* value
Sex			
Male	28 (42%)	61 (43%)	0.98
Female	37 (58%)	83 (57%)	
Other malignancy			
Yes	15 (23%)	73 (50%)	<0.01
Reason for diagnosis			
Incidental	27 (42%)	130 (90%)	<0.01
Symptomatic	38 (58%)	14 (10%)	
Location			
Head	20 (31%)	61 (42%)	
Body	17 (25%)	46 (32%)	<0.01
Tail	28 (44%)	37 (26%)	
Septated			
Yes	48 (75%)	40 (28%)	<0.01
Calcium with cyst			
Yes	10 (16%)	14 (9%)	0.18
Solid component			
Yes	34 (53%)	8 (5%)	<0.01
Factor	Mean (SD)		
Age (yr)	61 (13)	67 (11)	0.04
Cyst diameter (cm)	5.7 (3.8)	2.4 (2.0)	<0.01
Serum amylase (U/l) (n = 47)	6 (5)	55 (328)	0.50
Serum CA 19-9 (U/ml) (n = 32)	8 (0)	23 (19)	0.53

SD = standard deviation.

during follow-up for patients being managed nonoperatively was 0 cm (range 2.2 to 4.9 cm). The observed change in cyst diameter did not correlate with cyst diameter at the time of presentation (Fig. 1). While being followed radiographically, 6 (4%) of the 144 patients initially selected for nonoperative management underwent operative exploration. The median time from initial diagnosis to resection in these six patients was 10 months (range 3 to 72 mo). In four of these six patients, operative resection was recommended because of cyst growth. None of the six patients who underwent operative exploration were found to have a malignant lesion (serous cystadenoma, n = 3; pseudocyst, n = 2; IPMN without dysplasia, n = 1). Two patients with serous cystadenomas were followed radiographically for 5 and 6 years prior to resection with an associated increase in cyst diameter of 3 and 4 cm, respectively.

The operation performed and the histopathology of the resected specimens for the 65 patients initially selected for operative management is depicted in Table 3. The most common histologic diagnosis was serous cystadenoma (n = 35; 54%). Carcinoma or carcinoma in situ was identified in six cases (9%). The median follow-up for these six patients was 27 months, and all six patients were alive without evidence of disease at the time of last follow-up. The patient and cyst characteristics of these six patients are shown in Table 4.

The average length of stay for all patients undergoing operative exploration was 10 days (range 3 to 32 days), and in 19 patients (29%) significant postoperative complications were reported. A single patient (1.5%) who had undergone pancreaticoduodenectomy for an unsuspected pseudocyst died from the postoperative complications of pancreatic leak and pulmonary embolism.

DISCUSSION

Over the past decade the increased availability and subsequent increased use of high-quality CT and ultrasound has resulted in an increased number of patients diagnosed with cystic lesions of the pancreas.[6,7] At the Massachusetts General Hospital, cystic tumors of the pancreas comprised 16% of cases in 1991 and 30% of cases in 1998.[7] As the quality of radiographic imaging improves and its use increases, lesions are being discovered more frequently and are being detected at a smaller size. In the initial description of serous and mucinous lesions of the pancreas by Compagno and Oertel[8,9] in 1978, the average cyst diameter was 10.5 cm. More recent reports, including this study, have reported average cyst diameters of 4 to 6 cm.[10,11]

Because cystic lesions of the pancreas are diagnosed radiographically, the histologic entities included under this radiographic term have also changed

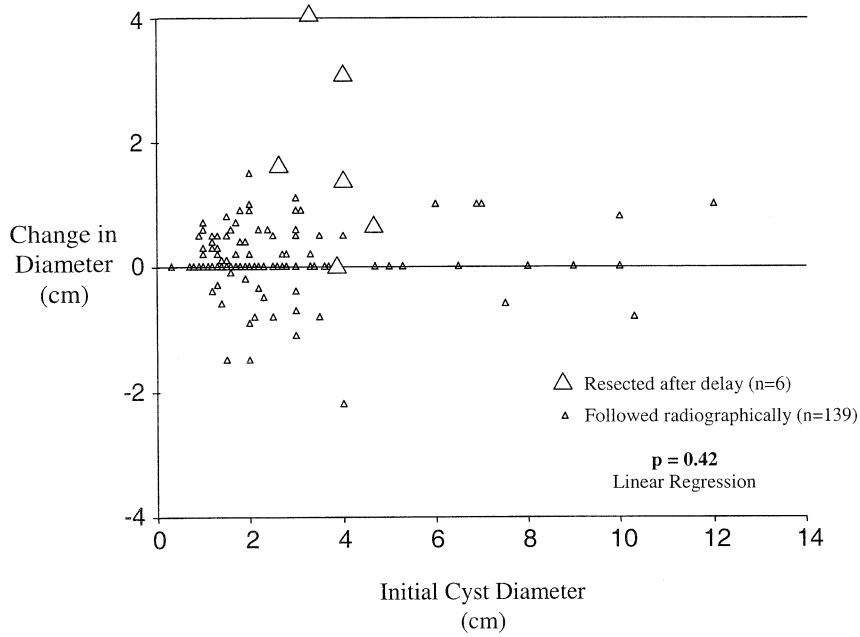

Fig. 1. Change in cyst diameter at time of last follow-up as a function of initial cyst diameter for patients initially managed nonoperatively (n = 144). No correlation was observed between initial cyst diameter and change in cyst diameter over time.

Table 3. Operation performed and pathologic results for 65 patients who underwent operative exploration*

	No. of patients	%
Operation		
Pancreaticoduodenectomy	16	24
Distal pancreatectomy ± splenectomy	34	52
Enucleation	5	8
Cystenterostomy	8	11
Unresectable, biopsy only	2	4
Histopathology		
Serous cystadenoma	35	54
Mucinous cystadenoma	8	11
Mucinous cystadenocarcinoma/ CIS	3	5
Intraductal papillary mucinous neoplasm (without dysplasia)	1	2
Intraductal papillary mucinous neoplasm with carcinoma/CIS	3	5
Neuroendocrine	1	2
True cyst	5	7
Pseudocyst	9	14

CIS = carcinoma in situ.
*Histologic diagnosis is unknown for the remaining 144 patients, as they have not undergone resection.

secondary to the improved quality of radiographic and endoscopic imaging. In the 1970s and 1980s, the quality of preoperative imaging was limited, and therefore the major preoperative concern was in distinguishing a pseudocyst from a cystic neoplasm, as the former might be internally drained and the latter resected.[12,13] Improved imaging techniques, as well as an improved understanding of the nature of these lesions, has made possible the preoperative distinction between pseudocyst and cystic neoplasm in the vast majority of cases.[3] In the 1990s, the focus of investigation has been in trying to preoperatively distinguish between the histologic variants of cystic neoplasms.[7,14] Currently, more than 90% of patients undergoing resection of a cystic neoplasm of the pancreas will have the histologic diagnosis of a serous cystadenoma, mucinous cystadenoma, mucinous cystadenocarcinoma, or intraductal papillary mucinous tumor.[10,15]

Because of their malignant or premalignant nature, most have recommended surgical resection for all cystic neoplasms except the serous cystadenoma.[14,15] Most consider a serous cystadenoma a benign entity, and therefore do not uniformly recommend surgical resection unless it is of such a size that it becomes symptomatic.[8,14] The principal difficulty in managing patients with cystic lesions of the pancreas is that there are currently no preoperative laboratory, radiographic, or endoscopic studies that can consistently predict histologic diagnosis. Therefore, to eliminate the risk of following a patient with a malignancy, many have recommended resection for all patients with cystic lesions of the pancreas.[3–5,16]

Because of the increasing incidence of smaller, asymptomatic cystic lesions of the pancreas, we have followed a more selective approach than operative resection in these patients. The current study was designed to identify, and report, on all patients evaluated at our institution with a cystic lesion of the pancreas, regardless of whether or not they ever underwent resection. In this study two thirds of the patients evaluated with a cystic lesion were managed nonoperatively, and with a median follow-up of 2.5 years the median change in cyst diameter was zero. Only 6 (4%) of the 144 patients selected for nonoperative management developed changes within the cyst that resulted in resection, and none of these patients had a malignant lesion. Within the one third of patients who were initially managed operatively, 54%

Table 4. Cyst characteristics of six patients who underwent resection and were found to have a malignant histology

Patient	Histopathology	Symptoms	Diameter (cm)	Septated	Solid component	Follow-up (mo)	Status
1	Mucinous cystadenoma with CIS	Yes	10.0	Yes	Yes	24	NED
2	Mucinous cystadenocarcinoma	Yes	7.0	Yes	Yes	36	NED
3	Mucinous cystadenocarcinoma	Yes	5.0	Yes	Yes	84	NED
4	IPMN with CIS	Yes	6.0	Yes	No	15	NED
5	IPMN with adenocarcinoma	Yes	7.5	Yes	Yes	18	NED
6	IPMN with adenocarcinoma	Yes	4.0	Yes	Yes*	30	NED

CIS = carcinoma in situ; IPMN = intraductal papillary mucinous neoplasm; NED = no evidence of disease.
*Lesion almost completely solid on radiographic imaging.

were found to have a serous cystadenoma and 9% were found to have a malignant lesion. None of these patients have had a recurrence or died of disease with a median follow-up of 30 months. Significant complications were reported in 29% of patients who underwent resection, and a single patient (1.5%) died from complications after resection of an unsuspected pseudocyst.

The willingness to nonoperatively manage patients with cystic lesions of the pancreas at our institution may be higher than at other institutions for several reasons. First, the patient population served at our institution is that of a cancer center. A current or past diagnosis of extrapancreatic malignancy was present in 42% of patients, with 24 of the 209 patients having documented metastatic disease at the time of cyst diagnosis. The presence of malignancy, as would be expected, was associated with the selection of nonoperative management, and this factor may not be as prevalent in a general hospital practice. The majority of patients in this study (75%) had lesions that were detected incidentally. Most other large studies of cystic lesions report an incidental detection rate of 30% to 50%.[4,10] The higher prevalence of incidental lesions in our study may be a result of increased radiographic imaging in patients with malignancy. Incidental detection of the cystic lesion was also associated with the decision to manage the patient nonoperatively.

Despite these limitations, similar results have also been reported from other studies where carefully selected patients with cystic lesions of the pancreas have been managed nonoperatively.[2,10] In a recent multi-institutional study from the French Surgical Association of 398 patients with cystic lesions of the pancreas, 26 patients were followed radiographically.[10] These patients were reported to have been followed because they presented with lesions that had "typical morphologic features" of a serous cystadenoma. These typical features are not described; however, with an average follow-up of 38 months, none of the patients developed cyst changes that warranted resection. In another recent prospective study from the Cleveland Clinic, 53 (61%) of 87 patients evaluated with a cystic lesion were managed nonoperatively. None of these 53 patients developed symptoms, or an increase in size, that resulted in operative resection.

Because of the current inability to reliably obtain a tissue diagnosis prior to operative resection, the recommendations regarding management must be based on laboratory, radiographic, and endoscopic findings. Laboratory and endoscopic evaluations were used infrequently in this study. Serum amylase and CA19.9 levels were performed in less than half of the patients and were almost uniformly normal; only one patient who had metastatic disease had an elevated CA19.9 of 10,200 U/ml. Endoscopy was performed in 14% of the patients and was generally used as a means for obtaining additional cyst information in patients with cysts that were of intermediate size. Cyst fluid analysis was not performed, and a biopsy was obtained in 11% of patients. Biopsy results were falsely positive for malignancy in two patients with serous cystadenomas. A biopsy was also performed on two patients with malignancy with the result being read as suspicious in one and inconclusive in the other. Given these results, we currently do not recommend biopsy of these lesions as the information is not reliable.

A variety of radiographic imaging studies have also been used in an attempt to delineate the histologic variants of pancreatic cysts. These studies have included endoscopic ultrasound, CT, MRI/MRCP, and most recently positron emission tomographic (PET) imaging. A study published by Sperti et al.[17] of 56 patients with cystic lesions of the pancreas reported a sensitivity of 94% and a specificity of 97% for detecting malignant cystic lesions. PET imaging, however, was unable to differentiate between histologic variants (mucinous vs. serous) of benign cysts. PET was not used as a diagnostic modality for patients in this study, but its use is currently being investigated.

The primary modality used for treatment recommendations in this study was radiographic imaging with CT and or MRI/MRCP. Cyst factors that were identified by these studies and associated with the selection of operative or nonoperative management included cyst location, size, and the presence of a solid component or septations. The average cyst diameter in patients managed nonoperatively was 2.4 cm. On the contrary, the average cyst diameter in patients who underwent resection was 5.7 cm, with an average diameter of 6.5 cm in patients with a malignant cyst. All six patients with a malignant cyst had symptoms and septations within the cyst, and all but one had a solid component. In addition, cyst location within the body or tail of the gland was associated with the choice of operative management. This finding is likely related to the decreased morbidity associated with a distal pancreatic resection as compared to a pancreaticoduodenectomy.

The natural history of unresected cystic neoplasms is unknown because the general approach to cystic neoplasms of the pancreas has been to perform operative resection. Since the time of the initial distinction of mucinous and serous lesions by Compagno and Oertel[8,9] in 1978, it has been assumed that nearly all

mucinous lesions, if left in situ, will undergo malignant transformation. This concept of transformation from cystadenoma to cystadenocarcinoma has been fostered because of two findings: malignant mucinous lesions will often have benign columnar epithelium directly adjacent to malignant epithelium, and there have been reported cases of cystadenomas diagnosed by biopsy recurring as cystadenocarcinomas several years after internal drainage.[9,12,18]

Even if the adenoma to carcinoma transformation occurs, present evidence would suggest that this process is one that takes many years and is associated with growth and other radiographic changes within the cyst. In Compagno and Oertel's studies[8,9] from 1978, several patients were reported who had been followed with a palpable abdominal mass for up to 15 years prior to resection. In this study, cyst growth warranting resection did occur in 4% of patients, none of whom had a malignant diagnosis. Two patients with serous cyst adenomas were followed for 5 and 6 years prior to resection with associated cyst growth of 3 and 4 cm, respectively. Other studies, also with limited follow-up, have not found significant changes in the cyst characteristics of selected patients being followed radiographically.[2,10]

CONCLUSION

As the use of high-quality radiographic imaging has increased, there has been an increase in the diagnosis of small incidental cystic lesions of the pancreas. The natural history of these lesions, and thus their significance to the patient, is unknown. The data presented in this study suggest that patients who present with small (<3 cm) asymptomatic cysts of the pancreas without a solid component may be safely followed radiographically. This group of patients represented nearly half of the patients in this study. Careful radiographic follow-up is necessary, as resection should be performed if changes occur within the cyst or symptoms develop. Continued efforts to distinguish patients with serous cystadenomas are needed to minimize the number of patients with this benign entity who are exposed to the risks of resection.

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Discussion

Dr. W. Traverso (Seattle, WA): I looked at your paper as an opportunity to provide guidelines to exclude patients from immediate operative intervention. The best item would be whether the lesion was most likely malignant or premalignant. I was also struck by the low incidence of IPMNs in your series. The majority of patients with pancreatic cystic neoplasm referred to me are those with IPMNs. These are all premalignant and they all should be resected. The best way to determine

if a lesion is an IPMN is with ERCP. If there is a connection to the main pancreatic ductal system with a cystic structure, then the lesion is an IPMN. Have you considered that in your analysis?

Dr. P. Allen: This study was comprised of patients who were at any time labeled with the ICD-9 code for pancreatic cyst. When we actually looked at our operative database over those same 5 years, certainly there were a greater number of patients in the operative database with IPMN who were resected, but indeed if you look at how those patients were coded in the clinic, they were not coded as having a cystic lesion. This made us realize that preoperatively we are able to frequently determine patients who have an IPMN, specifically by the characteristics of endoscopic and radiographic findings.

So, indeed, we are seeing more IPMNs than are reported in this study; however, very few of those patients were given the ICD-9 diagnosis of pancreatic cyst preoperatively. Often these patients were given the 157 ICD-9 code, which is pancreatic malignancy, islet cell vs. ductal by anatomic location in the body.

Dr. C. Fernandez-del Castillo (Boston, MA): I echo Dr. Traverso's concern about the very low proportion of intraductal and mucinous tumors in this series. I think that by selecting with the diagnosis code you end up with a universe of patients that by definition are going to be benign. This doesn't give the whole picture. The fact is that a lot of IPMNs and mucinous cystic neoplasms will present as small cystic lesions that can often be incidental. What made you operate on those versus the others is not clear. So the message to the community that we can safely watch these lesions may be erroneous.

In our experience, 37% of pancreatic cysts are incidental. If we look at these, even the ones that are less than 2 cm, 60% of them will be either intraductal papillary mucinous tumors or mucinous cystic neoplasms. Even though these may not be cancerous at the time that you are seeing them, they are, as of now, considered precancerous lesions, and therefore it may not be prudent to say it is safe to observe these patients. Endoscopic ultrasound has an important role in the evaluation of these patients.

Dr. Allen: With regard to the incidental findings, being that 75% of the patients in this study had an incidental lesion found with 92 patients having very small lesions discovered, I think that is a reflection of our institution being a cancer hospital. These patients are undergoing CT scans for screening for metastatic disease and they are discovered to have these small lesions. I think the applicability of our results to other institutions differs in this sense. So our incidence of asymptomatic lesions is much higher.

Certainly among the 144 patients we are following radiographically there are patients with small mucinous lesions. I do not think we know right now what the natural history is of 1 to 2 cm mucinous lesions. The tidbits of clinical information from the reports out of the AFIP, which was in an era of no CT scans, suggest that the progression of mucinous lesions may well be very, very prolonged. The reports from the Armed Forces Institute of Pathology actually mention patients who had been followed for up to 15 years with a palpable abdominal mass who then underwent resection. We are very interested in following these patients, and following them very carefully, and we are certainly setting up prospective studies to evaluate them in a uniform manner.

Dr. Fernandez-del Castillo: There may be a role for endoscopic ultrasound in the evaluation of these patients.

Dr. Allen: Certainly endoscopic ultrasound is being used more frequently.

Dr. R. Prinz (Chicago, IL): What I heard was that the distribution of these lesions was equal throughout all three parts of the pancreas, yet more than half of the lesions you operated on were in the tail. Do you think there is a bias in recommending removal of these lesions depending on their location and on the magnitude of the operation?

Also, smaller lesions in the tail can be removed laparoscopically. Do you have any experience with laparoscopic distal pancreatectomy for these?

Dr. Allen: To answer your first question, this is a retrospective study so it is difficult to draw firm conclusions; however, we feel there probably is the temptation to take out a smaller lesion in the tail of the pancreas in a young patient rather than do a Whipple procedure.

As to the second question, we are approaching selected patients with laparoscopic resection and are interested in further defining the role of that procedure.

Dr. M. Arregui (Indianapolis, IN): I enjoyed your presentation, and it makes it a little easier for us to follow some of these lesions. You did not mention very much about fluid analysis, carcinoembryonic antigen amylase, and mucin stains, which tend to be helpful in selecting out the lesions most likely to be benign, the serous cystadenomas, which I am inclined to watch if they are asymptomatic. Are you using this type of fluid analysis?

Also, you had a large proportion of serous cystadenomas that were resected. Were they resected because you were not sure what they were, or were they resected because they were symptomatic?

Dr. Allen: Of the patients with serous cystadenomas, a minority were resected because they were symptomatic. In terms of fluid analysis, the literature is certainly full of reports about analysis of cyst fluid and how the results may be useful. During the study time period, we were not doing cyst fluid analysis. We are interested in determining if this may be helpful, particularly in those patients who fall into the group we feel is this mid-range of size.

Dr. H. Pitt (Milwaukee, WI): I am a little confused about how you chose these patients. You said that you did it from your billing records, but then you said that many were found incidentally on CAT scans. Are you able to bill for incidental findings on CAT scans? We have reviewed all the CAT scans over a 7-year period and found that 1% to 1.5% of all of our patients have a cystic lesion in the pancreas but about half of them are pancreatitis related. So, my question is, how did you identify these patients if some of them were just incidental findings on CAT scans? Did you miss some patients who had CAT scans but were not billed for the incidental finding?

Dr. Allen: This is certainly an underestimate. These were all patients who were evaluated and billed at the time of evaluation either within the department of surgery or by gastroenterology for a cystic lesion of the pancreas. This is certainly an underestimation of what the true population is. But I think what we wanted to get a handle on was to try to somehow search for the patients who were not just being seen and then taken to the operating room.

Total Pancreatectomy and Autologous Islet Cell Transplantation as a Means to Treat Severe Chronic Pancreatitis

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Autologous islet cell transplantation after near-total or total pancreatic resection can alleviate pain in patients with severe chronic pancreatitis and preserve endocrine function. From February 2000 to February 2003, a total of 22 patients, whose median age was 38 years, underwent pancreatectomy and autologous islet cell transplantation. Postoperative complications, metabolic studies, insulin usage, pain scores, and quality of life were recorded for all of these patients. The average number of islet cells harvested was 245,457 (range 20,850 to 607,466). Operative data revealed a mean estimated blood loss of 635 ml, an average operative time of 9 hours, and a mean length of hospital stay of 15 days. Sixty-eight percent of the patients had either a minor or major complication. Major complications included acute respiratory distress syndrome (n = 2), intra-abdominal abscess (n = 1), and pulmonary embolism (n = 1). There were no deaths in our series. All patients demonstrated C-peptide and insulin production indicating graft function. Forty-one percent are insulin independent, and 27% required minimal amount of insulin or a sliding scale. All patients had preoperative pain and had been taking opioid analgesics; 82% no longer required analgesics postoperatively. Pancreatectomy with autologous islet cell transplantation can alleviate pain for patients with chronic pancreatitis and preserve endocrine function. (J GASTROINTEST SURG 2003;7:978–989)

KEY WORDS: Pancreatectomy, autologous islet transplantation, quality of life, chronic pancreatitis

Chronic pancreatitis is a disease that progressively destroys pancreatic exocrine tissue, causes pain syndromes that frequently require hospitalization, and can severely compromise quality of life. Medical management consisting of analgesics and pancreatic enzyme replacement rarely leads to acceptable relief of pain and can often act as a precursor to the abuse of narcotics.[1] Patients who suffer from chronic pancreatitis are at increased risk for pancreatic adenocarcinoma relative to the general population.[2,3]

At least 50% of patients who suffer from chronic pancreatitis will ultimately require some form of surgical intervention[4–6] secondary to persistent refractory pain and/or complications of the disease.[7,8] The selection of specific surgical procedures to treat chronic pancreatitis are generally based on the presenting characteristics of each patient, including baseline endocrine and exocrine function, pancreatic and ductal pathology, and intensity of pain.[9] Among patients undergoing surgery, 30% to 50% will develop recurrent symptoms or complications related to pancreatic disease, despite initially successful surgical intervention. Additional operative intervention for resection or drainage may then be indicated.[10–18] Another subset of patients who initially present with small duct disease are not thought to be suitable candidates

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for partial pancreatectomy or duct decompression procedures.

In these patients for whom both medical and standard surgical management is deemed inappropriate or has failed to provide relief, total pancreatecomy may alleviate symptoms of debilitating pain.[19] Although morbidity and mortality rates associated with this procedure have decreased markedly during the past 25 years,[20] most clinicians do not consider total pancreatectomy to be a therapeutic option because patients often develop "brittle" diabetes, which is extremely difficult to manage. Autologous islet cell transplantation after near-total or total pancreas resection, however, may offer a means to preserve endocrine function while alleviating the debilitating pain associated with severe chronic pancreatitis.[21,22] It is estimated that roughly 30% to 50% of patients who forgo surgery for chronic pancreatitis will become diabetic nonetheless.[5,23,24]

Autologous islet cell autotransplantation was first described 30 years ago as a means of preventing diabetes after total pancreatectomy,[25] with various sites of implantation attempted in animal and human models.[25–34] The feasibility of autologous islet cell transplantation after pancreatectomy was first demonstrated in 1977 in a 39-year-old female patient who suffered from familial pancreatitis. After the procedure, she remained insulin independent and pain free for the remainder of her life (6 years).[35] Since that time the body of literature on the subject has remained comparatively limited with only 15 centers worldwide reporting on their experiences to the International Islet Transplant Registry.[36] According to 2001 data, 140 islet cell autotransplants after pancreatectomy were performed between 1990 and December 31, 2000. Of these, 93 cases were performed at four institutions. The current approach involves infusion of islet cells isolated into the portal vein following pancreatectomy and distribution throughout the liver. In the best-case scenario, patients no longer need insulin and have normal glucose tolerance,[25,37] although some patients with less optimal outcomes require exogenous insulin to treat hyperglycemia.[31,32,35,38]

In this report we describe 22 consecutive cases of near-total to total pancreatectomy with simultaneous islet cell autotransplantation for patients suffering from chronic pancreatitis in which other treatment options were no longer considered feasible.

MATERIAL AND METHODS

Patients

From February 2000 to February 2003, a total of 22 patients (15 females and 7 males) were referred to the Pancreatic Disease Center (PDC) at the University of Cincinnati for treatment of chronic pancreatitis and subsequently underwent total, completion, or near-total pancreatectomy with immediate islet cell autotransplantation.

The diagnosis of chronic pancreatitis was based on the patient's history, results of laboratory tests, computed tomography (CT) scans, endoscopic retrograde cholangiopancreatography (ERCP), and in some patients, pathologic confirmation. Intractable pain was a symptom that was shared by all patients, and all of them used narcotics on a chronic basis for analgesia. All operations were performed under the guidance of one of three attending surgeons. All patients signed informed consent forms that had been approved by the institutional review board of the University of Cincinnati for the surgical procedure, autologous islet cell transplantation, and in most cases pre- and postoperative metabolic testing. All of the patients underwent CT scanning and chest roentography. For patients requiring splenectomy, vaccinations for *H. influenza* and *Pneumoccocus* was given.

The PDC database was reviewed for standard demographic data. The hospital course of each patient was reviewed to identify the indications for surgery, pathologic findings, perioperative complications, and length of hospital stay. Operative data recorded included estimated blood loss, which was determined jointly by the attending surgeon and staff anesthesiologist, and the length of the surgical procedures.

Operative Procedures

The technique used for near-total pancreatectomy involved removing the entire pancreas except for a small rim (<5%) along the duodenal C-loop that was left intact along with the common bile duct and pancreaticoduodenal arteries. The technique of total pancreatectomy involved removing the entire pancreas along with the spleen, duodenum, and distal common bile duct. Preservation of the pylorus was at the discretion of the attending surgeon. Gastrointestinal reconstruction involved either a side-to-side two-layer gastrojejunostomy or an end-to-side duodenojejunostomy. Bile duct continuity was usually restored by an end-to-side hepaticojejunostomy just proximal to the gastrojejunostomy.

During the operative procedure, the blood supply to the pancreas was preserved for as long as possible during the mobilization and resection process to minimize warm ischemia to the islet cells. Typically the distal portion of the pancreas was mobilized initially and divided, along with the splenic artery and vein, at the level of the superior mesenteric vein; this portion was then preserved and processed for islet cell harvest,

while the remainder of the pancreas was mobilized and resected.

An intravenous insulin drip was started immediately after the pancreatic resection to maintain blood glucose levels less than 120 mg/dl. This was done to prevent acute metabolic decompensation and to provide a more favorable environment for the return of the islet cells, inasmuch as the detrimental effects of hyperglycemia on islet cell engraftment have been demonstrated in animal studies.[39,40] Finally, gastrostomy and jejunostomy tubes were placed at the discretion of the attending surgeon.

Islet Cell Preparation

A separate back table was prepared in the operating room to handle the resected pancreas. The excised gland was placed in a bowl containing University of Wisconsin (UW) solution iced to 4° C to better preserve it and to minimize cold ischemic damage to the pancreas. While still submerged in cold UW solution, the pancreas was detached from the duodenum (if present), spleen, and any excess retroperitoneal fat. If patent, the pancreatic duct was then cannulated with an appropriate-gauge angiocatheter. The dissected gland was transported to the laboratory for further processing and distended with a solution containing Liberase (Roche Molecular Biochemicals, Indianapolis, IN).

Islet cells were liberated from the remaining exocrine tissue through the use of continuous cold enzymatic perfusion and digestion as previously described.[41] Briefly, pancreatic tissue was mechanically and enzymatically dissociated in a digestion chamber in the presence of a recirculating solution containing collagenase. The solution was recirculated using a roller pump, and the temperature of the fluid was maintained as close to 38° C as possible to sustain optimum digestion. When digestion was completed (islet cells were adequately liberated from the remaining exocrine tissue), the flow was rerouted to a separate collecting flask where the majority of enzymatic reactions were arrested by both diluting the islet-containing solution and lowering its temperature to 7° to 10° C.

Islet Cell Transplantation

While the islet cells were being harvested, the operating surgeons completed the pancreatic reconstruction. Approximately 3 hours after pancreatectomy, the recovered islet cells were transplanted into the liver. Islet cell infusion was performed in one of two ways: either through a middle colic venous tributary or directly into the portal vein. All of the patients received 5000 IU intravenous heparin immediately preceding infusion of pancreatic islet cells. Portal venous pressure was selectively measured, depending on the volume of digested pancreatic tissue and islet cells. Arterial and central venous pressures were monitored in all patients.

Metabolic Studies

Preoperatively, patients were referred to the Clinical Research Center at the Cincinnati Children's Medical Center for metabolic testing. After an overnight fast, intravenous catheters were placed into veins in both forearms. Following removal of fasting blood samples, subjects received 5 g of arginine as a bolus infusion over 60 seconds, and serial blood samples were obtained over a 15-minute period. After a 30-minute rest period to allow islet hormones to return to steady-state baseline, blood samples were again drawn, 75 g of glucose solution was then ingested over 5 minutes and blood samples were taken over 3.5 hours. Plasma was removed and stored at −30° C. Metabolic testing was repeated 3 to 4 months postoperatively. Plasma glucose level was measured using a glucose oxidase method, and insulin and C-peptide concentrations were determined using radioimmunoassay. Pre- and postoperative values for each subject were determined in the same assay.

The acute insulin/C-peptide response to arginine was computed as the area under the hormone response curve above fasting. The glycemia and insulin responses to the oral glucose load were calculated as the area under the glucose and insulin response curves over the period of sampling.

Post-Transplant Care

Postoperative care was undertaken in the surgical intensive care unit for the first 24 hours, with hourly glucose monitoring to ensure blood glucose levels between 100 and 120. Patients were given intermittent injections of insulin as required for glycemic control. Postoperative pain was controlled with either an epidural analgesic catheter or a patient-controlled analgesic pump. An oral diet was reinstituted when gastrointestinal motility returned.

Pain Assessment and Quality of Life

Follow-up data regarding narcotic usage, insulin regimens, pain assessment, and quality of life were collected during postoperative clinic appointments; information was obtained via a written questionnaire and by direct follow-up phone contact. The SF-36 Health Survey (SF-36)[42] was administered to assess quality of life. The McGill Pain Questionnaire

(MPQ)[43] was administered to evaluate pain. Because patients used a variety of analgesics for management of pain, for the sake of analysis and comparison morphine equivalent doses were obtained using the Pharmacokinetics of Narcotic Agonist Analgesics table as listed in *Drug Facts and Comparisons 2003* and the Narcotic Agonists Comparative Pharmacokinetics table listed in the *Drug Information Handbook 2002–2003*. *t*-tests were used to analyze pre- and postoperative information. These studies were approved by the institutional review board of the University of Cincinnati Medical Center, and written consent was obtained from all patients included in the study. The questionnaire included five general questions about the location and intensity of the pain, other analgesics or pancreatic enzymes being taken, and employment. In addition, patients were asked to answer 10 questions comparing pre- and postoperative levels in health, mood, activities, and pain (SF-36). Each response was graded as follows: −10 = increased/worsened pain level; 0 = no change; and +10 decreased/improved pain level.

The primary end points for the study included improvement in patients' quality of life and pain after pancreatectomy and autoislet infusion. A secondary outcome included decrease in the amount of analgesics and pancreatic enzymes used by the patients after surgery. Employment before and after surgery was also included as an outcome measurement.

Statistical Analysis

Distribution of quantitative measures was inspected graphically to verify parametric assumptions (e.g., normality) and results are summarized as the arithmetic mean, standard deviation, and range unless otherwise noted. Patients were classified dichotomously according to whether postoperative insulin independence was achieved. Potential associations of categorical patient attributes (e.g., sex) with this binary outcome were evaluated by means of Fisher's exact test. Differences with respect to quantitative measurements between patients who achieved insulin independence and those who did not were tested with *t*-tests. Associations between quantitative measures were graphically inspected for possible nonlinearities using scatter plot displays. Unless otherwise noted, the magnitudes of linear associations are summarized by the square of the Pearson correlation coefficient (R), which is interpreted as the proportion of the variation in one variable that can be predicted from the other variable (i.e., $R^2 = 1.0$ implies perfect prediction).

RESULTS

Patients

From February 2000 to February 2003, a total of 22 patients underwent partial or total pancreatic resection with immediate autoislet transplantation. Fifteen of these patients were females with a median age of 40 years (range 16 to 62 years), and seven were males with a median age of 36 years (range 22 to 53 years). Eighteen patients (82%) had idiopathic pancreatitis. Of these 18 patients, 32% (n = 7) were found to have pancreas divisum, but this could not be definitively linked as a cause of pancreatitis. Idiopathic pancreatitis was a diagnosis of exclusion after all other causes were rejected. Other causes of pancreatitis in our series included alcohol-induced pancreatitis in two patients, post-ERCP pancreatitis in one patient, and trauma-induced pancreatitis in one patient. Indications for surgery included pain that was refractory to high-dose opiod analgesics (all 22 patients) and recurrent acute pancreatitis (3 of 22 patients), with documented amylase and/or lipase elevations.

During this period 12 patients underwent total pancreatectomy as their initial procedure, seven patients underwent completion pancreatectomy, and three patients underwent partial pancreatectomy. Partial pancreatectomy procedures included subtotal pancreatectomy (n = 1) and pancreaticoduodenectomy (n = 2). Of the patients undergoing completion pancreatectomy, four had undergone a previous subtotal pancreatectomy and three had undergone a previous Whipple procedure. In addition, of the patients undergoing completion pancreatectomy, two had also undergone a lateral pancreaticojejunostomy. Of the 12 patients undergoing total pancreatectomy as their initial procedure, two had undergone a lateral pancreaticojejunostomy (Table 1). All patients with an intact sphincter of Oddi at the time of their islet cell transplantation (n = 17) had undergone an ERCP, and of these patients seven also had prior sphincterotomy and stent placement.

Perioperative Results

Analysis of operative data demonstrated a mean estimated blood loss of 635 ml (range 50 to 2200 ml). The average operative time was 9 hours (range 5 to 12 hours). The average length of hospital stay for these patients was 15.2 days, with a range of 5 to 40 days.

Sixty-eight percent of patients had postoperative complications. Major complications included pulmonary embolism (n = 1), acute respiratory distress syndrome (n = 2), and intra-abdominal abscess (n = 1). The patient who suffered a pulmonary embolism was

Table 1. Demographics, operative indications, and operative procedures

	Average	Number
Age	38 yr	Range 16–62 yr
Sex		
Female	68%	15
Male	32%	7
Operative indications		
Refractory pain	100%	22/22
Recurrent chronic pancreatitis	14%	3/22
Suspected etiology		
Idiopathic	82%	18
Alcohol	9%	2
Post-ERCP	4.5%	1
Trauma	4.5%	1
Total	100%	22
Operative intervention	Previous operation	
Partial pancreatectomy (N = 3)	Subtotal pancreatectomy	1
	Pancreaticoduodenectomy	2
Completion pancreatectomy (N = 7)	Subtotal pancreatectomy	4
	Whipple operation	3
	Lateral pancreaticojejunostomy	2
Total pancreatectomy (N = 2)	Lateral pancreaticojejunostomy	2
Other operative interventions prior to pancreatectomy		
ERCP		17
Sphincterotomy/stent placement		7

treated with anticoagulants and recovered with no sequelae. The two patients with acute respiratory distress syndrome were treated with ventilatory and standard intensive care unit support, and both recovered. Minor complications included delayed gastric emptying (n = 6), urinary tract infection (n = 2), and one case each of pneumonia, hyperglycemia, wound infection, intra-abdominal hematoma, cellulitis, and line infection. Seven patients were discharged without any complications. There were no deaths in this series (Table 2).

Islet Cell Preparation and Transplantation and Metabolic Studies

The mean number of islet cell equivalents (IE) harvested was 245,457 (range 20,850 to 607,466 ± 175,234). The mean total number of islet cells isolated was 350,428 (range 31,500 to 1,164,000 ± 299,321). Following pancreatecomy and autologous islet cell transplantation, 41% of the patients (9 of 22) were insulin independent. These patients received an average of 4611 IE/kg (range 287 to 10,419). Of those patients who needed insulin at the time of discharge, 27% (6 of 22) required less than 10 units of NPH insulin per day (range 3 to 9 units/day). In this group of patients, 2802 IE/kg (range 218 to 5827) were transplanted. Finally, 32% (7 of 22) required an average of 25 units of insulin per day (range 15 to 40 units/day). These patients were transplanted with 2326 IE/kg (range 611 to 4593) (Table 3). Three of these seven patients were being treated for diabetes prior to their pancreatectomy and islet cell transplantation procedures.

Table 2. Major and minor complications

Complications	No. of Patients
Major	
Acute respiratory distress syndrome	2
Intra-abdominal abscess	1
Pulmonary embolism	1
Minor	
Delayed gastric emptying	6
Urinary tract infection	2
Pneumonia	1
Wound infection	1
Line infection	1
Hyperglycemia	1
Hematoma	1
Cellulitis	1

Table 3. Comparison of patients who are insulin independent vs. patients who are non-insulin independent

	Patients (N = 22)			
	Insulin independent		Non-insulin independent	
Etiology of pancreatitis				
Alcohol	1		1	
Idiopathic	4		7	
Idiopathic (pancreas divisum)	4		3	
ERCP	0		1	
Trauma induced	0		1	
Total	9		13	
Islet isolation numbers	Mean	Standard deviation	Mean	Standard deviation
Islet number*	475,126	(322,569)	264,098	(260,164)
Islet equivalents†	302,178	(185,459)	206,187	(163,475)
Islet equivalents per patient body weight transplanted‡	4,611	(3,180)	2,583	(1,859)

P values for *t* tests of mean differences:
* < 0.1242
† < 0.23
‡ < 0.11

None of the patients required hospitalization for acute metabolic consequences of diabetes after their surgery.

Five patients have completed both pre- and postoperative metabolic testing. Of these subjects, two were insulin independent postoperatively, whereas three required small amounts of long-acting insulin for glycemic control (average 12.7 units/day; range 6 to 22 units/day). On average, these subjects had normal glucose tolerance preoperatively (Fig. 1, *A*). Following pancreatectomy and islet autotransplantation, the glycemic response after glucose ingestion increased in these subjects, although one patient continued to have normal glucose tolerance. The C-peptide response to a bolus of intravenous arginine after surgery was decreased to approximately 38% ± 13% of the preoperative response, whereas C-peptide secretion after oral glucose was decreased to 59% ± 22% of the prepancreatectomy level (Fig. 1, *B*). All five subjects, including those requiring exogenous insulin, had demonstrable insulin and C-peptide secretion in response to intravenous and oral beta cell stimuli.

Pain Assessment

Although all patients were evaluated for quality of life by means of a modified version of the SF-36 and McGill questionnaires, at the present time complete data are available for 11 patients. Of these patients, seven were females and four were males. All identified unremitting abdominal pain as their primary presenting symptom (Table 4). Patients were interviewed at a median follow-up 19 months (range 3 to 41 months) after their procedures. The vast majority of patients reported a significant decrease in frequency, duration, and intensity of pain, as well as a significant increase in quality of life since their procedures (Table 5).

Eighteen (82%) of the 22 patients who had resections are completely off narcotics. These patients required 78.4 (± 105.9) morphine equivalents (range 9 to 405) of pain medications preoperatively and only

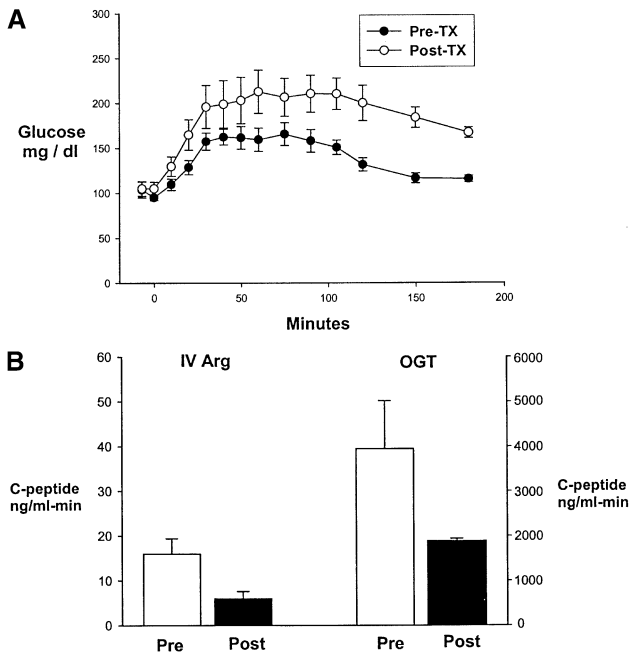

Fig. 1. Glucose tolerance test (**A**) and C-peptide secretion (**B**) in response to a bolus of intravenous arginine and oral glucose tolerance test before and after islet cell autotransplant in five patients undergoing pancreatectomy for unremitting pain from chronic pancreatitis.

Table 4. Demographics, presenting signs and symptoms, and medical history of patients who completed Pain Assessment and Quality of Life Questionnaires

	Study group (N = 11)	
	Number	Percentage
Age (median)	39.8 yr	
Sex		
Female	7	63.6%
Male	4	36.4%
Race/Ethnicity		
White	11	100%
Black	0	—
Other	0	—
Presenting signs and symptoms		
Abdominal pain	11	100%
Weight loss	2	18.2%
Nausea/vomiting	8	72.7%
Jaundice	0	—
Diarrhea	5	45.5%
Steatorrhea	1	9%
Fever/chills	0	—
Gastrointestinal bleeding	0	—
Past medical history		
Smoking	2	18.2%
Narcotic dependence	4	36.4%
Alcohol abuse	1	9%
Peptic ulcer disease	0	—
Hypertension	2	18.2%
Diabetes	2	18.2%
Myocardial infarction	0	—
Chronic obstructive pulmonary disease	0	—
Peripheral vascular disease	0	—
Presumed etiology of chronic pancreatitis		
Alcohol/drug induced	2	18.2%
Idiopathic	7	63.7%
Idiopathic (pancreas divisum)	2	18.2%
Trauma	0	—
Type of operation		
Total pancreatectomy	10	90.9%
Partial pancreatectomy	1	9%

9.5 ($\pm$ 24) morphine equivalents (range 0 to 90) postoperatively. Three patients (13.6%) showed a 34% decrease in their narcotic requirement. Only one patient demonstrated an increase from 15 to 90 morphine equivalents in the narcotic requirement; this patient had a history of polysubstance abuse, which was not known preoperatively (Table 6).

DISCUSSION

Management of chronic pancreatitis presents a challenge to physicians.[1,5,9,23,44,45] Although there remains no broad consensus as to the ideal treatment for chronic pancreatitis, there is general agreement that for a subset of patients, primarily those who suffer from small duct pancreatitis, and those whose previous surgical procedures, medical management and more conservative surgical intervention did not produce desirable outcomes. In the past, near-total or total pancreatecomy has been avoided as a treatment option for these patients primarily because of concerns regarding increased morbidity associated with the surgery and fear of brittle diabetes. More recently, however, it has been shown that total pancreatectomy can be accomplished with acceptable morbidity and mortality.[20] One of the most relevant factors that precludes pancreatic resection as a desirable therapeutic option is the potential for surgically induced diabetes, which poses a very real threat of leaving patients with the unenviable choice of intractable pain vs. extremely difficult to manage diabetes. Immediate autotransplantation of islet cells after near-total to total pancreatecomy offers both the potential pain relief as well as a means of preserving endocrine function.

In this series the primary objective was to provide pain relief for patients followed by the secondary but equally important objective of preserving endocrine function.[21,22,25,27,31,33,35,38,46] All of these patients presented with the primary symptom of refractory and unremitting pain, and three patients also had recurrent acute or chronic pancreatitis. The vast majority enjoyed dramatic and almost immediate relief of abdominal pain after their procedures, with 82% (n = 18) no longer requiring narcotics and 14% (n = 3) experiencing a marked decrease in the need for analgesics. Although the results are promising, this study represents a relatively short follow-up and cannot be used to draw definitive conclusions about long-term outcomes for this cohort of patients; clearly, further follow-up is required. Other groups have published their results comparing pain relief in patients undergoing pancreatectomy plus islet cell autotransplantation with a cohort of patients treated conventionally.[47,48] It is now appropriate for such a comparison to be made in a randomized controlled trial.

In this series, after pancreatectomy, none of the patients presented with the brittle diabetes that is often seen in persons undergoing total pancreatectomy *without* islet cell transplantation.[21,49] Forty-one percent of our patients are insulin free, 27% require less than 10 units per day of insulin, and the remaining seven patients require 15 to 40 units per day. Among the latter group, three of the patients were already receiving hypoglycemic agents and thus had antecedent diabetes as a result of either pancreatitis or concurrent type II diabetes. Closer analysis of these

Table 5. Results of McGill Pain Questionnaire

Item	Preoperative (N = 11)	Postoperative (N = 11)
Where is your pain?		
Internal (%)*	100	54.5
External (%)†	27.2	54.5
Pain rating index		
What does your pain feel like?		
Sensory (average)	19.4 ± 9.5	7.3 ± 6.6
Affective (average)	6.3 ± 3.9	0.9 ± 1.4
Evaluative (average)	4.2 ± 0.9	1 ± 1.3
Miscellaneous (average)	7.2 ± 2.1	1.2 ± 1.9
Pain-rating index total (average)‡	37 ± 13.4	11 ± 10.1
Present pain intensity (average)§	4.1 ± 0.8	0.7 ± 0.6
Number of words chosen (average)	13.3 ± 4.8	5.1 ± 4.4
How does your pain change with time?		
Continuous (%)	45.4	18.2
Steady (%)	18.2	9.1
Constant (%)	36.4	0
Rhythmic (%)	18.2	0
Periodic (%)	18.2	36.4
Intermittent (%)	27.3	9.1
Brief (%)	0	0
Momentary (%)	9.1	18.2
Transient (%)	27.3	18.2
How strong is your pain? (scale 1–5) 1 representing lowest and 5 representing highest		
At present (average)	4.1 ± 0.8	0.7 ± 0.6
At its worst (average)	5.0 ± 0	2.4 ± 2.2
At its least (average)	2.0 ± 0.8	1.2 ± 1.2
Worst toothache ever experienced (average)	4.3 ± 1	2.6 ± 2.3
Worst headache ever experienced (average)	4.5 ± 0.9	2.8 ± 2.4
Worst stomachache ever experienced (average)	4.4 ± 4.4	2.9 ± 2.5

* t-test showed $P < 0.01$.
† t-test showed $P = 0.08$.
‡ t-test showed $P < 0.01$.
§ t-test showed $P < 0.01$.

results demonstrates that improved pancreatic endocrine function is associated with the transplantation of more islet cell equivalents per kilogram, with a desired minimum of 3,000 IE per kilogram.[36] The patients in our series who are now insulin independent each received more than 3000 IE/kg, which compares favorably with other larger series.[32,35,36,38,47,50] Although there are many factors that affect the number and quality of islet cells isolated for transplantation, it is reasonable to conclude that the extent of pancreatic disease is one of these. This raises the possibility that in many patients with unremitting pancreatitis, temporizing procedures such as the use of stents, partial resections, and drainage procedures ultimately cause more islet cell damage by prolonging the exposure to inflammation and fibrosis. In fact, it is interesting to speculate that many patients may benefit from early pancreatectomy with transplantation of a better-preserved islet mass. In our series patients who underwent a total pancreatectomy as the initial procedure had an average IE of 337, 888 + 142,577 and 4281 IE/kg, whereas patients who underwent completion pancreatectomy had an average IE of 209,546 + 177,952 and 3589 IE/kg. Based on previous reports of patients who underwent a partial pancreatic resection for chronic pancreatitis, 72% who required an 80% to 95% pancreatectomy developed diabetes, whereas only 32% of those receiving 80% resection became diabetic.[51] Thus an important hypothesis for future consideration is that if pancreatic resection and islet cell autotransplantation were performed earlier in the course of treatment for chronic pancreatitis, islet cell function would be enhanced and rates of postoperative diabetes decreased. It seems likely that a major factor influencing beta cell function after pancreatectomy and islet cell autotransplantation is the severity of fibrosis.

Table 6. Pre- and postoperative morphine equivalent requirements

Patient	Preoperative	Postoperative
1	15	0
2	15	0
3	105	0
4	278	0
5	240	45
6	35	25
7	10	0
8	40	0
9	405	60
10	180	0
11	10	0
12	25	0
13	35	0
14	30	0
15	30	0
16	9	0
17	35	0
18	72	0
19	10	0
20	10	0
21	15	90
22	120	0
Average	78.4	9.5
SD	105.9	24

SD = standard deviation.

Simply put, a highly diseased pancreas will decrease the number and quality of islet cells available to transplant and therefore have a major impact on postoperative beta cell mass and glucose homeostasis.

In our series, although 68% of patients suffered complications, most of these complications were minor. None of the complications in our series were fatal, and they were similar to those in other series. Others have reported hepatic infarction, disseminated intravascular coaguation, splenic hemorrhage, and portal hypertension as being associated with intraportal infusion of islet cells.[52–54] Another complication associated with this procedure is portal vein thrombosis. We have not experienced these complications in our series. Traditionally, portal vein thrombosis has been thought to be related to the volume of infused islet cells.[55,56] More recently this concept has come under scrutiny, and there is now some debate over the most efficacious method of transplanting islet cells in terms of using a purified vs. an unpurified preparation. The use of unpurified preparations has the advantage of returning a larger mass of islet cells to the patient, thereby increasing the likelihood of improved metabolic function but increasing the risk of portal vein thrombosis.[57] Conversely, purified islet cell preparations reduce the volume of tissue infused and thus decrease the likelihood of portal vein thrombosis. However, with increased purification comes a decrease in the number and quality of these islet cells, as the gradients used for purification elicit a toxic effect on the islet cells.[58] Although in the past, portal vein thrombosis was thought to be related to the "final volume" infused, newer evidence suggests that tissue factor[59] released at the time of transplantation may be related to inducing a hypercoagulable state. In this series we used unpurified preparations to maximize the number and quality of islet cells and routinely administer heparin to these patients at the time of islet cell infusion.

Perhaps the primary limitation to the widespread application of autologous islet cell transplantation immediately after near-total to total pancreatic resection is that only a very limited number of facilities possess the expertise and technology to isolate and prepare pancreatic islet cells that are suitable for human transplantation. There are reports, however, demonstrating the feasibility of distance processing for both allo- and autoislets.[60,61] The feasibility of this approach is enhanced by improvements in preservation methods, specifically by the development of the two-layer method,[62] which allows for an extended preservation time in the ischemic pancreas, thereby increasing the islet cell yield and improving the viability of islet cells from suboptimal pancreata.[63] Although this method still needs validation in a clinical autotransplant setting, preliminary results with alloislet transplantation are significant,[64–66] and further studies should be pursued.

CONCLUSION

It has become increasingly clear during the past decade that islet cell autotransplantation is technically feasible. Short-term follow-up from our experience as well as long-term results from others demonstrate the feasibility of islet cell autotransplantation immediately after pancreatecomy as a means to alleviate pain from chronic pancreatitis and to prevent the development of surgically induced diabetes. As this technology improves, total pancreatecomy and autologous islet cell transplantation may become a standard therapy for patients suffering from refractory chronic pancreatitis.

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Discussion

Dr. M. Sarr (Rochester, MN): In the patients who had a partial reduction in their insulin dosage, did they have C-peptide in their blood?

Dr. H. Rilo: We are currently conducting a study with Dr. Dave D'Alessio, who has worked previously with Dr. Paul Robertson in Minnesota. We are testing the patients before and after transplantation. We have demonstrated that all resected and islet autotransplanted patients produce C-peptide.

Dr. Sarr: The question is, do they lack a total number of beta cells that is sufficient to lower their insulin level? In other words, did they have successful engraftment of the transplanted cells but just need to have more beta cells?

Dr. Rilo: Yes, a larger islet beta cell mass would be better. We see a decrease in the acute insulin response, and clearly there is a decrease in C-peptide that is evident on the arginine test and also on the oral glucose tolerance test.

Dr. L. Stewart (San Francisco, CA): Can you give me a better idea of what the preoperative opioid dose in these patients was across the board?

Also, what kinds of procedures had these patients had prior to your procedure, how many had a Puestow or a Whipple procedure, and so forth?

Dr. Rilo: Several of the patients had previous surgeries: seven had completion pancreatectomies, four had subtotal pancreatectomies, two had pancreaticojejunostomies, and three had whipple procedures. These are often patients with long-standing disease who come to the Pancreatic Disease Center after having been referred by other surgeons and physicians; these patients have already gone through a sequence of stenting procedures, or have been exposed to drainage operations and resections before we complete the pancreatectomy and autotransplant.

As to your other question, we converted the doses to a morphine equivalent, because all of these patients, as you know, are taking an unaccountable amount of opioid analgesics. On average, the overall reduction was from 78 morphine equivalent requirements preoperatively to 9.5 postoperatively. Eighteen of the patients, representing 82% of the study population, no longer required narcotics.

Dr. A. Warshaw (Boston, MA): This is excellent work and clearly a major advance. I have one question and that is concerning the use of the insulin dosage to quantitate survival of the transplanted islets. People who are off of insulin entirely represent unequivocal success, but it is an observed fact, and also our experience, that patients after total pancreatectomy may require less insulin than those who still have some functional pancreas left because of the absence of glucagon production as well as insulin. So I am not sure that

the numerical insulin requirement is proportional to relative insulin independence or whether it is a valid measure of the success of the transplant.

Dr. Rilo: I am aware of the impaired glucoregulation that is the result of the pancreatic glucagon deficiency, although there may be enteric sources of glucagon production present. I think this will be determined in the long term by the avoidance of complications. There is evidence that 50% of those patients who do not have any C-peptide will have a greater chance of developing complications. So the presence of a small amount of insulin being secreted will help to avoid those complications. We also see it in cases of so-called failed allotransplants where there is a very small amount of insulin and C-peptide being secreted, and those patients develop fewer complications. Although many patients with diabetes secondary to pancreatectomy take lower doses of insulin than individuals with type I diabetes, they almost always require multiple injections of mixed insulins daily. Many of our patients take only long-acting insulin and in smaller doses than is typical for pancreatic diabetes. We believe this is because of the preservation of some islet function.

Dr. T. Sielaff (Minneapolis, MN): We are obviously very interested in this sort of work and have been doing it since Drs. Sutherland and Najarian described it in the late 1970s.

Would you comment on the negative impact of previous surgery on islet yield and the effects of achieving insulin independence, and would you advocate this type of surgery for patients with idiopathic small duct chronic pancreatitis, similar to the presentation you were describing?

Dr. Rilo: I think that it will be one of the indications because the thinking is that if we can operate earlier, we can eventually preserve greater beta cell mass. We did an analysis of islet equivalence per gram of pancreas and islet equivalence per kilogram, and we clearly correlate the success with the amount of islets isolated per gram of pancreas. So if you eventually have more tissue, you will have greater success.

Dr. H. Reber (Los Angeles, CA): Your second conclusion indicates that you are recommending earlier surgical intervention, and I would like you to give us some sense of what you would recommend now with your experience.

Let's say that you have a patient who is 40 years of age and has idiopathic chronic pancreatitis. The patient does not have a dilated duct or diabetes. Would you suggest that a patient like that, with chronic recurrent intractable pain, go straight to total pancreatectomy and islet transplantation?

Dr. Rilo: These are patients that you say have failed—by that I mean they are left with intractable pain, correct?

Dr. Reber: Yes.

Dr. Rilo: Yes. I do not know of any resection that will improve their endocrine function, so why wait?

Dr. Reber: The patient is not diabetic.

Dr. Rilo: No, no, but I mean if we wait, we have nothing to offer that patient. The condition is likely to get worse and the patient's endocrine function will deteriorate. We do not really evaluate the deterioration of the islet cells as part of the process of cycles of inflammation. Clearly the islets are also affected.

Dr. C. Ulrich (Lewisville, TX): One of the things that I have been continuously approached about since the technology became available is for patients with hereditary pancreatitis, possibly to offer this to them in a setting where they are having recurrent episodes of acute pancreatitis; most investigators in the field believe that this probably needs to be done before they progress to advanced chronic pancreatitis because of the islet yield issue.

How long do you think it will be before this is ready for patients with hereditary pancreatitis, and is it really a viable option?

How many more years do we need to follow these patients to see how long the insulin independence lasts?

Dr. Rilo: The longest report in terms of a patient being free of insulin with autotransplantation comes from the Minnesota group and was published by Dr. Paul Robertson and that was more than 13 years ago, but this is just one case. Many improvements have been made with the procedure over the past several years.

First of all, we are now using a different collagenase that is called Liberase (Roche Applied Sciences, Indianapolis, IN). There are also new recombinant collagenases that look very promising, so we can perhaps improve the islet yield of these preparations. These new techniques will allow centers with greater experience in processing islets to receive a pancreas that has been resected at another center, by using the two-layer method with oxygenated perfluorcarbon, isolate and ship the islet preparation back to the center performing the pancreatectomy to be infused into the patient to prevent the development of diabetes. There are several centers now that are able to do that, and I think we are definitely one of those centers.

Dr. Ulrich: How many years do you think it will be before I have patients that come to me with recurrent acute pancreatitits, no obvious chronic pancreatitis, but they probably have low-grade chronic pancreatitis if they have recurrent acute disease, and this could actually be discussed with them by me as a viable option so that I could send them to Cincinnati or Minnesota or wherever? Next year, 2 years, 3 years, 5 years, is it ready now?

Dr. Rilo: It is ready now. I think the scientific community needs to come to an agreement.

Surgical Management of Hypertensive Lower Esophageal Sphincter With Dysphagia or Chest Pain

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Hypertensive lower esophageal sphincter (LES) is an uncommon manometric abnormality found in patients with dysphagia and chest pain, and is sometimes associated with gastroesophageal reflux disease (GERD). Preventing reflux by performing a fundoplication raises concerns about inducing or increasing dysphagia. The role of myotomy in isolated hypertensive LES is also unclear. The aim of this study was to determine the outcome of surgical therapy for isolated hypertensive LES and for hypertensive LES associated with GERD. Sixteen patients (5 males and 11 females), ranging in age from 39 to 89 years, with hypertensive LES (>26 mm Hg; i.e., >95th percentile of our control population) who had surgical therapy between 1996 and 1999 were reviewed. Patients with a diagnosis of achalasia and diffuse esophageal spasm were excluded. All patients had dysphagia or chest pain. Eight of 16 patients had symptoms of GERD, four had a type III hiatal hernia, and four had isolated hypertensive LES pain. Patients with hypertensive LES and GERD or type III hiatal hernia had a Nissen fundoplication, and those with isolated hypertensive LES had a myotomy of the LES with partial fundoplication. Outcome was assessed as follows: *excellent* if the patient was asymptomatic; *good* if symptoms were present but no treatment was required; *fair* if symptoms were present and required treatment; and *poor* if symptoms were unimproved or worsened. All patients were contacted by telephone for symptom assessment at a median of 3.6 years (range 3 to 6.1 years) after surgery. Patients with hypertensive LES and GERD or type III hiatal hernia had significantly lower LES pressure than those with isolated hypertensive LES (29.9 vs. 47.4 mm Hg; $P = 0.013$). Dysphagia and chest pain were relieved in all patients at long-term follow up. Outcome was excellent in 10 of 16, good in 3 of 16, and fair in 3 of 16. All patients but one were satisfied with their outcome. Patients with hypertensive LES are a heterogeneous group in regard to symptoms and etiology. Treatment of patients with hypertensive LES should be individualized. A Nissen fundoplication for hypertensive LES with GERD or type III hiatal hernia relieves dysphagia and chest pain suggesting reflux as an etiology. A myotomy with partial fundoplication for isolated hypertensive LES relieves dysphagia and chest pain suggesting a primary sphincter dysfunction. (J GASTROINTEST SURG 2003;7:990–996)

KEY WORDS: Hypertensive lower esophageal sphincter, dysphagia, antireflux surgery

The hypertensive lower esophageal sphincter (LES) was first described by Code et al.[1] in 1960. It is classified as a primary disorder of esophageal motility characterized by a resting pressure in the LES that exceeds the upper limit measured in a series of normal volunteers. It is often associated with elevated residual pressure in the LES during swallow-induced relaxation, and an elevation of the intrabolus pressure in the body of the esophagus.[2] Hypertensive LES is distinguished from achalasia and diffuse esophageal spasm, which can have similar findings on LES manometry, by normal esophageal body peristalsis.

Dysphagia and chest pain are the most common symptoms in patients with a hypertensive LES, occurring with a frequency of 33% to 100%.[2–10] Emphasis on these obstructive symptoms has led to treatment recommendations focusing on reducing the pressure in the LES by medical and surgical means. However, the recent demonstration that acid reflux often accompanies the hypertensive LES[2,7,11] raises questions regarding the wisdom of treatment focused

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solely on reducing the pressure of the hypertensive LES. On the other hand, performing a fundoplication raises concerns about inducing or worsening outflow resistance, resulting in more severe dysphagia and/or chest pain. A variety of surgical approaches to this disorder have been recommended, although large series reporting surgical outcomes are lacking. In this report we describe our experience with an individualized approach to surgery in patients with a hypertensive LES.

PATIENTS AND METHODS

Over a period of 3 years between September 1996 and October 1999, a total of 1390 patients were evaluated by esophageal manometry in the Esophageal Laboratory at the University of Southern California Department of Surgery. Hypertensive LES was diagnosed when the resting pressure in the LES exceeded the ninety-fifth percentile values obtained in our healthy volunteer control subjects (>26 mm Hg), and after excluding achalasia, diffuse esophageal spasm, and previous surgery of the LES. Of the 1390 patients, 100 (7.2%) were identified as having a hypertensive LES. Dysphagia and/or chest pain occurred in 82%, and heartburn in 71% of these patients. A subgroup of 16 patients with hypertensive LES, all of whom had severe dysphagia and/or chest pain and underwent surgical treatment, formed our study group. They consisted of five males and 11 females, with a median age of 63 years (IQR 53-69). The primary indication for surgical treatment in these patients was severe dysphagia unresponsive to conservative therapy.

Eight of the 16 patients had hypertensive LES in association with GERD. Six had evidence of increased acid exposure (DeMeester score >14.7) on 24-hour pH monitoring, and two had typical GERD symptoms with excellent response to preoperative proton pump inhibitor therapy. Three patients with abnormal DeMeester scores in this group also had esophagitis on endoscopy. In 4 of the 16 patients, hypertensive LES was associated with a type III hiatal hernia. The median size of the hernia was 4.5 cm, and none of the patients had esophagitis or evidence of increased acid exposure on 24-hour pH monitoring. Four of the 16 patients had isolated hypertensive LES, and none had esophagitis or evidence of increased esophageal acid exposure on 24-hour pH monitoring.

The 12 patients with hypertensive LES associated with GERD or type III hiatal hernia were treated with a full Nissen fundoplication. The four patients with isolated hypertensive LES were treated with a myotomy of the LES and a partial fundoplication. Standard operative techniques previously described were used in all of them.[12,13]

Stationary Esophageal Manometry

Esophageal manometry was performed with a water-perfused catheter system as previously described.[14] The resting LES pressure was measured at the respiratory inversion point (RIP), using a catheter with 0.8 mm openings located 5 cm apart and an infusion rate of 0.5 ml/min. LES relaxation was measured by the technique previously described.[15] Manometry of the esophageal body was done by placing the manometry catheter such that the proximal sensor was 1 cm below the lower border of the upper esophageal sphincter. The patient was asked to swallow 5 ml of water at room temperature to assess the esophageal body response. Hypertensive esophageal body contractions (nutcracker esophagus) were identified when the mean contraction amplitude in any of the distal esophageal channels was greater than 180 mm Hg or the duration of contractions was greater than 6 seconds. Ineffective esophageal motility was identified if 30% or more of the swallows had a distal esophageal contraction amplitude of less than 30 mm Hg or were not transmitted in the distal esophagus.

Symptom Assessment

A review of the clinical records was performed to determine the presence and severity of symptoms in all 16 patients. In addition to physician assessment of patient symptoms, a structured questionnaire for foregut symptoms was administered when the patient was seen for manometric evaluation. Outcome was assessed by completion of a similar questionnaire via telephone contact by one of us (A.P.T.). The median long-term follow-up was 3.6 years (range 3.0 to 6.1).

Outcome Scores

Symptomatic outcome was categorized as excellent, good, fair, or poor. Outcome was considered excellent if the patient was asymptomatic, good if mild symptoms were present that did not require treatment, fair if symptoms were present and treatment was required, and poor if symptoms were unimproved or became more severe. Patients were also asked if they were satisfied with their surgical treatment.

RESULTS

Preoperative Motility

Table 1 shows the preoperative motility assessment of the LES and esophageal body. Patients with

Table 1. Comparison of preoperative parameters in both patient groups

Preoperative parameters	HLES + GERD/type III hernia (n = 12)	HLES only (n = 4)	*P* value
LES pressure: Median (mm Hg)	29.9 (range 27.3–46.8)	47.4 (range 39.4–56.8)	0.013
LES relaxation: Partial or incomplete	6/12	3/4	NS
Hypertensive body contractions (>180 mm Hg)	5/12	2/4	NS
Ineffective esophageal motility	0/12	0/4	NS

GERD = gastroesophageal reflux disease; HLES = hypertensive lower esophageal sphincter; NS = not significant.

hypertensive LES and GERD or type III hernia had significantly lower LES pressures than those with an isolated hypertensive LES (29.9 vs. 47.4 mm Hg; $P = 0.013$) (Fig. 1). LES relaxation and esophageal body contractility were similar in both groups.

Post-surgical Outcome

At a median of 3.6 years after Nissen fundoplication, all 12 patients with GERD and type III hernia had complete relief of their dysphagia and chest pain (Fig. 2). Three patients developed recurrent symptoms of heartburn or reguritation at 6 or more months after surgery (Figs. 3 and 4). Despite this all but one patient in this group were satisfied with the outcome of their surgery.

All four of the patients with isolated hypertensive LES had complete relief of their symptoms at a median of 3.1 years after myotomy and partial fundoplication (see Fig. 2). One patient developed new-onset heartburn that required acid suppression therapy with proton pump inhibitors (see Fig. 3). All four patients were satisfied with the outcome of their surgery.

Post-surgical Symptomatic Scores

An excellent or good outcome occurred in 83.3% of patients with hypertensive LES and GERD or type III hernia and in 75% of patients with isolated hypertensive LES (Table 2). The outcome scores were related to the recurrence of symptoms of heartburn or regurgitation, as all patients in both groups were free of their preoperative dysphagia or chest pain.

Patient Satisfaction

Ninety-two percent of the patients with hypertensive LES associated with GERD or type III hiatal hernia and all patients with isolated hypertensive LES were satisfied with their treatment.

DISCUSSION

The treatment of symptomatic hypertensive LES has been controversial. Medical management has been largely limited to the use of nifedipine, sildenafil, and botulinum toxin to reduce LES pressure.[16–18] The results have been variable, often short-lived, and can be associated with severe side effects. Often the transient manometric improvement that occurs with these drugs was not associated with equivalent clinical improvement. Dilatation has been tried infrequently with similar disappointing results.[19] Acid suppression therapy has been tried, particularly for coexisting

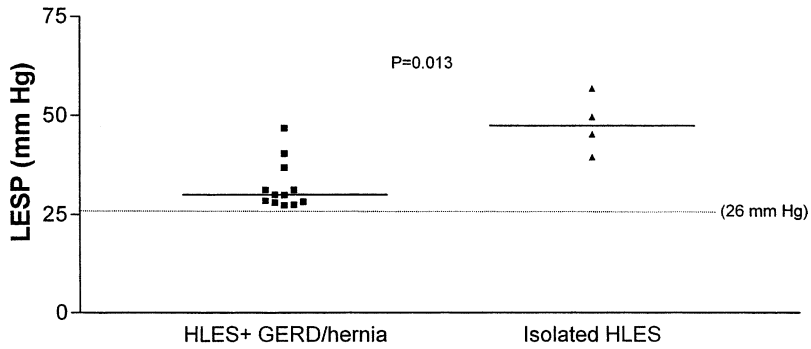

Fig. 1. LES pressure was significantly higher in the isolated hypertensive LES group (*HLES*). The dotted line at 26 mm Hg represents the upper limit of normal LES pressure. GERD = gastroesophageal reflux disease.

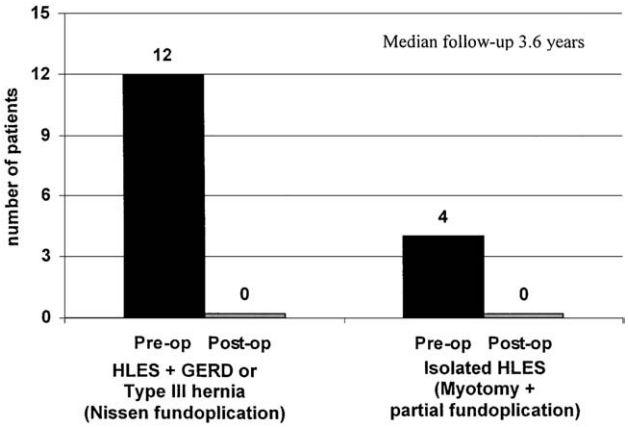

Fig. 2. Relief from dysphagia and chest pain at long-term follow-up. Y axis represents the number of patients with dysphagia/chest pain. Abbreviations as in Fig. 1.

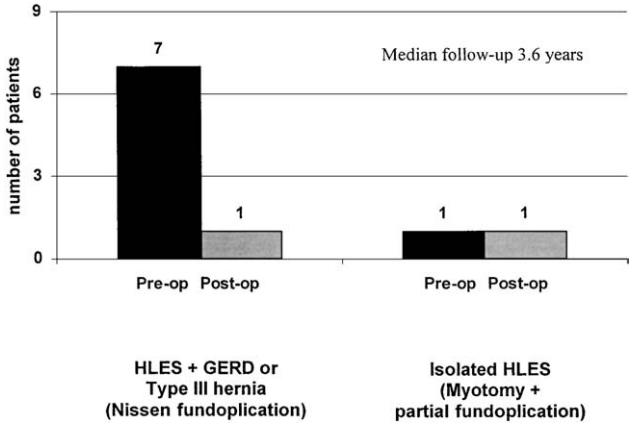

Fig. 4. Relief from regurgitation at long-term follow-up. Y axis represents the number of patients with regurgitation. Abbreviations as in Fig. 1.

reflux symptoms, but their effect on dysphagia and chest pain was poorly documented.[7]

Reports on surgical treatment of hypertensive LES also have been sporadic and limited to a small number of patients because of the relative rarity of the condition.[1,3,4,5,7,9,20,21] Most of the reports are marred by the use of the same surgical procedure for all patients with hypertensive LES under the assumption that there is a single etiology for the disease. Our study shows that hypertensive LES is an abnormal manometric finding associated with more than one disease, and treatment should be individualized on the basis of pH and motility studies.

We have reported that hypertensive LES constitutes 7.2% of the patient population referred for motility studies and causes significant symptoms in patients with a relatively uncommon manometric problem.[2] Both dysphagia and chest pain were present in 82% of patients diagnosed with hypertensive LES and is likely due to the high prevalence of partial LES relaxation associated with a raised intrabolus pressure suggesting the presence of outflow resistance at the LES.[2] Paradoxically, more than 70% of patients with hypertensive LES complain of heartburn and 26% have increased esophageal acid exposure on 24-hr pH monitoring.[2] This clearly suggests that hypertensive LES occurs in a variety of disease processes and may have more than one etiology causing the motility abnormality—that is, primary motor disorder or secondary to acid exposure. Evidence supporting this hypothesis is the observation that patients with isolated hypertensive LES had higher LES pressures compared with hypertensive LES associated with GERD or type III hernias (see Fig. 1). Because of this finding we tailored our surgical approach on the basis of clinical and manometric findings. In contrast to the manometric findings of the LES, the prevalence of hypertensive contractions in the esophageal body was similar in patients with isolated hypertensive LES and those associated with GERD or type III hernias and did not affect our therapeutic approach.

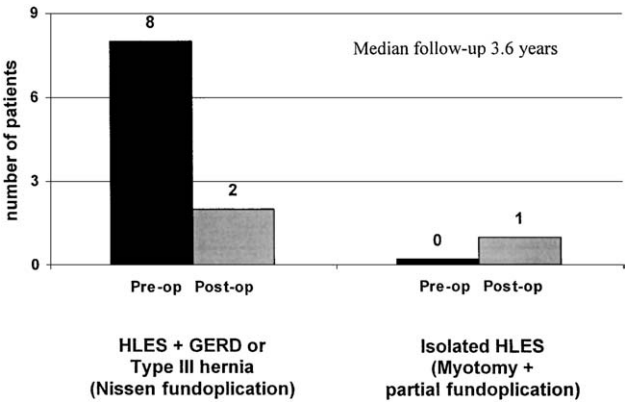

Fig. 3. Relief from heartburn at long-term follow-up. Y axis represents the number of patients with heartburn. Abbreviations as in Fig. 1.

The presence of preoperative dysphagia is always a concern if a Nissen fundoplication is contemplated. Herron et al.[22] have shown that preoperative dysphagia predicts the risk of dysphagia after a fundoplication. Further, Blom et al.[23] have shown that new-onset dysphagia after Nissen fundoplication is more common with increasing preoperative LES pressure. Faced with these concerns it was rewarding that all of our patients were free of dysphagia or chest pain after surgery. This experience suggests that until hypertensive LES is more completely understood, affected

Table 2. Outcome scores

Control of reflux symptoms	HLES + GERD/type III hiatal hernia (%)	Isolated HLES (%)
Excellent	66.6	50
Good	16.7	25
Fair	16.7	25
Poor	0	0

Excellent or good score occurred in 83.3% of the patients in the HLES + GERD / type III hiatal hernia group and in 75% of the isolated HLES group.
Abbreviations as in Table 1.

patients should be treated on the basis of their coexisting pathology, and myotomy should be restricted to patients with the isolated motility abnormality.

One might argue that patients with isolated hypertensive LES represent the normal 5% of the population above the ninety-fifth percentile. The presence of dysphagia and chest pain would indicate that LES pressure is not just the extreme range of normality but true pathology. Further, the relief of symptoms after myotomy would strengthen this opinion. In contrast, the lower sphincter pressure seen in hypertensive LES with GERD or type III hernia could be the 5% population above the ninety-fifth percentile of normality. As such they could be considered as having a normal LES. The relief of their symptoms of dysphagia and chest pain after a Nissen fundoplication without a myotomy would support this concept. Consequently, isolated hypertensive LES may be the only real primary disorder in our study group. Of interest, Champion et al.[24] have shown that patients with hypertensive LES lacked ganglion cells and were similar in this respect to achalasia. Isolated hypertensive LES may be a precursor of achalasia or *forme fruste* of achalasia.

Previous reports on myotomy for hypertensive LES lacked uniformity because they included patients with a variety of complaints and consisted of small numbers.[3–5,7,9,21] In the present study we have made a specific attempt to identify patients with isolated hypertensive LES and found that the LES pressures were significantly higher than those associated with GERD or type III hernia. We tailored the surgical procedure for isolated hypertensive LES by performing a myotomy of the LES with a partial fundoplication and were pleased with the long-term results. Although we cannot be sure that an antireflux procedure without a myotomy would result in the same outcome, wisdom would indicate that it would be unlikely since the patients had an exceptionally hypertensive sphincter without clinical or pH evidence of increased esophageal acid exposure.

CONCLUSION

Patients with a hypertensive LES are a heterogeneous group with regard to symptoms and etiology. Isolated hypertensive LES may be a primary motor abnormality and hypertensive LES with GERD or type III hiatal hernia could be the 5% of the population that is above the ninety-fifth percentile for normal LES pressure. Symptoms may be secondary to motor abnormality in isolated hypertensive LES and to reflux or alteration of the geometry of the cardia in hypertensive LES associated with GERD or type III hiatal hernia.

A Nissen fundoplication in patients with hypertensive LES with GERD/type III hiatal hernia relieves dysphagia and chest pain supporting reflux as an etiology. A myotomy with partial fundoplication for isolated hypertensive LES relieves dysphagia and chest pain supporting a primary sphincter dysfunction. Surgical management requires that the etiologies be determined prior to surgical intervention. Surgical myotomy should be reserved for patients with a primary motor etiology, that is isolated hypertensive LES.

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Discussion

Dr. D. Oleynikov (Omaha, NE): I want to congratulate you on a very thought-provoking study. By my calculation it appears that roughly 90 patients or so in your group had a hypertensive LES diagnosed, but only 16 or so underwent surgery. Would you please comment on how you selected which patients had surgery and which did not.

Please comment on how you made the diagnosis of hypertensive LES. Did you use a manometry device on everyone? We see that many referred patients with hypertensive LES, in fact, do not have the disease when we repeat manometry.

In those few patients who underwent myotomy with a very high LES pressure, did you find that their manometric studies were at all different from those in the other group? I know you mentioned there were no esophageal motility disorders, but we believe that some of these very high LES pressures are due to an early achalasia-type picture. Perhaps we are wrong.

Dr. A. Tambankar: To answer your first question, hypertensive LES patients with severe symptoms, who are unresponsive to conservative treatment, were evaluated for surgical therapy. We agree with you that most patients can be treated conservatively.

As to your next question regarding the motility pattern in the esophageal body of patients with very high LES pressures, obviously we looked very carefully at this, and there was no abnormality in the body motility to suggest early achalasia. There were no differences in body motility patterns between those with very high sphincter pressures and those with hypertensive sphincters with relatively lower pressures. However, we have seen early achalasia, as you mentioned, in a few patients, but they were excluded from this study population because of the diagnosis of achalasia.

Your third question was regarding the measurement of LES pressure. In our laboratory we use a water-perfused system with five single ports located 5 cm apart, and we then perform a station pull-through and average all five of the readings. We also perform a motorized pull-through utilizing four radial ports located at the same level, and then combine the results of both studies to get our results. On the whole, with the use of this system, we have consistent and reproducible results.

Dr. M. Patti (San Francisco, CA): In order to talk about the hypertensive LES as a primary motility disorder, you need to have normal peristalsis. Two of your patients had nutcracker esophagus. So I suggest that it was not an isolated hypertensive LES.

Can you speculate on the reason why in type III hiatal hernia and in patients with reflux you found an elevated pressure? Second, what was the radiographic picture in the four patients with a hypertensive LES?

Dr. Tambankar: Your comment regarding nutcracker esophagus is very interesting. Nutcracker esophagus and hypertensive LES may have a lot in common with regard to their etiologies, and there will be some degree of overlap. There may be a relationship here, but we often see hypertensive distal esophageal contraction amplitudes in patients and volunteers who do not have symptoms. We are not convinced that nutcracker esophagus is in itself a primary motility disorder with the same confidence that we have regarding isolated hypertensive LES.

Regarding the question on hypertension in patients with GERD and in type III hernia, we have no definite

answers. We believe that some patients with type III hernia develop high pressure from the geometric distortion of the cardia and extrinsic pressure from the hernia components. This may mean that the sphincter is not primarily high pressure in terms of its own squeeze, but it only reflects the extrinsic pressure secondary to type III hernia.

The reason why some patients with GERD have a hypertensive LES is still unclear. There may be a spastic component secondary to acid reflux in some patients perhaps early in the disease process. Another explanation for this, since these patients did not have very high pressures, is that they may represent the 5% of patients above the ninety-fifth percentile for LES pressure.

Dr. Patti: What was the x-ray feature in these four patients?

Dr. Tamhankar: The four patients with isolated hypertensive LESs had absolutely normal videoesophagograms. The four patients with type III hernias also had normal motility on their videos. The only abnormality in these patients was the presence of a standard type III hernia. The median size of the sliding component was 2 cm and the median size of the paraesophageal component was 4.5 cm.

Invited Discussion—Expert Commentary

Carlos A. Pellegrini, M.D. (Seattle, WA): This paper analyzes the results of surgery in 16 patients with hypertensive lower esophageal sphincter (LES). The operation was selected on the basis of the underlying condition determined by careful preoperative studies. Thus patients with GERD and hypertensive LES underwent a Nissen fundoplication, those with a paraesophageal hernia and hypertensive LES underwent a repair (and Nissen fundoplication presumably to anchor the repair), and those without either cause had a Heller myotomy and a partial fundoplication to facilitate empyting of the esophagus and prevent abnormal reflux. The results at a mean of more than 3 years were excellent. We have reported similar results using the same approach in patients with hypertensive LES and GERD.

This paper brings to the attention of the surgeon the following three important elements:

(1) The importance of a thorough preoperative workup in all patients with esophageal disease—that is, clearly defining the problem in terms of esophageal function before the initiation of treatment;
(2) thoughtful planning in terms of corrective surgery with the aim of dealing with the problems identified during the workup; and
(3) careful and detailed follow-up of patients by surgeons.

Incidence and Outcome of Anastomotic Stricture After Laparoscopic Gastric Bypass

Ninh T. Nguyen, M.D., C. Melinda Stevens, B.S., Bruce M. Wolfe, M.D.

Anastomotic stricture is a frequent complication after Roux-en-Y gastric bypass (GBP). We evaluated the frequency of anastomotic stricture following laparoscopic GBP using a 21 mm. vs. a 25 mm circular stapler for construction of the gastrojejunostomy and the safety and efficacy of endoscopic balloon dilation in the management of anastomotic stricture. We reviewed data on 29 patients in whom anastomotic strictures developed after laparoscopic GBP. All strictures were managed with endoscopic balloon dilation using an 18 mm balloon catheter under fluoroscopic guidance. Main outcome measures were the number of anastomotic strictures in patients in whom the 21 mm (vs. 25 mm) circular stapler was used to create the gastrojejunostomy, time interval between the primary operation and symptoms, complications of endoscopic balloon dilation, the number of patients with resolution of obstructive symptoms, and body weight loss. There were 28 females with a mean age of 39 years and a mean body mass index of 48 kg/m^2. Anastomotic stricture occurred significantly more frequently with the use of the 21 mm compared to the 25 mm circular stapler (26.8% vs. 8.8%, respectively; $P < 0.01$). The median time interval between the primary operation and presentation of stricture was 46 days. After the initial dilation, recurrent stricture developed in 5 (17.2%) of 29 patients. These five patients underwent a second endoscopic dilation, and only one of these five patients required a third endoscopic dilation. None of the 29 patients required more than three endoscopic dilations. The mean percentage of excess body weight loss at 1 year for patients in whom the 21 mm circular stapler was used for creation of the gastrojejunostomy was similar to that for patients in whom the 25 mm circular stapler was used (68.2% vs. 70.2%, $P = 0.8$). In this series the rate of anastomotic stricture significantly decreased with the use of the 25 mm circular stapler for construction of the gastrojejunostomy without compromising weight loss. Endoscopic balloon dilation is a safe and effective option in the management of anastomotic stricture following laparoscopic GBP. (J GASTROINTEST SURG 2003;7:997–1003)

KEY WORDS: Gastric bypass, anastomotic stricture, balloon dilation, laparoscopy

Roux-en-Y gastric bypass (GBP) is the most commonly performed operation for the treatment of morbid obesity in the United States. Recently there has been an increase in the demand for bariatric surgery, which has generated an increase in the number of surgeons interested in performing bariatric surgery. It is important for surgeons who want to learn the technique of bariatric surgery to also have an understanding of the possible postoperative complications and how to manage them. A frequent late complication following Roux-en-Y GBP is the development of an anastomotic stricture at the gastrojejunostomy. Anastomotic stricture has been reported with all techniques for creation of the gastrojejunostomy (circular stapler, hand-sewn technique, or linear stapler)[1–3]; however, the most frequently performed technique reported in the literature is the circular stapler.[4–7] The aim of this study was to review (1) the rate of anastomotic stricture with the use of two different sized circular staplers (21 mm vs. 25 mm) for creation of the gastrojejunostomy and (2) outcomes of balloon dilation performed for the treatment of gastrojejunostomy stricture after laparoscopic GBP.

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MATERIAL AND METHODS

Study Population

A retrospective chart review was performed for 185 patients, 29 of whom developed anastomotic strictures after laparoscopic GBP. The diagnosis of anastomotic stricture was determined primarily on the basis of the clinical presentation of frequent vomiting immediately after meals. The following data were collected: demographics, the number of anastomotic strictures in patients who had the 21 mm vs. 25 mm circular stapler for creation of the gastrojejunostomy, time interval between the primary operation and symptoms, complications of endoscopic balloon dilation, the number of patients with resolution of obstructive symptoms, and body weight loss. This retrospective study was approved by the institutional review board of the University of California, Irvine Medical Center.

Surgical Treatment

Laparoscopic GBP was performed according to the technique previously described.[8] In short, a 15 to 20 ml gastric pouch was created. The Roux limb length was measured at 75 cm for morbidly obese patients and 150 cm for super-obese patients. The Roux limb was routed via a retrocolic, retrogastric path. In the first 71 cases, we used the 21 mm circular stapler (U.S. Surgical Corp., Norwalk, CT) for creation of the gastrojejunostomy. We noted a high frequency of anastomotic stricture and subsequently changed to the 25 mm circular stapler (U.S. Surgical Corp.). The gastrojejunostomy anastomosis was reinforced circumferentially with interrupted sutures. Intraoperative flexible endoscopy was performed in all cases for inspection of the anastomosis and to test for air leaks.

Flexible Endoscopy With Balloon Dilation

All patients underwent endoscopic balloon dilation performed by a single surgeon (N.T.N.). All flexible endoscopy procedures were performed under either intravenous sedation or general anesthesia. Flexible endoscopy was performed using a GIF-Q160 gastroscope (Olympus America, Inc., Melville, NY). The diagnosis of an anastomotic stricture was confirmed endoscopically if the 9.5 mm diameter gastroscope could not be passed through the gastrojejunostomy (Fig. 1). Once the stricture was confirmed, an 18 mm balloon catheter (Microvasive; Boston Scientific, Watertown, MA) was placed through the side channel of the gastroscope, and its tip was inserted through the stricture (Fig. 2). The entire balloon was outside of the gastroscope channel before dilatation. The position of the balloon catheter was confirmed under fluoroscopic guidance, and the midportion of the balloon was positioned endoscopically to traverse the "waist" of the stricture. The balloon was inflated with water-soluble contrast medium up to 3 or 4 atmospheres (atm) under fluoroscopic control. Pressure was monitored with an in-line pressure gauge. A waist appeared in the midportion of the balloon, thus confirming the correct position. The balloon was left inflated until the waist of the balloon disappeared and for an additional 30 to 60 seconds thereafter. After deflation of the balloon, the balloon was withdrawn into the gastroscope, and the endoscope was advanced through the anastomosis into the jejunum (Fig. 3). In certain cases a second dilation was performed using the same balloon if the first dilation was inadequate to allow passage of the endoscope. All procedures were performed on an outpatient basis. Patients were instructed to maintain a full liquid diet for 2 days postoperatively and then resume a regular diet. Patients

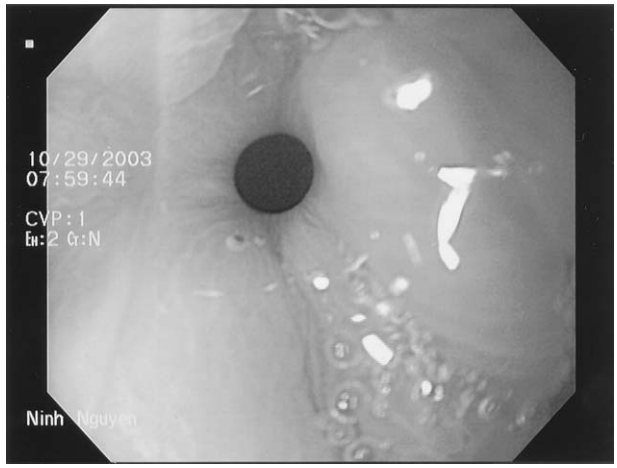

Fig. 1. Endoscopic view of anastomotic stricture.

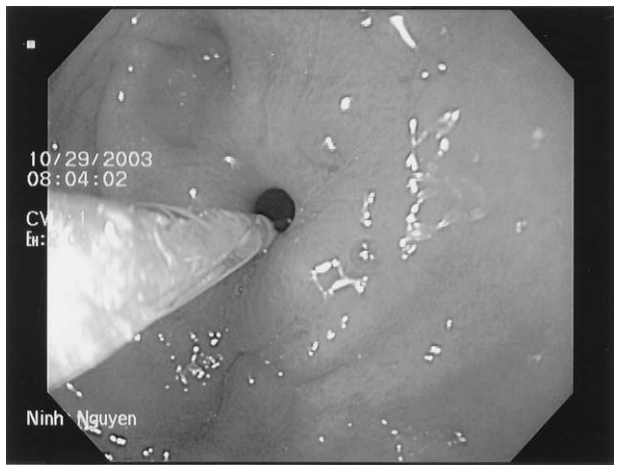

Fig. 2. Insertion of a balloon catheter through the anastomotic stricture.

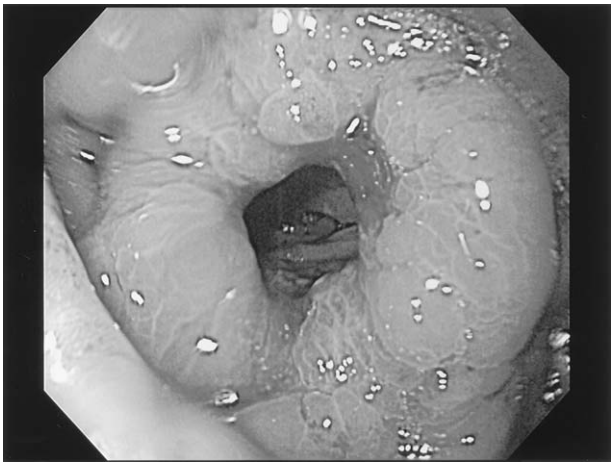

Fig. 3. Endoscopic view of anastomotic stricture following dilation with an 18 mm balloon.

were followed up at 7 to 10 days after the procedure. The endoscopic dilation was considered successful when there was complete resolution of obstructive symptoms and no recurrence of symptoms in the follow-up period.

Statistical Analysis

All values for continuous variables are expressed as means ± standard deviation. The mean percentage of excess body weight loss was compared between groups (21 mm vs. 25 mm stapler) using a two-tailed Student's *t* test. Chi-square or Fisher's exact tests were performed for categorical data. Statistical analysis was performed using SPSS software (SPSS Inc., Chicago, IL). A value of $P < 0.05$ was considered statistically significant.

RESULTS

Twenty-nine (15.7%) of 185 patients (164 females) who underwent laparoscopic GBP developed postoperative anastomotic stricture of the gastrojejunostomy. There was a significantly greater number of anastomotic strictures in patients who underwent laparoscopic GBP with the use of a 21 mm circular stapler compared to the 25 mm circular stapler (26.8% vs. 8.8%, respectively; $P < 0.01$). Of the 29 patients, there were 28 females with a mean age of 39 ± 7 years (range 22 to 54 years) and a mean body mass index of 48 ± 5 kg/m^2. There was no significant difference in the male/female ratio for the entire patient population compared to the male/female ratio for those in whom anastomotic stricture developed (11.3% vs. 3.4%, respectively; $P = 0.09$, Fisher's exact test). All patients presented with symptoms of dysphagia, nausea, and vomiting without systemic evidence of peritonitis. The median time interval between the primary operation and the initial presentation of symptoms was 46 days (range 24 to 505 days). The majority of patients (93.1%) developed the anastomotic stricture 30 days or more postoperatively.

The mean stoma size observed at endoscopy was 3.7 ± 1.3 mm. Endoscopic balloon dilation was successful in all cases. There were no intraoperative or postoperative complications.

All procedures were performed on an outpatient basis, and only 1 (3.4%) of 29 patients required hospital admission for 23 hours. At a mean follow-up of 37 ± 14 months, 24 (82.7%) of 29 patients had complete resolution of symptoms after the initial dilation. Five (17.2%) of 29 patients developed recurrent obstructive symptoms at a median time interval of 2.5 months after the initial dilation and required a second endoscopic balloon dilation. Four of these five patients had complete resolution of symptoms, and only one (20%) of five patients developed recurrent symptoms requiring a third endoscopic dilatation (Table 1). Endoscopic balloon dilation was equally effective for resolution of symptoms after a single dilation in both the 21 mm and the 25 mm anastomoses (79% vs. 90%, respectively; $P = 0.33$, Fisher's exact test).

The mean percentage of excess body weight loss at 1 year for patients in whom anastomotic strictures developed was 68.7% ± 14.8% compared to 69.8% ± 15.0% for patients who did not have strictures ($P = 0.7$). The mean percentage of excess body weight loss at 1 year for patients in whom the 21 mm circular stapler was used for creation of the gastrojejunostomy was 68.2% ± 14.8% compared to 70.2% ± 16.3% for patients in whom the 25 mm circular stapler was used ($P = 0.8$).

DISCUSSION

Anastomotic stricture of the gastrojejunostomy is a frequent complication after both open and laparoscopic GBP (Table 2).[1,3,4,6,8–20] In a comparative study

Table 1. Endoscopic balloon dilation of anastomotic stricture in 29 patients following laparoscopic Roux-en-Y gastric bypass

No. of dilations	No. of patients
1	24 patients
2	4 patients
3	1 patient

Table 2. Anastomotic stricture in selected series of laparoscopic and open gastric bypass procedures

Reference	Rate of stricture
Laparoscopic GBP	
Schauer[4] et al. (2000)	13/275 (4.7%)
Wittgrove and Clark[6] (2000)	8/500 (1.6%)
Nguyen[8] et al. (2001)	9/79 (11.4%)
Higa[3] et al. (2001)	73/1500 (4.9%)
Dresel[10] et al. (2002)	3/100 (3.0%)
DeMaria[11] et al. (2002)	18/281 (6.4%)
Abdel-Galil and Sabry[1] (2002)	18/90 (20%)
Papasavas[12] et al. (2002)	4/116 (3.4%)
Oliak[13] et al. (2002)	6/300 (2.0%)
Gould[14] et al. (2002)	12/223 (5.4%)
Open GBP	
Sanyal[15] et al. (1992)	23/191 (12%)
Oh[16] et al. (1997)	6/194 (3.1%)
Curry[17] et al. (1998)	1/85 (1.1%)
Fobi[18] et al. (1998)	4/705 (0.6%)
Balsiger[19] et al. (2000)	2/191 (1.0%)
Nguyen[8] et al. (2001)	2/76 (2.6%)
Brolin[20] et al. (2002)	5/198 (1.7%)

of laparoscopic vs. open GBP, DeMaria et al.[9] reported no significant difference in the rate of stomal stenosis (24% vs. 20%) between the two techniques. Factors affecting the development of anastomotic strictures include technical factors (tension on the anastomosis, ischemia), techniques for construction of the gastrojejunostomy (i.e., circular stapler, the size of the circular stapler, linear stapler, whether the anastomosis was hand sewn, placement of additional reinforcement sutures), and the healing capacity of individual patients.

Techniques play a major role in the development of anastomotic strictures. There are a variety of techniques for construction of the gastrojejunostomy. These techniques include the circular stapler, the linear stapler, or the hand-sewn technique. The choice of technique is often based on the surgeon's preference and training. The initial description of the technique for laparoscopic creation of the gastrojejunostomy was reported by Wittgrove and Clark, [6] who used a 21 mm circular stapler. These investigators reported an anastomotic stricture rate of 1.6% in 500 cases; however, others have reported a stricture rate as high as 31% with the use of the circular stapler.[2] Anastomotic stricture has also been reported after both the hand-sewn and the linear stapler techniques. Higa et al.[3] reported a gastrojejunostomy stenosis rate of 4.9% in their first 1500 hand-sewn cases, and Abdel-Galil and Sabry[1] reported a stricture rate of 10% in 30 patients who underwent the linear stapler technique. In a study comparing the gastrojejunostomy stricture rates in the three techniques for creation of the gastrojejunostomy, Gonzalez et al.[2] reported that the circular stapler technique had the highest rate of stricture (31%) compared to the hand-sewn (3%) or linear stapler (0%) technique. In contrast, Abdel-Galil and Sabry[1] reported that the hand-sewn technique had the highest rate of stricture (33%) compared to the circular (16%) or linear (10%) stapler technique.

Currently the circular stapler technique is the most commonly reported technique in the literature for creation of the gastrojejunostomy.[4–8,12,13] The majority of laparoscopic GBP series reported the use of the 21 mm circular stapler.[4–8] Our study demonstrated a high rate of stricture using the 21 mm compared to the 25 mm circular stapler. We routinely perform a retrocolic and retrogastric gastrojejunostomy and do not believe that tension plays a significant role in the development of stricture. However, reinforcement sutures were placed circumferentially on the gastrojejunostomy anastomosis as a preventive measure against anastomotic leaks. Our higher rate of stricture formation, however, could be related to this technique. The use of a larger circular stapler (25 mm) in this series reduced the rate of stricture but did not compromise weight loss. Stahl et al.[21] also reported that there were no significant differences in weight loss between patients who had a gastrojejunostomy performed using the 21 mm vs. the 25 mm circular stapler.

The primary treatment of anastomotic stricture following Roux-en-Y GBP is balloon dilation. There are two basic types of balloon dilation techniques: endoscopic balloon dilation and fluoroscopically guided balloon dilation. In this series we demonstrated that endoscopic balloon dilation is a safe and effective treatment for anastomotic stricture. We performed endoscopic balloon dilation under fluoroscopic guidance to ensure a full expansion of the stricture. Using this technique the majority of our patients (83%) who underwent endoscopic dilation had complete resolution of symptoms after a single dilation. Five patients required a second dilation, and only one of these five patients required a third dilation. Surgical revision of the gastrojejunostomy was not necessary in any patient. Patients who developed anastomotic stricture in this series had a weight loss pattern similar to that in patients who did not develop strictures. Other investigators have reported similar success.[7,15] Sanyal et al.[15] reported complete resolution of symptoms in 18 (90%) of 20 patients who underwent endoscopic dilation for stomal stenosis after Roux-en-Y GBP. Barba et al.[7] reported that

67% of patients who developed an anastomotic stricture after Roux-en-Y GBP required a single dilation and 30% required a second dilation; there were no complications in their series. The results of fluoroscopically guided balloon dilation after surgery for morbid obesity have not been as successful.[22,23] Vance et al.[22] reported that only 14 (50%) of 28 patients who underwent fluoroscopically guided balloon dilation had complete relief of symptoms after the initial treatment.

The main concerns with dilation of the gastrojejunostomy stricture are bleeding and perforation. Perforation is certainly the most worrisome concern and can occur at the anastomosis or the jejunal Roux limb. Perforation of the anastomosis is related to the size of the balloon and the amount of circumferential force (atmospheres) exerted on the stricture, which is related to the initial narrowing and length of the stricture. Perforation of the jejunal Roux limb is related to traumatic iatrogenic manipulation of the tip of the balloon. Although the tip of the balloon is flexible, it is important to avoid forcing the balloon through the stricture opening as the tip of the balloon can perforate the jejunal Roux limb. Perforation can also be related to the timing of dilation after GBP. In most of the patients in this series, stricture developed after 30 days postoperatively and our earliest dilation was performed at 24 days after surgery. We do not advocate the use of balloon dilation for anastomotic stricture during the first 3 weeks after Roux-en-Y GBP. If a patient presents with obstructive symptoms during the first 3 weeks postoperatively, we recommend maintaining the patient on a full liquid diet or if necessary total parenteral nutrition until the 3-week mark. If endoscopic dilation is performed during the first 3 weeks postoperatively, a smaller balloon (l2 mm) should be used and the anastomosis should be dilated at a lower pressure (<2 atm).

Endoscopic balloon dilation for the treatment of anastomotic stricture following Roux-en-Y GBP is often performed by the gastroenterologist at most institutions.[7,15] Depending on the experience of the surgeon performing therapeutic endoscopy, we believe that the bariatric surgeon should take an active role in performing flexible endoscopy for intraoperative and postoperative management of bariatric patients. The Society of American Gastrointestinal and Endoscopic Surgeons is an organization that has developed a major focus in educating surgeons in the practice of flexible endoscopy and incorporating endoscopy into the practice of laparoscopy. In this series we demonstrated that endoscopic balloon dilation can be performed safely in the context of a bariatric surgery practice. The surgeon is in a unique position to understand the surgically altered gastrointestinal tract and should be able to diagnose and treat complications of Roux-en-Y GBP. In our practice endoscopy is commonly performed both intraoperatively as an adjunct to a laparoscopic procedure and postoperatively for management of complications after laparoscopic gastrointestinal surgery. Schirmer et al.[24] echo our recommendations and reported that upper endoscopy is a useful tool that may be used by the surgeon for diagnosis and treatment of postoperative complications after Roux-en-Y gastric bypass.

CONCLUSION

Anastomotic stricture is a common late complication after Roux-en-Y GBP. The rate of anastomotic stricture significantly decreased with the use of the 25 mm compared to the 21 mm circular stapler for construction of the gastrojejunostomy without compromising weight loss. Endoscopic balloon dilation is a safe and effective option in the management of anastomotic stricture following laparoscopic GBP. The bariatric surgeon, with knowledge and experience in endoscopy, is the best qualified person to treat stricture complications after Roux-en-Y GBP.

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Discussion

Dr. M. Murr (Tampa, FL): Do you truly think that the small increase in the staple size, from 21 mm to 25 mm, reduced your anastomotic stricture rate threefold? For many years the literature on open gastric bypass with the 21 mm circular stapler has shown the incidence of anastomotic strictures to be between 1% and 2%. Why is the stricture rate for laparoscopic procedures in your study so much higher than the rate for open procedures?

Dr. N. Nguyen: Even though you are changing only a few millimeters in the circular stapler diameter size, the cross-sectional area is approximately 40% larger with the 25 mm compared to the 21 mm circular stapler. The incidence of anastomotic strictures after open gastric bypass ranges from 0.6% to 12%. In this series we reported an overall stricture rate of 15.7%. I am not sure why we are seeing a higher rate of stricture with the laparoscopic approach. However, I believe that we try to be proactive in diagnosing the problem early to avoid complications from nutritional deficiency and hence are probably diagnosing more strictures.

Dr. T. Magnuson (Baltimore, MD): I also enjoyed your presentation. Because there was no difference in weight loss with a 25 mm vs. a 21 mm stapler, do you think that the actual size of the anastomosis matters, and if not, why not go with a larger circular stapler to eliminate the problem of stricture similar to doing an open hand-sewn procedure during open gastric bypass?

Dr. N. Nguyen: The size of the anastomosis has been a topic of continuing controversy and will continue to be debated until a prospective randomized study evaluating stoma size is carried out. I believe that to an extent the size of the stoma does affect weight loss. The optimal stoma size is unknown but most surgeons aim for a stoma size of 1.2 to 1.5 cm. From our data we demonstrated that weight loss was equivalent between the 21 mm and 25 mm circular stapler, and the stricture rate was lower with the 25 mm circular stapler. A disadvantage of a larger circular stapler (i.e., 29 mm) is the technical difficulty of introducing the stapler through the abdominal wall.

Dr. J. Eagon (St. Louis, MO): My question regards the definition of stricture. How often do your patients have to vomit before you bring them in for an endoscopy to determine the diagnosis of stricture? Also, what percentage of patients with symptoms of stricture did not have a stricture?

Dr. N. Nguyen: The diagnosis of anastomotic stricture is based on clinical presentations. These patients tend to have symptoms of persistent vomiting after meals. Initially, vomiting occurs after ingestion of solid food but can progress to occurring after liquids and occasionally even after swallowing saliva. There is no hard and fast rule about the number of episodes of vomiting after meals before making a diagnosis of stricture, but if vomiting persists for more than 48 hours, we will bring the patient in for endoscopy. There were two instances in which we performed endoscopy in a patient with these symptoms and did not find a stricture. These two patients had obstruction of the jejunojejunostomy and had similar presentations. The diagnosis of obstruction at the jejunojejunostomy can be made on the basis of an upper gastrointestinal contrast study.

Dr. B. Krevsky (Philadelphia, PA): After dilatation, do you find that weight loss decelerates? In other words, have you made the lumen larger? Have you followed up on these patients who have undergone stricture dilatation?

Dr. N. Nguyen: I do not believe that there is deceleration of weight loss after dilation. Although the size of our balloon is 18 mm, the estimated stoma size after dilation is between 1.2 and 1.5 cm. Weight loss after Roux-en-Y gastric bypass is related to a partly restrictive and partly malabsorptive component. By opening the stoma, I do not believe that we are altering the restrictive component. Surgeons should not delay treatment for an anastomotic stricture in order to avoid deceleration of weight loss. Delay in therapy can lead to dehydration, malnutrition, and occasionally Wernicke-Korsakoff encephalopathy.

Invited Discussion—Expert Commentator

Bruce D. Schirmer, M.D. (Charlottesville, VA): This paper addresses the subject of the gastrojejunostomy created during performance of laparoscopic Roux-en-Y gastric bypass (GBP). The authors used a circular stapler for this anastomosis, a common technique. Not surprisingly, the 21 mm stapler was associated with a higher incidence of stricture than the 25 mm stapler. Their incidence of postoperative stricture was 15.7%, which although higher than in many series of Roux-en-YGBP reported in the literature, is consistent with our own institution's findings using a circular stapled technique. We also experienced an unacceptably high incidence of stricture after using the 21 mm stapler, and found a similar incidence for the 25 mm stapler during our experience with open Roux-en-YGBP. The timing of postoperative stricture in this series is also similar to that seen in previously reported open series.

The authors used endoscopic balloon dilatation successfully to treat all patients, with only one patient requiring three dilatations. This is an excellent treatment success rate. We have found that these patients will often do well with a fluoroscopic dilatation as a second procedure, since the size of the dilating balloon can be larger than 18 F. However, we agree with the authors' approach of using an endoscopic dilatation as the initial procedure, because that allows the surgeon to evaluate the pouch, assess the severity of the stricture, and dilate those particularly tight strictures most safely as a single diagnostic and therapeutic intervention. It also goes without saying, and I strongly agree with the authors' opinion as stated in the manuscript, that such endoscopy should be performed by the operating surgeon, who best understands the anatomy and is most qualified to determine appropriate treatment.

Finally, the authors have also contributed data to the growing but still unconfirmed opinion that the size of the gastrojejunostomy anastomosis probably has little to do with the amount of weight lost by patients after Roux-en-Y GBP. Although some surgeons have gone to great lengths to band or control such anastomotic size, there are no data in the literature that have ever shown a correlation between anastomotic size and weight loss. Indeed, if the vertical banded gastroplasty serves as an historic example, that procedure suggested that an overly tight gastric outlet could lead to unhealthy dietary changes and poor weight loss.

Finally, although it is just an impression that requires data as documentation, in our own experience it seems that using a linear stapler to perform the gastrojejunostomy during laparoscopic Roux-en-YGBP is associated with a lower incidence of anastomotic stricture than when we use a circular stapler.

Tumor Suppressor Gene Hypermethylation as a Predictor of Gastric Stromal Tumor Behavior

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The growing understanding of the epigenetic changes associated with cancer, including aberrant promoter methylation of tumor suppressor genes that afford selective growth advantages to human neoplasms, suggests that the characterization of gene methylation patterns among gastrointestinal stromal tumors (GISTs) may be useful for predicting tumor behavior. Thirty-eight c-kit–positive gastric stromal tumors were subjected to methylation-specific polymerase chain reaction (MSP) to detect promoter methylation associated with 11 candidate tumor suppressor genes (*p16/INK4a*, *APC*, *MGMT*, *hMLH1*, *p73*, *E-cadherin*, *RAR-β*, *RASSF1A*, *RB*, *ER*, and *DAPK*), established to have a role in tumorigenesis of several solid human organs. Aberrant methylation of any of the 11 candidate tumor suppressor genes was detected in 84% of all GISTs. In decreasing order of frequency, the six most commonly methylated genes were: *MGMT* (47%), *p16* (45%), *RASSF1A* (40%), *E-cadherin* (37%), *hMLH1* (34%), and *APC* (31%). For all of the GISTs, promoter methylation was less reliable than tumor mitotic rate in predicting 5-year tumor-free survival for the GISTs; however, *E-cadherin* methylation was a multivariate prognostic factor for early recurrence of GISTs (50% at 2 years; $P = 0.030$). Among the mitotically active (>5 per 50 high-power field), histologically indistinguishable GISTs, *E-cadherin* methylation was an independent predictor of tumor-related mortality: 5-year disease-free survival was worse for the *E-cadherin* methylated GISTs (19%) compared to the *E-cadherin* unmethylated tumors (71%; $P = 0.010$). Detection of methylation within selected genes may afford a reliable and accurate molecular marker system for predicting neoplastic behavior among GISTs. This study supports the methylation status of *E-cadherin* as a prognostic marker for early GIST recurrence and survival. (J GASTROINTEST SURG 2003;7:1004–1014)

KEY WORDS: Methylation, GIST, tumor suppressor genes

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumor of the gastrointestinal tract and affect approximately 5000 patients in the United States each year.[1] In the past, GISTs have been characterized clinically and pathologically as leiomyomas and leiomyosarcomas. Over the past several years, however, studies have delineated the likely etiology of GISTs from early progenitor cells of the gastrointestinal tract. Furthermore, these putative progenitor cells hold the capacity for differentiating into peristaltic pacemaker cells within the gut smooth muscle, the so-called interstitial cells of Cajal (ICC).[1–3] In fact, the immunohistochemical detection of the *KIT* tyrosine kinase receptor (c-KIT), normally expressed by the ICC, has now become the diagnostic criterion for GISTs.[1,2]

Although GISTs may arise from any portion of the foregut to hindgut, two thirds of stromal tumors originate from the stomach. Because it is generally accepted that GIST behavior varies significantly according to

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the site of origin along the gastrointestinal tract, any development of novel prognostic markers must be studied with specific attention to anatomic location.[1,3]

Predicting the clinical behavior of GISTs, namely, benign vs. malignant, requires an experienced pathologist with a vast exposure to gastrointestinal mesenchymal tumors. In the past, conventional clinical and histologic criteria for characterizing GISTs, including tumor size, histologic subtype, mitotic rate, and cellularity, have been used to predict tumor behavior.[1] Recently new cytogenetic and genetic molecular markers have been studied for their respective roles in GIST pathogenesis, and for their prognostic value in predicting GIST behavior. In a recent study by Singer et al.,[2] particular *KIT* mutations within exon 11 were described as independent predictors of tumor-free survival in addition to established histologic features. Certainly a greater understanding of GIST biology will promote the development of novel molecular markers for GIST diagnosis and prognosis in addition to further improvements in therapy, such as the recent application of the phenylaminopyrimide derivative, STI571 (Norvartis, Basel, Switzerland), to patients with GISTs carrying a constitutively active form of the *KIT* receptor.[3–6]

There is growing evidence that the biology of several solid human tumors results from epigenetic as well as genetic alterations within the cell. Of the several epigenetic mechanisms that play a role in tumorigenesis, including genomic heterochromatization, histone deacetylation, and loss of genomic imprinting, DNA methylation of tumor suppressor gene promoter sites has gained particular attention in recent years.[7–11] Silencing of tumor suppressor gene expression by promoter hypermethylation at CpG-rich islands is common among several human malignancies.[12–17] The hypermethylation of promoter regions for *p16/INK4a*, *p15/INK4b*, *E-cadherin*, *VHL*, and *hMLH1* correlates directly with the loss of transcription of these tumor suppressor genes in a variety of tumors.[13,15,18,19] Methylation has been found for several tumor suppressor genes (i.e., *p16*, *RAR-β*, *TIMP3*, *E-cadherin*, *DAPK-1*, *hMLH1*, *cyclin G*, *and ppENK*) involving cancers of the pancreas, breast, and stomach.[16,17,20]

Characterization of the methylation patterns of tumor suppressor genes, established to play a role in human tumorigenesis, may afford helpful insight into the peculiar biology associated with GISTs. The methylation profile for a histologically defined GIST may be predictive of GIST behavior including time to early tumor recurrence and disease-free survival following a margin-negative surgical resection.

MATERIAL AND METHODS

Human Tissue Samples

Tumor samples were obtained from 38 completely resected gastric stromal tumor specimens that were presented to the department of pathology at The Johns Hopkins Hospital between 1989 and 2001. Permission for cataloging and processing all samples for this study was obtained in accordance with the guidelines set forth by the institution's review board and joint committee for clinical research. The GIST specimens were paraffin embedded and sectioned sequentially at a thickness of 4 to 10 μm each. Characterization of the GIST samples, including tumor size, histology, cellularity, mitotic rate, necrosis, and pleomorphism, was carried out by two separate gastrointestinal pathologists examining the tissue sections. Tissue margins were found to be free of disease in all of the collected specimens. Histologic grade was assigned to the GISTs according to previously published criteria.[21,22] Low-grade criteria included a mitotic rate of less than 5 per 50 high-power fields (hpf), low cellularity, and absence of pleomorphism and necrosis. High-grade tumors displayed a high mitotic rate (>5 per 50 hpf), high cellularity, pleomorphism/atypia, and necrosis. Parameters falling between these criteria designated intermediate-grade GISTs. To establish the definition of pathologic diagnosis of GIST, immunohistochemical analyses were performed for each of the tumors using the avidin-biotin-peroxidase method (Ventana/Biotek Solutions, Tucson, AZ) incorporating monoclonal antibodies for CD117/c-kit (Dakko Corp., Carpinteria, CA) and CD34 (Immunotech/Coulter, Westbrook, ME). All of the gastric GISTs in this cohort stained positively for CD117/c-kit by this assay. Table 1 lists the tumor characteristics and associated patient demographics

Table 1. Gastrointestinal, stromal tumor characteristics

Total number of tumors	N = 38
Tumor sites	Gastric
Median patient age (yr)	60.5 (range 30–86)
Male:female ratio	23:15
Mean tumor size (cm)	10.8 (0.6–34)
Median follow-up (mo)	40
Tumors >5 cm	N = 24 (63%)
Tumor mitotic index >5/50 hpf	N = 18 (47%)
Tumor with necrosis	N = 17 (45%)
Tumor cellularity	
High	N = 17 (45%)
Intermediate	N = 20 (53%)
Low	N = 1 (3%)
Tumor grade	
High	N = 7 (18%)
Intermediate	N = 26 (68%)
Low	N = 5 (13%)
Tumors with synchronous mets	
Hepatic	N = 2 (5%)
Regional	N = 5 (13%)

hpf = high-power fields; mets = metastases.

for the samples included in this study. Overall, 60% of the tumors were derived from male patients and 80% from Caucasian patients.

Several samples of resected stomach from pancreaticoduodenectomy specimens were designated as normal tissue.

DNA Preparation

Two sequential 10 μm sections from each gastric stromal tumor and normal stomach were deparaffinized with xylene and digested overnight at 50C with proteinase K buffered in 1% sodium dodecyl sulfate (pH = 8). DNA was isolated by phenol-chloroform extraction and ethanol precipitation.[23] Approximately 10 μg of DNA was partially purified from the two 10 μm tissue sections.

Methylation-Specific Polymerase Chain Reaction

The methylation status of the promoter regions for 11 tumor suppressor genes was determined by the methylation-specific polymerase chain reaction (MSP) method further modified as a nested two-step approach in order to increase the sensitivity of detecting allelic hypermethylation at targeted sequences and to facilitate the examination of multiple gene loci.[23–25] Initially, 1 μg of tissue DNA was treated with bisulfite according to previously described protocols to render unmethylated cytosines to uracil.[24] The bisulfite-treated DNA was column purified over Wizard cleanup resin (Promega Corp., Madison, WI) and ethanol precipitated. Step 1 of the nested MSP was carried out with primer sets (sense and antisense) for five individual genes in each reaction. Step 1 primers flanked the CpG-rich promoter regions of the respective targeted genes. Hence these primers did not discriminate between methylated and unmethylated nucleotides after bisulfite treatment. Polymerase chain reaction (PCR) products of step 1 were diluted 1:1000 and subjected to step 2 of the MSP that incorporated one set of primers for each gene (labeled as unmethylated [U] or methylated [M]) that were designed to recognize bisulfite-induced modifications of unmethylated cytosines. All of the primer sequences and PCR conditions for this nested-MSP approach have been published previously.[26] Both steps of the nested MSP used a 25 μl reaction volume, 0.5 μl of Jump Start Red *Taq* DNA polymerase (Sigma, St. Louis, MO), and 1 μl of DNA template. DNA isolated from normal peripheral lymphocytes from healthy individuals served as a negative methylation control. Human placental DNA was treated in vitro with SssI methyltransferase (New England Biolabs, Beverly, MA) to create completely methylated DNA at all CpG-rich regions. In vitro methylated DNA served as the positive methylation control. MSP products were analyzed on 6% polyacrylamide gel electrophoresis.

Statistical Analysis

Fisher's exact probability test was used to analyze the univariate differences in methylation status of tumor suppressor genes among gastric GISTs subdivided according to histologic characteristics (Stata Release 6, College Station, TX). A two-tailed *P* value of less than 0.05 was considered statistically significant. A univariate Cox analysis was applied to the differences among individual variables relating to survival or early tumor recurrence. Recurrence-free survival was measured from the time of diagnosis to the time of first recurrence or last follow-up. Kaplan-Meier curves were plotted to display graphically the prognostic value of methylation findings and histologic features in recurrence and survival.[27] The log-rank test was used to compare the differences between survival groups. Cox regression multivariate analysis of survival, adjusting for age, sex, histologic findings, and methylation differences, was used to compare the recurrence-free distributions of the various subgroups in order to identify independent predictors of recurrence-free survival.[28]

RESULTS

Gastrointestinal Stromal Tumor Hypermethylation

Using a nested approach of MSP, we examined the methylation status of 11 candidate tumor suppressor genes, *p16/INK4a*, *APC*, *O^6-methylguanine methyltransferase (MGMT)*, *hMLH1*, *p73*, *E-cadherin*, *retinoic acid receptor beta 2 (RAR-β)*, *ras association domain family protein 1 isoform A (RASSF1A)*, *retinoblastoma (RB)*, *estrogen receptor (ER)*, and *death-associated protein kinase 1 (DAPK)*, established to have roles in human cancer formation and progression.[29,30] Fig. 1

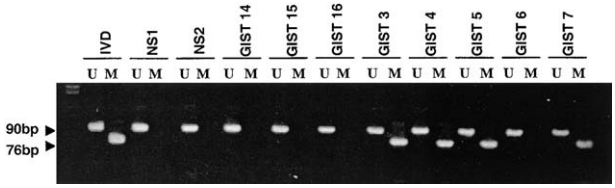

Fig. 1. The amplified products following step 2 of the nested MSP for MGMT. Lanes marked *U* contain products derived from unmethylated alleles; whereas products amplified from methylated alleles are found in lanes marked *M*. In vitro methylated DNA (*IVD*) served as the positive control. Tissue samples of normal stomach (*NS*) showed no methylation for MGMT or any of the 11 tumor suppressor genes. MGMT methylation was found for some of the gastrointestinal stromal tumor samples (*GIST*).

is representative of the promoter methylation findings for *MGMT* among GISTs of differing histologic grade. Five cases of normal stomach were included in our MSP studies and demonstrated no methylation relating to the candidate group of genes in this study. The absence of any promoter methylation within the normal gastric tissue supports the widely accepted paradigm that aberrant promoter hypermethylation of specific genes is likely a neoplastic-related or neoplastic-predisposing process.[12,29,30] Overall, we detected aberrant promoter methylation of at least one gene in 32 (84%) of the 38 gastric stromal tumors. Fig. 2 summarizes the methylation results for each of the 38 gastric stromal tumors, arranged according to methylation frequency. The frequency of methylation for each gene varied considerably. In decreasing order of frequency, the five most commonly methylated genes were: *MGMT* (47%), *p16* (45%), *RASSF1A* (40%), *E-cadherin* (37%), and *hMLH1* (34%). Multigene methylation, defined as methylation involving more than three gene promoters, was present in 42% of the tumors. However, distinct phenotypes were not readily apparent between tumors categorized according to multigene methylation. Differences in tumor mitotic rate, cellularity, necrosis, or overall grade did not correlate with the total number of genes methylated for each tumor.

When individual or multiple gene promoter methylation status was compared to differences in patient age (</>60 years), GIST size (with a cutoff at 5 cm), tumor mitotic rate (</>5 per 50 hpf), presence (or absence) of tumor necrosis, and locally advanced (or contained) disease, no significant multivariate differences in methylation were found between the tumor subgroups, except for *E-cadherin*. As shown in Fig. 3, *E-cadherin* methylation showed a marked, albeit nonsignificant, association with various traditional components of the clinicopathologic grading system for GISTs.[21] In general, *E-cadherin* methylation was found more commonly among more advanced, aggressive gastric stromal tumors.

Prognostic Factors for Gastrointestinal Stromal Tumor Behavior

In an effort to predict GIST behavior with improved reliability, we performed a univariate analysis of several factors with regard to clinical outcome. These studies were carried out to distinguish the tumor characteristics that afford the greatest prognostic value. Two-year follow-up data were available and complete for 87% (33 of 38) of the original patients. Of the 33 patients included for analysis, 27% experienced a recurrence of their GISTs within 24

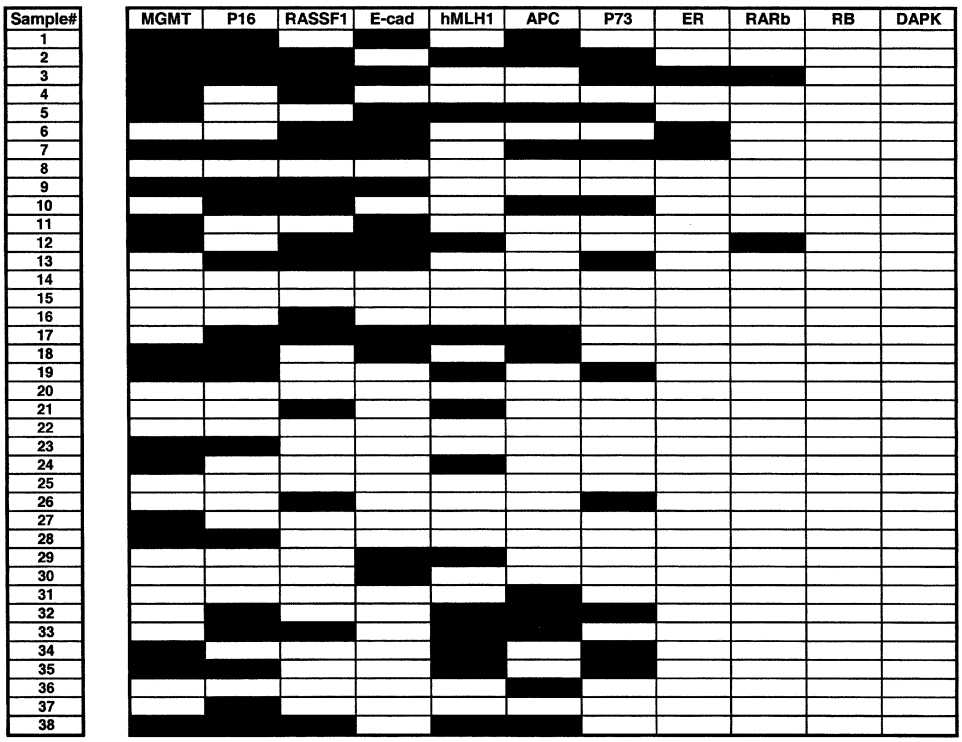

Fig. 2. Methylation profile for 11 tumor suppressor gene promoter regions involving 38 gastric stromal tumors. *Black grid squares* represent postive methylation. *Open squares* denote no detectable methylation.

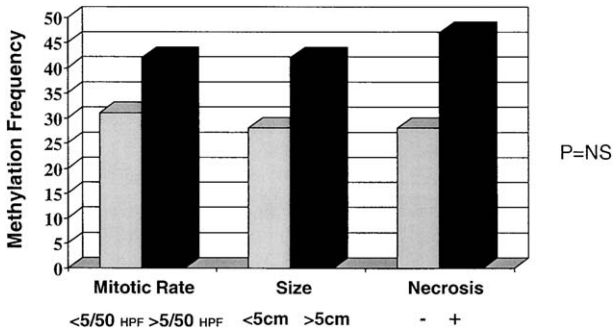

Fig. 3. The frequency of *E-cadherin* methylation among 38 gastric stromal tumors subdivided according to mitotic rate, tumor diameter, and presence of tumor necrosis. Fisher's exact probability test failed to show significant differences between the groups.

months from the time of surgery. In fact, all of the stromal tumors that recurred did so within the first 24 months after surgery. Among the five classic histologic features predictive of tumor behavior, mitotic rate greater than 5 per 50 hpf was associated with early tumor recurrence compared to mitotically dormant tumors (Table 2). In addition to tumor mitotic rate, high tumor grade and the presence of necrosis within a gastric stromal tumor were predictive of 2-year recurrence. Table 2 also presents the correlative findings for several prognostic histologic and methylation variables. As shown, trends in early recurrence are consistent with established indicators of malignant GIST behavior; however, statistical significance with a univariate Cox regression analysis was reached only for tumor necrosis and *E-cadherin* gene methylation. The association between regional GIST invasion of adjacent tissues and early recurrence is not shown. Of the nine tumors that recurred within 24 months, seven were found to have regional or hepatic metastases at the time of the original surgical resection; however, the presence of synchronous metastases was not a significant predictor of early GIST recurrence. In all of the grossly advanced GISTs, malignant histologic features were found.

As displayed in Fig. 3, aberrant promoter methylation (i.e., *E-cadherin*) correlated with malignant histologic findings; however, tumor suppressor gene methylation did not necessarily coincide with the presence of locally or regionally advanced disease. We therefore studied promoter methylation as an independent predictor for early recurrence after gastric stromal tumor resection. Only the presence of *E-cadherin* methylation and the absence of *hMLH1* methylation correlated with early tumor recurrence. Table 2 indicates that 50% of the GISTs with *E-cadherin* methylation recurred within 24 months compared to 14% of the unmethylated tumors. This difference in early tumor recurrence, based on *E-cadherin* methylation, was also significant during multivariate analysis adjusting for constant variables and histologic grade, $P = 0.030$. On the contrary, the presence of an unmethylated *hMLH1* promoter was associated with a worse outcome.

Long-term follow-up of 60 months or longer was achieved for 60% (23 of 38) of the original GISTs. The main reason for this substantial lack of follow-up data relates to the short median time from operation for this study (40 months). No significant study

Table 2. Univariate analysis* for 2-year recurrence

Factor	N	2-year recurrence	Hazard ratio	*P* value
Tumor size				
<5 cm	11	1 (9%)	REF	
>5 cm	22	8 (36%)	5.1	0.129
Tumor mitotic rate				
<5/50 hpf	15	0 (0%)	REF	
>5/50 hpf	18	9 (50%)	NA	NA
Tumor cellularity				
Low/intermediate	16	3 (19%)	REF	
High	17	6 (35%)	2.1	0.206
Tumor necrosis				
Absent	14	1 (7%)	REF	
Present	19	8 (42%)	14.2	0.011
Tumor grade				
Low	4	0 (0%)	REF	
Intermediate	22	5 (23%)	NA	NA
High	7	4 (57%)	NA	NA
Synchronous metastases				
Absent	26	4 (16%)	REF	
Present	7	5 (71%)	4.7	0.185
APC methylation				
Negative	24	8 (33%)	REF	
Positive	9	1 (11%)	0.43	0.213
P16 methylation				
Negative	20	6 (30%)	REF	
Positive	13	3 (23%)	0.79	0.738
MGMT methylation				
Negative	17	6 (35%)	REF	
Positive	16	3 (19%)	0.6	0.473
hMLH1 methylation				
Negative	23	9 (39%)	REF	
Positive	10	0 (0%)	NA	NA
E-cadherin methylation				
Negative	21	3 (14%)	REF	
Positive	12	6 (50%)	4.9	0.025
RASSF1 methylation				
Negative	20	4 (20%)	REF	
Positive	13	5 (38%)	2.0	0.283

NA = no events in reference cohort.
*Cox regression analysis.

variables related to the loss or inclusion of patients during follow-up. After adjusting for age, sex, and histologic variables, only tumor mitotic rate, tumor necrosis, and *E-cadherin* methylation were found to be significant predictors of disease-free survival at 5 years (Table 3). The presence of *E-cadherin* methylation within a gastric stromal tumor at the time of surgical resection was associated with a survival disadvantage at 5 years, 55% vs. 83% for patients with unmethylated *E-cadherin* tumor alleles, $P = 0.028$ (Fig. 4). The promoter methylation statuses of the other 10 tumor suppressor genes included in this study were not significant as prognostic markers.

Given the association of *E-cadherin* methylation with GIST malignant histology, 5-year tumor-free survival was significantly lower in patients with mitotically active tumors (>5/50 hpf) compared to GISTs without this malignant feature (52% vs. 94%, $P = 0.036$). Among the patients at risk for early tumor recurrence and mortality, namely, those with primary GISTs with a mitotic rate greater than 5/50 hpf, *E-cadherin* methylation was able to stratify the risk for disease progression (Fig. 5). On multivariate Cox regression analysis, *E-cadherin* methylation was associated with a higher disease-specific mortality among the mitotically active GISTs compared to the mitotically active, *E-cadherin* unmethylated tumors (hazard ratio = 5; $P = 0.030$).

Table 3. Multivariate analysis* for 5-year survival

Factor	N	5-year tumor-free survival	Hazard ratio	*P* value
Tumor size				
<5 cm	8	7 (88%)	REF	
>5 cm	15	8 (53%)	2.5	0.26
Tumor mitotic rate				
< 5/50 hpf	9	8 (89%)	REF	
>5/50 hpf	14	7 (50%)	4.1	0.036
Tumor cellularity				
Low/Intermediate	11	8 (73%)	REF	
High	12	7 (58%)	1.7	0.36
Tumor necrosis				
Absent	8	7 (88%)	REF	
Present	15	8 (53%)	10.6	0.002
Tumor grade				
Low	2	2 (100%)	REF	
Intermediate	15	12 (80%)	NA	NA
High	6	1 (16%)	NA	NA
Synchronous metastases				
Absent	18	14 (78%)	REF	
Present	5	1 (20%)	5.5	0.205
APC methylation				
Negative	16	10 (63%)	REF	
Positive	7	5 (71%)	0.98	0.986
P16 methylation				
Negative	13	8 (62%)	REF	
Positive	10	7 (70%)	3.9	0.067
MGMT methylation				
Negative	12	7 (58%)	REF	
Positive	11	8 (73%)	1.3	0.62
hMLH1 methylation				
Negative	18	10 (56%)	REF	
Positive	5	5 (100%)	0.37	0.21
E-cadherin methylation				
Negative	13	10 (77%)	REF	
Positive	10	5 (50%)	3.4	0.048
RASSF1 methylation				
Negative	13	7 (54%)	REF	
Positive	10	8 (80%)	1.1	0.86

NA = no events in reference cohort.
*Cox regression analysis.

DISCUSSION

Despite the fact that the pathologic criteria for defining GISTs have been refined over recent years, especially with the routine application of CD117/c-kit immunohistochemical analysis, there continues to be difficulty in predicting the clinical outcome of patients with GISTs after surgery. In 2000 DeMatteo et al.[31] reported a 54% 5-year survival for patients who underwent complete resection of a primary GIST at Memorial Sloan-Kettering Cancer Center. In that single-institution study, only tumor size was predictive of GIST recurrence, typically within the abdominal cavity and/or liver. Other retrospective studies have shown prognostic value for both tumor size and margin of resection; however, tumor mitotic rate continues to be the most reliable indicator of GIST malignant behavior.[32,33] Although mitotic rate has proved itself to be a reliable prognostic indicator for GIST behavior, the lack of universal guidelines for calculating the tumor mitotic rate and threshold for malignant mitotic activity mandates a more objective set of molecular criteria for predicting GIST outcomes.

A recent study by Singer et al.[2] explored the use of specific *c-kit* mutations as a predictor of GIST outcome. Although the characterization of *c-kit* mutations was no more reliable than tumor mitotic activity as an indicator of malignant potential, patients with missense exon 11 mutations experienced an improved 5-year tumor-free survival compared to patients with other *c-kit* mutation types (89% vs. 40%; $P = 0.03$). Several other molecular markers have been studied for their prognostic value in a variety of human cancers.[34–40] *MGMT* promoter methylation has been linked to increased *K-ras* mutations, and subsequently

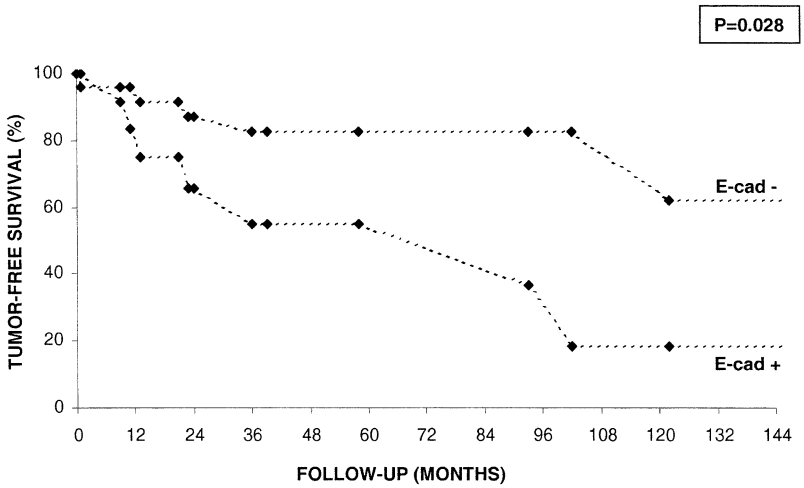

Fig. 4. Disease-free survival at 5 and 10 years for patients with (*E-cad+*) or without (*E-cad−*) *E-cadherin* tumor methylation at the time of surgical resection (time *0*). Statistical significance between the two survival groups was determined by the log-rank test.

poor prognosis, in patients with gastric cancer.[40] Likewise, *p53* genetic mutations have been used to predict outcomes for surgically treated gastric cancer patients.[36] Clearly a greater understanding of GIST biology will help to elucidate novel molecular markers for predicting malignancy.

In characterizing the methylation status of multiple tumor suppressor genes in these gastric stromal tumors, we sought to determine some of the epigenetic changes contributing to the molecular events responsible for the unique clinical and biological behavior of GISTs. Particular methylation patterns of tumor, margins, and adjacent tissue can be indicative of occult regional and/or systemic metastasis, and methylation at specific genomic sites may be predictive of early tumor recurrence after complete surgical extirpation. From the 11 tumor suppressor genes that we studied, only the methylation status of two promoter sites was predictive of early tumor recurrence within 24 months of the time of surgery. The presence of methylated *E-cadherin* alleles or the absence of methylated *hMLH1* alleles correlated with increased early tumor recurrence (50% and 39%, respectively) compared to the overall 27% recurrence rate for the GISTs in this study. Although *E-cadherin* methylation was associated with malignant histologic

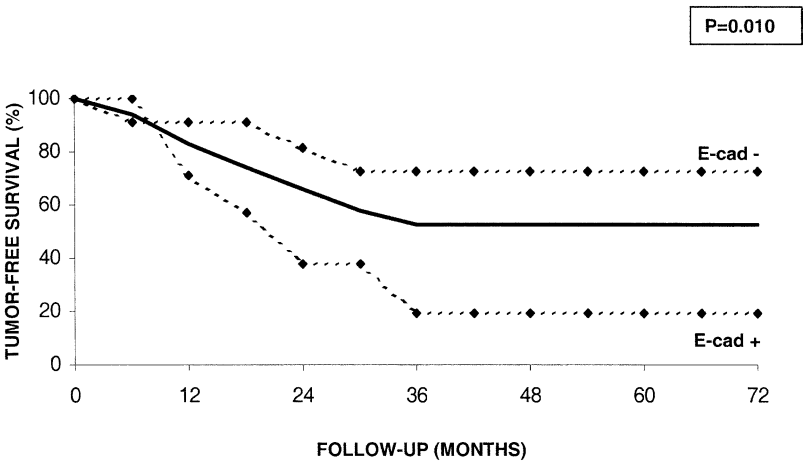

Fig. 5. Disease-free survival at 6 years for patients with gastric stromal tumors with a high mitotic rate (>5 per 50 hpf). The disease-free survival for all patients with a tumor mitotic rate (>5 per 50 hpf) is shown (*solid line*). The long-term survival of patients with mitotically active tumors is shown according to the presence (*E-cad+*) or absence (*E-cad−*) of *E-cadherin* methylation (*dashed lines*).

features including size, mitotic activity, and necrosis, there were no significant differences in either *E-cadherin* or *hMLH1* methylation between GIST subpopulations divided according to the presence of advanced neoplastic disease, that is, adjacent tissue invasion.

Aberrant hypermethylation of specific promoter regions is associated with the loss of *E-cadherin* expression. In this study we examined the methylation status of the exact *E-cadherin* promoter region (approximately 185 base-pairs in size) that has been shown to coincide directly with *E-cadherin* expression in a variety of normal and neoplastic human tissues.[24] Methylation-induced suppression of promoter activity in neoplastic cells, and the loss of *E-cadherin* expression, has a causal role in the progression from benign to malignant cancer.[41] The loss of E-cadherin expression presumably potentiates a neoplastic cell's transition to an invasive phenotype as a result of disrupted epithelial organization and cell-cell growth inhibition. Although these phenotypic changes are not directly responsible for increased cell proliferation and tissue invasion, loss of *E-cadherin*–mediated cell adhesion may affect other signaling pathways with a more direct role in the neoplastic phenotype. Presumably, tumors that have lost *E-cadherin* expression secondary to aberrant promoter methylation would be expected to carry greater malignant potential.

Clinical follow-up 60 months or longer was limited to 23 of the original 38 patients included for study; however, no differences in methylation findings or histologic parameters were observed between tumors included and tumors excluded from the 5-year survival studies. This reduction in population size caused difficulty for statistical analyses with Cox regression, correlating long-term disease-free survival to tumor suppressor gene methylation patterns or even histologic features, to demonstrate any statistically significant differences between defined subgroups. For example, *E-cadherin* methylation predisposed patients to a poorer 5-year disease-free survival compared to tumors without *E-cadherin* methylation (55% vs. 83%). The converse was found for *hMLH1* methylation, whereby promoter methylation was associated with improved outcome. Other reports have found that *hMLH1* methylation frequently coincides with a microsatellite unstable phenotype that carries an improved clinical outcome for colorectal cancer.[42] Given the survival advantage afforded by microsatellite instability in colon cancer, our finding of *hMLH1* methylation and improved survival is consistent with previous studies demonstrating an improved clinical outcome resulting from a loss of mismatch repair gene expression.[42,43]

This study has shown that *E-cadherin* methylation within gastric stromal tumors can be used as a clinical marker to discriminate patients at highest risk for disease recurrence and mortality. Among the tumors with a high mitotic rate greater than 5 per 50 hpf, and thus at risk for disease progression after curative resection, the presence of *E-cadherin* methylation was associated with a poorer 5-year survival (19%) compared to mitotically active GISTs without methylation at this genomic site (71%; $P = 0.010$). Disregarding *E-cadherin* methylation status, patients with mitotically active gastric stromal tumors experienced a 52% 5-year survival. Thus the methylation patterns of specific genes may be able to identify molecularly distinct tumors with histologically indistinguishable features. As a result, tumor methylation status may be useful for stratifying patients, with the highest risk for disease progression, to different adjuvant treatments.

The results of this study encourage future investigations into understanding GIST biology from a genetic, epigenetic, and proteomic standpoint. A greater appreciation of the molecular events that underlie the behavior of this interesting tumor will afford even further novel strategies for developing diagnostic, prognostic, and therapeutic systems.

CONCLUSION

Detection of methylation within specific tumor suppressor genes affords a reliable and accurate molecular marker system for predicting neoplastic behavior for GISTs. Furthermore, the methylation patterns of specific genes can identify molecularly distinct tumors with histopathologically indistinguishable features.

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Discussion

Dr. M. Zenilman (Brooklyn, NY): I have two questions. First, do you think that your DNA methylation assay is going to be applicable to other tumors in the gastrointestinal tract, specifically colon cancer, and which specific genes do you think are going to be the ones to look at in that tumor?

Second, this study of GISTs begs the question of how this finding of genetic manipulation correlates with changes in other genes, which we know are involved with gastrointestinal stromal tumors, specifically the c-kit oncogene mutations.

Dr. M. House: This study was carried out to develop a simple PCR-based molecular marker system that would be able to stratify GISTs into high- vs. low-risk groups for recurrence. Although this study focused exclusively on *c-kit*–positive GISTs arising from the stomach, our group has applied this identical methylation-specific PCR methodology to malignancies arising all along the alimentary tract.

For colorectal cancer, we have aimed to use methylation-induced silencing of several tumor suppressor genes, specifically the *hMLH1*, *O*^{6}*-MGMT*, and *p*16 genes, as a predictor of not only recurrence and survival but also as a predictor of response to adjuvant therapy. Recently our group has shown that O^6-methylguanine methyltransferase, the *MGMT* gene, is an independent marker for predicting a positive response to alkylating chemotherapy in gliomas. So we would like to be able to use individual (and potentially multiple) tumor suppressor gene methylation profiles as a reliable prognostic marker system for several gastrointestinal malignancies.

For this study of GISTs, we originally wanted to determine whether an individual neoplasm's gene methylation pattern could predict a response to ST1571. Although we have a large clinical database with nearly 100 GISTs over a 15-year period, only eight of the patients in this study have received Gleevec on an adjuvant basis. Consequently our data are not mature enough to determine if any of these methylation markers would be predictive of a response to Gleevec.

Dr. J. Deutsch (Duluth, MN): Is there any clinical predictor of which patients are more likely to be methylated? If you had a program of 70-year-old patients vs. 50-year-old patients or patients who present with 2 cm tumors vs. 1 cm tumors, is there any way you can determine who is more likely to have a methylated GIST?

Dr. House: That is an excellent question, and it sounds like you may have performed some methylation studies in your own laboratory. One limitation of methylation studies is that gene methylation is not an entirely neoplastic-specific phenomenon. Many genes at one point during a cell's life span will develop aberrant methylation, even during benign processes that may or may not lead to complete gene silencing. The patients that really plague methylation studies are older patients, and by "older" I mean over 60 years of age (the age group at risk for developing cancer) as well as chronic smokers. Smokers, in particular, have frequent methylation of multiple genes even in benign lesions. For these two groups of patients, screening biopsies of the colon, for example, will reveal methylation of multiple genes, and particularly O^6-methylguanine methyltransferase, and p16.

Dr. S. Marcus (New York, NY): Typically hypermethylation offers an improved prognosis. I think you demonstrated, earlier, at the Pancreas Club meeting, that this was true with neuroendocrine tumors, and it is known to offer a better prognosis with colorectal cancer. Why are the results opposite with gastrointestinal stromal tumors, namely, that hypermethylation seems to have a worse prognosis in these patients?

Dr. House: Let me clarify this confusion. Methylation of the hMLH1 gene is associated with a replication error phenotype, manifested as microsatellite instability. The finding that hMLH1 methylation and microsatellite instability seem to cluster in patients with improved survival is consistent with other reports, namely, studies of colon cancer. Aside from hMLH1 methylation, methylation of other candidate gene markers, as well as multiple markers, is typically associated with a poorer prognosis and a more malignant phenotype.

Dr. L. Rikkers (Madison, WI): How discriminating was methylation? In the group of patients who had a high number of mitoses, was there a significant difference in the number of mitoses within that group between the methylated and nonmethylated subgroups?

Dr. House: Dr. Rikkers' question addresses the subgroup analysis of patients with mitotically active GISTs, with mitotically active being defined as greater than 5 per 50 high-power fields. Within this group, there were differences in gene methylation, specifically for E-cadherin methylation, that seemed to be associated with patients with very high mitotic rates greater than 25 per 50

high-power fields. Because there is tremendous variability in reporting an accurate mitotic index for GISTs, it is difficult to apply this histologic feature as a reproducible marker of malignant behavior. Also, the accuracy of the mitotic index depends on the amount of the pathologist's exposure to GISTs. Accordingly, we wanted to develop objective criteria, regardless of histologic features, to stratify patients into high- and low-risk groups.

Invited Discussion—Expert Commentator

David Fromm, M.D. (Detroit, MI): The study by Dr. House and colleagues provides another sophisticated means for determining the prognosis of GISTs. A number of genes have been proposed to be involved in cancer initiation of progression, and among these are promoter hypermethylation resulting in gene silencing. Hypermethylation is a consequence of DNA methyltransferase catalyzing the transfer of a methyl group from S-adenosyl-thionine to cytosine residues to form 5-methylcytosine. Several genes are selectively methylated in many cancers, but it is not yet known why certain genes are selected over others. In general, aberrant methylation is more often, but not exclusively, detected in cancer cells, as also noted in the present study. What has not been sorted out yet is the significance of hypermethylation of epithelial cells in focal patches of histologically normal tissues of cancer-free patients. Hypermethylation is not an isolated event but is linked to other events of epigenetic control. It would be interesting to know what the relationship is between the events described by Dr. House to the activation of c-kit, a growth factor receptor with tyrosine kinase activity that is an important target of current adjuvant therapy for GISTs.

Complications of Gastrectomy Following CPT-11–Based Neoadjuvant Chemotherapy for Gastric Cancer

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Potential benefits of neoadjuvant therapy for locally advanced gastric cancer include tumor downstaging and an increased R0 resection rate. Potential disadvantages include increased surgical complications. This study assesses postoperative morbidity and mortality by comparing patients undergoing gastrectomy with and without neoadjuvant chemotherapy. From October 1998 to July 2002, a total of 34 patients with locally advanced gastric cancer were placed on a phase II neoadjuvant chemotherapy protocol consisting of two cycles of CPT-11 (75 mg/m^2) with cisplatin (25 mg/m^2). Demographic, clinical, morbidity, and mortality data were compared for these patients (CHEMO) versus 85 patients undergoing gastrectomy without neoadjuvant chemotherapy (SURG). The CHEMO patients were more likely to be less than 70 years of age ($P \leqslant 0.01$), have proximal tumors ($P \leqslant 0.01$), and undergo proximal gastrectomy ($P \leqslant 0.025$). Fifty-two percent of SURG patients had T3/T4 tumors compared to 19% of CHEMO patients, consistent with tumor downstaging. The R0 resection rate was similar (80%). Morbidity was 41% in CHEMO patients and 39% in SURG patients. There were five postoperative deaths (4.4%), two in the CHEMO group and three in the SURG group (P = NS). It was concluded that neoadjuvant chemotherapy with CPT-11 and cisplatin is not associated with increased postoperative morbidity compared to surgery alone. CPT-11–based neoadjuvant chemotherapy should be tested further in combined-modality treatment of gastric cancer. (J GASTROINTEST SURG 2003;7:1015–1023)

KEY WORDS: Stomach neoplasms, gastrectomy, antineoplastic combined chemotherapy protocols, irinotecan, antineoplastic agents

Complete surgical resection of tumors with negative margins (R0 resection) is the most effective treatment for gastric cancer and is associated with improved long-term survival.[1,2] Strategies to increase the R0 resection rate and long-term survival in patients with gastric cancer include an emphasis on early diagnosis, improved preoperative clinical staging, extended lymph node dissection, and adjuvant and neoadjuvant chemotherapy. In the United States, routine screening for gastric cancer is not widely practiced. Consequently most patients present with advanced disease with a poor prognosis.[1] Improvements in preoperative staging, including the use of endoscopic and laparoscopic ultrasound imaging, may increase the R0 resection rate by decreasing the number of patients undergoing unnecessary laparotomy.[3] Unfortunately, the survival benefits of extended lymphadenectomy for gastric cancer seen in Japanese reports have not been realized in Western series. These techniques have been associated with increased morbidity and mortality and have not been widely adopted.[4,5] Until recently, adjuvant therapy following gastrectomy has not been shown to be beneficial to patients.[6,7] A significant survival benefit was recently reported for good performance status patients receiving adjuvant chemoradiotherapy following R0 gastrectomy compared to surgery alone.[8] Although this regimen is being widely embraced, patients

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with poor performance status or an incomplete resection were not included in the trial.

Various regimens of neoadjuvant chemotherapy in gastric cancer have been shown to induce tumor responses that may benefit patients with locally advanced gastric cancer by increasing their chances of being able to undergo a complete resection.[9–11] In addition, patients who may not be eligible to receive postoperative adjuvant therapy because of poor performance status secondary to postoperative complications may benefit from receiving systemic therapy first. There are limited data available regarding postoperative morbidity and mortality in patients receiving neoadjuvant chemotherapy for gastric cancer. If neoadjuvant chemotherapy is going to be considered as a therapeutic option in patients with locally advanced gastric cancer, it is necessary to verify that it can be delivered safely without an increase in postoperative morbidity and mortality.

We have previously shown in our institutional phase II trial that neoadjuvant chemotherapy with CPT-11 and cisplatin can downstage locally advanced gastric cancer.[12] The current study was undertaken to assess the postoperative morbidity and mortality in this group of patients receiving neoadjuvant chemotherapy prior to gastrectomy for gastric cancer in comparison to a group of patients undergoing gastrectomy without neoadjuvant chemotherapy during the same time period, in the same institutions, by the same surgeons.

METHODS

Thirty-four patients with histologically proved gastric adenocarcinomas were entered into a phase II trial of neoadjuvant CPT-11/cisplatin chemotherapy followed by surgery and then postoperative intraperitoneal chemotherapy between October 1998 and May 2002 (CHEMO group). Of these, two withdrew during induction chemotherapy and three others did not come to resection, leaving 29 patients who were evaluable for postoperative morbidity and mortality. Eighty-five patients undergoing gastric resection for gastric adenocarcinoma without neoadjuvant chemotherapy by the same surgeons in the same hospitals during this same time period were identified, and their medical records were retrospectively reviewed for clinical and pathologic data (SURG group). Patients at the following three affiliated hospitals of the New York University (NYU) School of Medicine were included in the study: NYU Tisch Hospital, which is a private urban teaching hospital; Bellevue Hospital, which is an urban public teaching hospital with a large immigrant population; and the Manhattan Veteran's Administration Hospital.

Before beginning therapy, patients were clinically staged on the basis of history and physical examination, upper endoscopy, abdominal and pelvic CT scans, and chest x-ray examination. Patients with lesions at the gastroesophageal junction had CT scans of the chest. Adenocarcinomas at the gastroesophageal junction that were not associated with Barrett's epithelium were treated as gastric cancers and are included in this analysis. Patients with cancers with Barrett's epithelium were treated as esophageal cancers and are not included in this study. All CHEMO patients and many SURG patients underwent endoscopic and/or laparoscopic ultrasonography. The eligibility criteria for the phase II neoadjuvant chemotherapy trial have been described in detail previously.[12] Patients were required to sign informed consent forms as approved by the institutional review board of New York University School of Medicine. Patients needing urgent surgery for obstruction, perforation, or bleeding were not eligible to receive neoadjuvant chemotherapy.

Induction chemotherapy with cisplatin and CPT-11 was given for two 6-week cycles consisting of 4 consecutive weeks of therapy followed by a 2-week break.[12] Gastric resection was performed in a similar fashion for both groups. Briefly, patients received perioperative antibiotics, usually a second-generation cephalosporin. Type of gastrectomy, performance of multivisceral resection, and extent of lymph node dissection were left to the discretion of the operating surgeon. R0 resection is defined as en bloc removal of the primary tumor with draining lymph nodes and omentum, with negative margins. Extended (D2) lymph node dissection is often practiced at our institutions and was encouraged for CHEMO patients. For patients receiving neoadjuvant chemotherapy, surgical resection was planned for approximately 4 weeks following completion of induction chemotherapy. All CHEMO patients were restaged with abdominal and pelvic CT scans immediately prior to planned resection. At the completion of resection in CHEMO patients, an intraperitoneal catheter (Mediport; Bard Access Systems, Salt Lake City, UT) was implanted subcutaneously over the costal margin, for the delivery of postoperative intraperitoneal chemotherapy with floxuridine (FUDR) and cisplatin.

Demographic, clinical, and pathologic characteristics of the two groups were analyzed. Mann-Whitney U test was used to compare median values. Comparison of observed vs. expected results in two by two tables was performed with chi-square anlysis. Univariate linear regression analysis was performed to determine factors predictive of complications. P values are reported for a two-tailed test, with $P < 0.05$ accepted as significant.

Table 1. Demographics

	CHEMO (n = 34)*	SURG (n = 85)	*P* value
Median age (years)	58 (37–78)	66 (21–92)	NS
⩾70 yr	4 (12%)	35 (41%)	<0.01
<70 yr	30 (88%)	50 (59%)	
Sex			
Female	12 (35%)	31 (36%)	NS
Male	22 (65%)	54 (64%)	
Race			
Asian	11 (32%)	24 (28%)	NS
Caucasian	13 (38%)	47 (55%)	
Hispanic	7 (21%)	7 (8%)	
African-American	3 (9%)	7 (8%)	
Hospital			<0.01
Bellevue	18 (53%)	22 (26%)	
NYU-Tisch	14 (41%)	62 (73%)	
Manhattan VA	2 (6%)	1 (1%)	

NS = not significant.
*Includes all patients registered in the phase II neoadjuvant chemotherapy trial.

RESULTS

Patient demographics, including all registered CHEMO patients, are summarized in Table 1. The median age for the entire group was 64 years. CHEMO patients tended to be younger than SURG patients (58 years vs. 66 years; $P = 0.07$). Although median age was not significantly different between the two groups, 41% of SURG patients were older than 70 years compared to 12% of CHEMO patients ($P < 0.01$). Of note, the protocol included a high proportion of minority patients, which is consistent with the diverse population of patients seen at our institutions, especially Bellevue Hospital, where a significantly higher proportion of patients were entered ($P < 0.01$).

Clinical characteristics are outlined in Table 2. CHEMO patients were more likely to have proximal tumors with 44% being located at the gastroesophageal junction/cardia compared to 20% in the SURG group. Conversely, SURG patients were more likely to have distal lesions ($P \leqslant 0.01$). This was reflected in the surgery performed, with 38% of CHEMO patients undergoing distal subtotal gastrectomy compared to 60% of SURG patients ($P \leqslant 0.025$). Partial proximal gastrectomy was performed more often in the CHEMO group (31% vs. 13%; $P \leqslant 0.025$). Although pathologic staging (according to the American Joint Committee on Cancer [AJCC] system) at the time of resection was similar in the two groups, more T2 tumors were seen in the CHEMO group as compared to the SURG group, which is consistent with a tumor downstaging effect ($P < 0.025$). Eighty

Table 2. Clinical characteristics

	CHEMO (n = 32)*	SURG (n = 85)	*P* value
Tumor location			⩽0.01
Gastroesophageal junction/cardia	14 (44%)	17 (20%)	
Body	9 (28%)	19 (22%)	
Antrum	9 (28%)	47 (55%)	
Gastric remnant	0 (0%)	2 (2%)	
Type of resection			⩽0.025
None	3 (9%)	0 (0%)	
Total	7 (22%)	23 (27%)	
Partial, proximal	10 (31%)	11 (13%)	
Partial, distal	12 (38%)	51 (60%)	
Extent of resection			NS
Unresectable	3 (9%)	0 (0%)	
R0	25 (78%)	69 (81%)	
R1	3 (9%)	7 (8%)	
R2	1 (3%)	9 (11%)	
Multivisceral resection	6 (19%)	20 (24%)	NS
En bloc	1	10	
Spleen	1	3	
Distal pancreas/spleen	0	1	
Distal pancreas/ spleen/colon	0	2	
Colon	0	3	
Partial liver	0	1	
Other	5	10	
Pathologic tumor (T) stage			⩽0.025
Not staged	3 (9%)	0 (0%)	
T0	1 (3%)	0 (0%)	
T1	4 (13%)	14 (16%)	
T2	18 (56%)	27 (32%)	
T3	5 (16%)	38 (45%)	
T4	1 (3%)	6 (7%)	
Pathologic node (N) stage			NS
Not staged	3 (9%)	0 (0%)	
N0	8 (25%)	33 (39%)	
N1	10 (31%)	31 (36%)	
N2–3	11 (34%)	21 (25%)	
Pathologic metastasis (M) stage			NS
M0	26 (81%)	74 (87%)	
M1	6 (19%)	11 (13%)	
AJCC stage			NS
Stage 0	1 (3%)	0 (0%)	
Stage I	8 (25%)	28 (33%)	
Stage II	8 (25%)	17 (20%)	
Stage III	7 (22%)	23 (27%)	
Stage IV	8 (25%)	17 (20%)	
No. of nodes resected (median)	20 (1–121)	18 (2–48)	NS

NS = not significant.
*Excludes three patients who withdrew from the phase II chemotherapy trial during induction chemotherapy.

percent of the entire group underwent potentially curative R0 resection, and this was similar in the two groups.

Multivisceral resection was performed in 24% of SURG patients as compared to 19% of CHEMO patients (P = NS). In the SURG group, 10 patients underwent en bloc multivisceral resection for locally advanced tumors, including splenectomy in three, distal pancreatectomy/splenectomy in one, distal pancreatectomy/splenectomy/colectomy in two, colectomy in three, and partial liver resection in one. Ten SURG patients underwent additional organ resections at the time of gastrectomy, including cholecystectomy in seven, small bowel resection in one, liver wedge resection in one, and cholecystectomy/liver wedge resection in one. One CHEMO patient had an en bloc splenectomy for an intraoperative splenic injury as well as a cholecystectomy. Five CHEMO patients had additional organs resected: cholecystectomy in three, liver wedge resection in one, and right colectomy in one.

Complications occurred in 45 (39%) of the 114 patients undergoing resection and were not significantly different between the two groups (Table 3). In the SURG group, complication rates were similar—39% for patients older and younger than 70 years of age. In the CHEMO group, only one of the four patients older than 70 had morbidity (pulmonary edema). Overall, nonsurgical complications were more common than surgical complications. The most common nonsurgical complications were pneumonia and urinary tract infection. Wound infection and postoperative obstruction or ileus were the most common surgical complications. SURG patients were more likely to have multiple complications.

Of the 114 patients undergoing gastrectomy, there were five deaths (4.4%) and seven patients needed early reoperation (6.8%). Neoadjuvant chemotherapy did not significantly increase the risk of postoperative complications, mortality, or the need for reoperation. The two deaths in the CHEMO group were the result of cardiac tamponade on postoperative day 4 and presumed pulmonary embolus on postoperative day 9. This latter patient presented with superior vena cava syndrome associated with a central venous catheter following the completion of neoadjuvant chemotherapy. The clot dissolved after removal of the catheter and 48 hours of thrombolytic therapy. This patient underwent gastrectomy 1 week later and had a sudden respiratory arrest on postoperative day 9. He died shortly after undergoing emergency reexploration, which failed to reveal an intra-abdominal source of his sudden death. Two additional patients in the CHEMO group underwent late reexploration: one on postoperative day 49 for resection of a stricture following an esophagojejunal anastomotic leak and one for completion gastrectomy for gastric atony following a proximal gastrectomy on postoperative day 48.

In the SURG group there were three postoperative deaths, all of them the result of multiorgan failure following respiratory/septic complications on postoperative days 18, 42, and 138, respectively. Four SURG patients underwent reexploration. One was for postoperative hemorrhage in a patient who eventually died, and one for completion gastrectomy after a positive proximal margin was identified on a distal gastrectomy specimen. Two patients were readmitted in the early postoperative period with small bowel obstruction and underwent reexploration for enterolysis.

By linear regression analysis, there was no significant association found between the development of complications and the following variables: age, sex, race, hospital, tumor location, type of resection, extent of resection (R0), multivisceral resection, nodal dissection, pathologic AJCC stage, and whether or not the patient received neoadjuvant chemotherapy.

DISCUSSION

With the exception of patients diagnosed with early gastric cancer, most patients with gastric cancer suffer a recurrence and eventually die of their disease, including patients undergoing R0 resection. Until recently there has been little evidence supporting the use of adjuvant therapy in these patients.[6–8] Neoadjuvant chemotherapy for gastric cancer has been used with varying success to downstage locally advanced gastric cancer.[12–24] This study was designed to examine postoperative morbidity and mortality in patients receiving neoadjuvant CPT-11–based chemotherapy compared to a group of patients undergoing surgical resection only during the same time frame by the same surgeons. Our results indicate that CPT-11–based neoadjuvant chemotherapy does not significantly increase the risk of postoperative complications in patients undergoing gastrectomy for gastric cancer.

Surgical morbidity and mortality following gastrectomy can be substantial. The most frequent complications following gastrectomy for gastric cancer are pulmonary, anastomotic leakage, intra-abdominal abscess, and wound infection.[25–35] In patients with gastric cancer receiving neoadjuvant chemotherapy followed by resection, postoperative morbidity ranges from 23% to 40% and mortality from 0% to 10%.[14,15,18–20,22,23] This is similar to reports of morbidity and mortality in patients undergoing gastric resection without neoadjuvant chemotherapy.[25–35]

Table 3. Morbidity and mortality

	CHEMO (n = 29)*	SURG (n = 85)	*P* value
Patients with complications	12 (41%)	33 (39%)	NS
No complications	17 (59%)	52 (61%)	
Postoperative LOS (days)	9 (3–75)	7 (4–138)	NS
Postoperative LOS with complications (days)	12 (4–75)	11 (5–138)	NS
Postoperative LOS without complications (days)	7 (3–10)	7 (4–12)	NS
Nonsurgical complications			
Arrhythmia	0	5	
Ascites	0	1	
Bacteremia	1	3	
Cardiac tamponade	1	0	
Colitis, *C. dificile*	0	3	
Deep venous thrombosis	0	1	
Gastrointestinal hemorrhage	1	1	
Jaundice	0	2	
Mental status changes	0	1	
Pleural effusion	1	3	
Pneumonia	2	6	
Poor intake by mouth	1	2	
Pulmonary edema	1	2	
Pulmonary embolus	1	0	
Renal Insuffciency	0	1	
Urinary tract infection	0	7	
Total nonsurgical complications	9	38	
No. of patients	7 (24%)	25 (29%)	NS
Surgical complications			
Anastomotic leak	1	0	
Enterocutaneous fistula	0	1	
Intra-abdominal abscess	0	2	
Postoperative bowel obstruction/ileus	2	6	
Postoperative hemorrhage	0	1	
Wound infection	5	9	
Total surgical complications	8	19	
No. of patients	7 (24%)	12 (14%)	NS
Reexploration	3 (10.3%)	4 (4.7%)	NS
Mortality	2 (6.9%)	3 (3.5%)	NS

LOS = length of stay; NS = not significant.
*Excludes two patients who withdrew from the phase II neoadjuvant chemotherapy trial during induction chemotherapy and three patients not coming to resection.

Unfortunately, most studies providing detailed analysis of postoperative complications in patients receiving neoadjuvant chemotherapy have not included a comparative group of patients undergoing surgery alone.[15,18,20,22,23]

Similar to our study, Kelsen et al.[19] compared operative morbidity and mortality in 50 patients undergoing surgical exploration following neoadjuvant therapy with fluorouracil, doxorubicin, and methotrexate to a concurrent group of 159 patients undergoing curative or palliative resection without neoadjuvant therapy during the same time period. Overall morbidity and mortality was 30.6% and 5.7%, respectively, and was not significantly different between the two groups. These results are similar to findings in our study.

Factors reported to influence morbidity in patients undergoing gastrectomy for gastric cancer include multiorgan resection, especially splenectomy and distal pancreatectomy, age greater than 70 years with underlying cardiopulmonary or renal disease, and extended lymph node dissection.[26–28,30–32] In the current study, no significant factors were found to be associated with the development of complications. Although SURG patients had a higher proportion of patients older than 70, increasing age was not associated with the development of postoperative complications. Only four patients older than 70 were included

in the current phase II trial. It is possible that accrual of geriatric patients was low because of physicians' concerns about toxicity with neoadjuvant chemotherapy. Further study is necessary to confirm the safety of this regimen in patients older than 70. Although extended lymphadenectomy was often performed in our patients, multivisceral resection, especially distal pancreatectomy and splenectomy, was rarely necessary. The low rate of major surgical complications and mortality secondary to surgical complications in the current series may partially relate to our limited use of multivisceral resection.

Relaparotomy for complications of gastrectomy is necessary in 2% to 12% of cases.[27–30,34] In a large series of 700 gastrectomies reported by Shchepotin et al.,[34] 40 patients (5.7%) underwent reoperation with an associated mortality of 62.5%. Anastomotic leakage and pancreatic necrosis were the most common indications for reoperation. Seven patients (6.8%) in the current series, including one patient with an anastomotic leak, underwent reexploration. Postoperative pancreatitis was not seen in our series. The single leak was managed initially nonoperatively with antibiotics, jejunal feedings, nasogastric tube, and chest tube drainage. Reoperation was necessary for resection and reanastomosis of a resultant anastomotic stricture. With improved surgical techniques, anastomotic leaks appear to be decreasing in incidence. We agree with the opinion that leaks should be managed conservatively and reoperation reserved for patients in whom conservative management is unsuccesful.[35] In the current series two patients (29%), one in each group, undergoing reoperation died. The patient in the neoadjuvant chemotherapy group who died had a negative laparotomy and likely suffered a pulmonary embolism. No association was identified between neoadjuvant chemotherapy and reoperation.

Operative mortality rates following gastrectomy range from 0% to 10%.[1,25–33] Our overall postoperative mortality of 4.4% is within this range. In our study CPT-11–based neoadjuvant chemotherapy was not associated with an increase in mortality. This is consistent with results in other series in which various regimens of neoadjuvant chemotherapy were used.[15,19–21,23] Others have reported increasing age and extended lymphadenopathy with multivisceral resection to be associated with increasing mortality.[27,30,32] Again, the younger age of our CHEMO patients and our limited use of multivisceral resection may also be partially responsible for the low mortality rate following gastrectomy observed in the present study. We agree that extended lymphadenectomy combined with multivisceral resection, specifically splenectomy with or without distal pancreatectomy, should be avoided unless there is direct extension of the tumor mandating resection in order to achieve negative margins.[5,29,36]

For a therapeutic option to be considered practical, it must show efficacy and not increase the risk. Overall, response rates to neoadjuvant chemotherapy vary widely from 24% to 70%[12–24]; however, responders appear to have improved survival compared to nonresponders.[13,18,37] The Dutch Gastric Cancer Group reported no effect on overall survival in a prospective randomized trial comparing surgery alone to neoadjuvant 5-fluorouracil, doxorubicin, and methotrexate (FAMTX) followed by surgery.[21] We have previously reported that the novel regimen of neoadjuvant CPT-11 and cisplatin can be delivered with acceptable toxicity and is associated with significant downstaging of locally advanced disease.[12] We believe that the preponderance of T2 tumors in the CHEMO group as opposed to T3 tumors in the SURG group is the result of such downstaging.

Rates of R0 resection following neoadjuvant chemotherapy range from 56% to 83% for high-risk patients who start therapy with potentially resectable tumors[15,17,18–22] to 9% to 45% for patients who start with unresectable tumors.[14,16,23] Seventy-eight percent of our patients receiving neoadjuvant chemotherapy underwent R0 resection, similar to our patients who had surgery alone. Although this regimen does not significantly increase operative risk, identification of more effective regimens that increase the response rate may improve the R0 resection rate and be more likely to result in improved overall long-term survival. A prospective randomized trial would be necessary to best determine any impact on overall survival as compared to surgery alone.

In the United States, stage III disease is the most common presentation of gastric cancer, and long-term survival for these patients in less than 20%.[1] Current management for suitable patients is surgical resection followed by adjuvant chemoradiation therapy as demonstrated in the Intergroup 0116 trial.[8] Disadvantages of this regimen include the following: delay in treatment for patients with postoperative complications; no proven benefit for patients with noncurative resections; incomplete therapy secondary to toxicity in the adjuvant setting; and complexity of postoperative radiation therapy planning.[38,39] One of the theoretical advantages of neoadjuvant therapy is the enhanced ability to deliver multimodality therapy to all suitable patients and not deny patients therapy because of a prolonged recovery from surgery or inadequate resection. In summary, we have shown that neoadjuvant chemotherapy with CPT-11 and cisplatin can be delivered without increasing surgical morbidity and mortality compared with gastrectomy

alone. Further investigation is warranted to determine the most efficacious and least toxic combination regimen of neoadjuvant/adjuvant therapies for gastric cancer.

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Discussion

Dr. H. Chen (Madison, WI): You focused on the perioperative complications in this paper. Were there any complications from the chemotherapy itself?

Dr. S. Marcus: Yes, there were complications from the chemotherapy, and the early results were published in the Journal of Gastrointestinal Surgery. They will be updated in 2 weeks at the American Society of Clinical Oncology meeting. Most toxicity was grade 2 and 3 in the patients receiving chemotherapy. Five patients required hospitalization during the induction period—less than 25%. Most of the symptoms of toxicity were fever, neutropenia, diarrhea, and dehydration, as you would expect. About 75% of the total planned dose of chemotherapy was delivered.

Dr. G. Mishra (Winston-Salem, NC): These are very interesting findings, and it is nice to see the sort of confirmatory results that you are getting from other studies. You mentioned that most of your patients were T3. You didn't mention how these patients were staged. Did every patient undergo endoscopic ultrasound imaging, or how was that staging performed?

Dr. Marcus: Preoperative staging of these patients is difficult, and the accuracy is certainly a problem. Our patients were staged with CT scans of the abdomen. If it was a proximal tumor, they also had a CT scan of the chest. The majority of patients had endoscopic ultrasound examinations. There were some patients who underwent laparoscopic ultrasound imaging for staging, but all protocol patients had one or the other.

Dr. A. Lowy (Cincinnati, OH): This is a nicely done study. I congratulate you and Dr. Newman. It is difficult to accrue that many patients for a gastric cancer trial. I have two questions. First, although it wasn't significant, the reoperation rate in your neoadjuvant group was 10%, which is somewhat high, and you indicated there was one anastomotic leak. I was interested in knowing what the indications were for reoperation in those patients.

Second, is your survival analysis an intent-to-treat analysis, since you had a 20% progression rate, or is it based on only those patients who were resected, because obviously that may tend to bias your results toward looking a little bit better?

Dr. Marcus: I will answer your second question first. This was an intent-to-treat analysis. There were 32 evaluable patients out of 34; two patients withdrew without receiving all of their neoadjuvant therapy and never went to surgery, but everyone else, including patients with disease progression, was counted in the survival analysis.

Regarding the indications for reoperation, in the chemotherapy group there were three reoperations. One of them was for the leak. That patient was treated conservatively. The leak resolved but the patient ended up with a stricture and underwent surgery on day 49 to redo the anastomosis. One of the patients who died of a presumed pulmonary embolism underwent an emergency laparotomy, the results of which were negative, to rule out an intra-abdominal disaster. That probably could have been averted. The other one was a patient who developed a gastric ileus and atonic stomach postoperatively and underwent a completion gastrectomy.

As an aside, your report on neoadjuvant chemoradiation therapy for gastric cancer is interesting. I would like to see a prospective trial using neoadjuvant chemo/radiation therapy. For those of you who are not aware, the next Intergroup trial being planned is the treatment arm of the prior trial compared to an arm in which epirubicin and cisplatin will be added. Only patients with R0 resection will be eligible for that trial. I think there are many patients out there who could benefit from additional therapy who may never receive it in the adjuvant setting. This is discussed further in our article.

Invited Discussion—Expert Commentary

David G. Fromm, M.D. (Detroit, MI): The additional data presented by Dr. Marcus and his associates strengthens their conclusion reported last year in the Journal of Gastrointestinal Surgery that neoadjuvant chemotherapy with CPT-11 (or irinotecan) and cisplatin combined with postoperative intraperitoneal FUDR and cisplatin does not result in an increase in postoperative complications. An important question is the efficacy of their neoadjuvant protocol to downstage adenocarcinoma of the stomach. The problem here, as the authors have previously stated, is the accuracy of preoperative compared to postoperative pathologic staging. Another question, of course, is whether or not neoadjuvant therapy with subsequent apparent R0 resection will result in greater rates of morbidity-free survival. It would be interesting to know if there were any patients in the present study with high alpha-fetoprotein levels. Shimada et al.[40] have reported two cases of gastric adenocarcinoma with multiple liver metastases associated with high alpha-fetoprotein levels who showed complete responses to combined treatment with CPT-11 and cisplatin.

Novel Combination of Cyclooxygenase-2 and MEK Inhibitors in Human Hepatocellular Carcinoma Provides a Synergistic Increase in Apoptosis

C. Max Schmidt, M.D., Ph.D., Yufang Wang, M.S., Chad Wiesenauer, M.D.

Cyclooxygenase-2 (COX-2) and ERK-MAPK mitogenic signaling pathways are important in human hepatocellular carcinoma. We investigated the effect of COX-2 inhibition on ERK-MAPK signaling and the effect of combining MEK (MAPK kinase) and COX-2 inhibitors in human hepatocellular carcinoma in vitro. COX and ERK expression were determined by immunoblot in HepG2 and Hep3B cells. COX-2 and MEK activity were determined by prostaglandin E_2 assay and phosphospecific immunoblot, respectively. Cell growth was determined by cell proliferation and cell counts. Apoptosis was determined by DNA fragmentation enzyme-linked immunosorbent assay and flow cytometry. Cell cycle was determined by flow cytometry. HepG2 and Hep3B cells do not express COX-1 or COX-2. Correspondingly, basal and agonist (arachidonic acid, lipopolysaccharide)–stimulated COX-2 activity is undetectable. Treatment of HepG2 and Hep3B cells with NS398 resulted in an increase in ERK1/2 phosphorylation (MEK activity) in a concentration-dependent fashion (NS398, 1 to 100 μmol/L). Treatment with the COX-2 inhibitor NS398 in the presence of U0126 (MEK inhibitor) effectively suppressed ERK1/2 phosphorylation as determined by phosphospecific ERK1/2 immunoblot. Total ERK1/2 and COX-2 were unchanged with NS398 and U0126 treatments. In HepG2 cells, NS398 (1 to 100 μmol/L) decreased apoptosis as determined by DNA fragmentation enzyme-linked immunosorbent assay. Relative apoptosis was increased with U0126 alone or in combination with NS398 (9 to 10 times the control value), eliminating the anti-apoptotic effect of NS398. In Hep3B cells, apoptosis was unchanged with NS398 (1 to 50 μmol/L) or U0126 (1 to 10 μmol/L) alone. The combination of NS398 and U0126 in Hep3B cells resulted in a synergistic increase in apoptosis (10 times the control value). Relative apoptosis in both cell lines strongly correlated with changes in the expression of the antiapoptotic protein Bcl-xL. Cellular growth was assessed by colorimetric proliferation assay and cell counts. HepG2 and Hep3B cells had concentration-dependent inhibition of cell growth with NS398 or U0126 treatment alone. The combination of NS398 and U0126 resulted in complementary inhibitory effects on growth. Growth inhibitory effects in HepG2 and Hep3B cells with combination treatment appear to be, in part, secondary to the induction of G_0/G_1 and G_2/M cell cycle arrest, respectively, as determined by flow cytometry. Despite differential signaling in HepG2 and Hep3B cells, the sum effect of combining the COX-2 inhibitor NS398 and the MEK inhibitor U0126 results in enhanced antitumor actions. This novel combination may be useful for in vivo studies of hepatocellular carcinoma. (J GASTROINTEST SURG 2003;7:1024–1033)

KEY WORDS: NS398, U0126, Bcl-xL, hepatocellular carcinoma, MAPK

Hepatocellular carcinoma is the most common cause of solid organ malignancy in the world.[1] Surgical treatment through surgical resection and/or transplantation can provide a cure for this cancer, but the number of individuals who are candidates for surgical treatment is minimal. Systemic treatment with chemotherapy has been largely ineffective in the treatment of this tumor. The reason for this is unclear.

Cyclooxygenase-2 (COX-2) inhibitors have been shown to have anticancer effects in hepatocellular

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carcinoma in vitro[2–4] and in vivo.[5] It is unclear whether these effects depend on the expression of COX-2.[2,3] Other effects of COX-2 inhibitors may exist to explain these anticancer effects. COX-2 inhibitors have recently been shown to inhibit ERK phosphorylation in colon cancer cells.[6] We investigated the effect of COX-2 inhibition on ERK-MAPK signaling and the effect on growth, apoptosis, and cell cycle of combining MEK (MAPK kinase) and COX-2 inhibitors in human hepatocellular carcinoma in vitro.

MATERIAL AND METHODS

Cell Culture

Human hepatocellular carcinoma cell lines (Hep3B and HepG2) and a human pancreatic adenocarcinoma cell line (BxPc-3) were obtained from American Type Culture Collection (ATCC; Manassas, VA). The cells were cultured in 10% fetal calf serum containing minimum essential media alpha 12571 (Gibco Invitrogen, Carlsbad, CA) with 100 U/ml penicillin and 100 μg/ml streptomycin (Bio Whittaker, Walkersville, MD). They were fed with fresh media three times a week. For experiments, cells were counted with a hemacytometer (Fisher Scientific, Pittsburgh, PA) and plated in consistent densities depending on the experimental method (see below). After 24 hours, they were examined by light microscopy (Olympus CK2) and then treated with the MEK enzyme inhibitor U0126 (CalBiochem, La Jolla, CA), the COX-2 enzyme-specific inhibitor NS 398 (Biomol, Plymouth Meeting, PA), or a combination of the two for various time courses. Doses employed were based on logarithmic concentration response curves.

Proliferation Assay and Cell Counts

Hepatocellular carcinoma cells were counted with a hemacytometer and plated in 96-well plates (5×10^3 cells per well for HepG2 and 2.5×10^3 cells per well for Hep3B,). After 24 hours' incubation at 37C (5% CO_2/95% O_2), cells were examined by light microscopy and then treated with U0126, NS398, or a combination for 48 hours. At 24 and 48 hours the cells were observed for light microscopic evidence of growth effects. At 48 hours 20 μl of Celltiter 96 aqueous one-solution reagent (Promega, Madison, WI) was instilled into each well. After incubation at 37C for 1 hour, absorbance was measured at 490 nmol/L. Each proliferation assay was performed in triplicate. Cell counts were used to confirm results of proliferation assay. Cell counts were performed in six-well plates. Cells were plated at 5×10^5 cells per well for HepG2 and 1×10^5 cells per well for Hep3B and similarly allowed to grow for 24 hours to approximately 75% confluence. Cells were then treated with U0126, NS398, or the combination for 48 hours. The cells were then trypsinized, stained with trypan blue, and the cells that excluded trypan blue were counted with a hemacytometer.

Prostaglandin E_2 Assay

We employed the prostaglandin E_2 (PGE_2) enzyme immunoassay (EIA) system (Amersham Pharmacia Biotech, Piscataway, NJ), which is based on competition between unlabeled PGE_2 and a fixed quantity of peroxidase-labeled PGE_2 for binding to a PGE_2-specific antibody bound to a plate coated with goat antimouse immunoglobulin. The amount of the bound PGE_2 peroxidase can be measured by the addition of the substrate. Specifically, hepatocellular carcinoma cells or control (COX-2 positive) BxPC-3 pancreatic cancer cells were counted with a hemacytometer and plated in 96-well plates (5×10^4 cells per well for HepG2, 1×10^4 cells per well for Hep3B, and 2×10^4 cells per well for BxPC-3). After 24 hours' incubation at 37C (5% CO_2/95% O_2), the cells were examined by light microscopy and then treated with vehicle, arachidonic acid (Sigma, St. Louis, MO), NS398, or a combination for 3 hours. Cells were washed with phosphate-buffered saline solution, and 100 μl/well of diluted lysis reagent (0.25% dodecyltrimethyl ammonium bromide) was added. The microtiter plate, which contained 12×8 well strips coated with goat antimouse IgG, was then set up to run all blanks, standards, and unknowns in duplicate. Fifty microliters of each unknown sample was pipetted into the appropriate wells, followed by 50 μl of diluted PGE_2 antibody and 50 μl of diluted PGE_2 horseradish peroxidase conjugate. The microtiter plate was then incubated for 1 hour at room temperature on a shaker. The wells were then washed four times with wash buffer consisting of 0.01 mol/L phosphate buffer (pH 7.5) containing 0.05% Tween 20. Next, 150 μl of room temperature equilibrated enzyme substrate (3,3′,5,5′tetramethylbenzidine/hydrogen peroxide in 20% (vol/vol) dimethylformamide) was pipetted into each well. After 30 minutes on a shaker, 100 μl of 1M sulfuric acid halted the reaction. The microtiter plate was then read at 450 nmol/L.

MAPK Signaling Assays

MAPK signaling was determined by detection of the degree of phosphorylation of the MEK substrates ERK1 and ERK2. MAPK signaling assays were performed by immunoblot with a specific phospho-

p44/42 MAP kinase (Thr202/Tyr204) antibody (Cell Signaling, Beverly, MA). Cells were lysed in radioimmune precipitation buffer (RIPA PVS, 1% nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium docecyl sulfate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 1 mmol/L sodium vanadate), and supernatants were obtained after centrifugation. Cell lysates (10 μg of total protein) were resolved by electrophoresis on 4% to 20% gradient sodium dodecyl sulfate–polyacrylamide gels (Invitrogen, Carlsbad, CA). Separated proteins were electrophoretically transferred to Immobilon-P membranes (Millipore Corporation, Bedford, MA) prior to incubation for 1 hour in blocking solution (TBS Tween with 5% nonfat dry milk). Membranes were washed three times in TBS Tween and incubated with the phosphorylated ERK-1/2 antibody overnight at 4C. After washing with TBS Tween, they were incubated with secondary antibody solution (horseradish peroxidase–conjugated IgG) for 60 minutes at room temperature. Membranes were washed again with TBS Tween prior to detection using an enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ). Equal loading of the samples was confirmed by stripping and reprobing the blots for actin.

Immunoblot Analysis

Changes in protein expression as a result of treatments were determined with immunoblot in the same manner as described in MAPK signaling assays outlined earlier. The different primary antisera employed included total ERK1/2 (K-23), COX-1 (C-20), COX-2 (C-20), COX-2 (N-20), cyclin B (GNS-1), and p53 (DO-1) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Other antibodies included Bcl-xL (Trevigen, Gaithersburg, MD), survivin (R&D Systems, Minneapolis, MN), p21, cyclin D1, cyclin A, (Neomarkers, Inc., Fremont, CA), p27, and CPP-32 (BD Pharmingen, San Diego, CA).

Apoptosis Assay

Apoptosis was measured with a DNA fragmentation assay. The Cell Death Detection ELISA kit (Roche Diagnostics, Indianapolis, IN) allows for quantitative determination of the amount of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) induced by cells undergoing apoptosis. Cells were plated in 96-well plates (1×10^4 cells well for HepG2; 4×10^3 cells per well for Hep3B) and then treated for 24 to 48 hours with NS398, U0126, or the combination. Cell lysates were prepared and placed into streptavidin-coated microtiter plates. They were incubated for 2 hours at room temperature with antihistone biotin and anti-DNA proxy antibodies. The wells were washed and then incubated with substrate. The 96-well plates were read at 405 nmol/L to quantitate the amount of nuclear histones bound to the plate.

Cell Cycle Studies

To determine the effects of cell cycle phase distribution, we used flow cytometric analysis. Cells (5×10^5 per well for HepG2 and 2×10^5 for Hep3B) were plated in six-well plates and treated the following day with U0126, NS398, or the combination. After 24 hours, the cells were trypsinized, washed, pelleted, and then treated with RNase A (final concentration: 40 μg/μl) in phosphate-buffered saline solution for 30 minutes at 37C and stained with propidium iodide at 20 μl on ice for 30 minutes. Samples were immediately analyzed by flow cytometry. Cell cycle phase distribution was determined using Modfit software (Verity Software House, Inc., Tupshin, ME) to analyze DNA content histograms.

Statistics

Student's *t* test was used for comparison of two groups and analysis of variance for comparison of multiple groups where $P < 0.05$ was considered significant.

RESULTS

COX-1 and COX-2 Enzyme Expression Undetectable in HepG2 and Hep3B Cells

Human hepatocellular carcinoma cell lines HepG2 and Hep3B were probed for COX-1 and COX-2 protein via immunoblot (Fig. 1). Neither HepG2

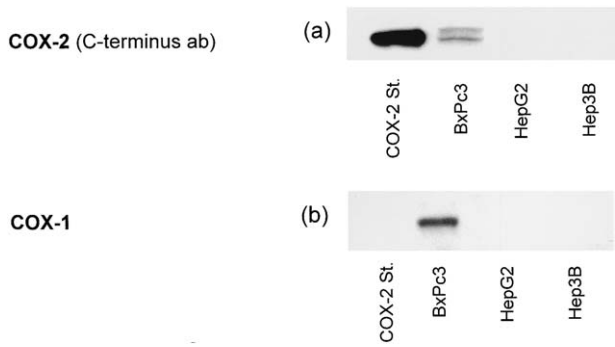

Fig. 1. Cyclooxygenase-1 (*COX-1*) and cyclooxygenase-2 (*COX-2*) enzyme expression in HepG2 and Hep3B cells. (a), Expression of COX-2 in HepG2 and Hep3B cells as determined by immunoblot analysis and detected by C-terminus–directed primary antibody; COX-2 control peptide and COX-2 positive control BxPC-3 cells are also shown. (b) Expression of COX-1 in HepG2 and Hep3B cells as determined by immunoblot; COX-1–positive control BxPC-3 cells are also shown.

nor Hep3B cells had detectable COX-2 expression as determined by immunoblot with a C-terminus COX-2 antibody (see Fig. 1, *A*). As a control, COX-2 purified peptide and BxPC-3 human pancreatic adenocarcinoma cells demonstrated clear COX-2 expression (see Fig. 1, *A*). As confirmation of these findings, can N-terminus COX-2 antibody was employed, which also failed to demonstrate COX-2 expression, and lipopolysaccharide was unable to induce the expression of COX-2 in these cell lines (data not shown). The expression of the COX-1 isoenzyme was also investigated. Although BxPC-3 cells demonstrated COX-1 expression, HepG2 and Hep3B cells again did not demonstrate detectable levels of COX-1 (see Fig. 1, *B*).

Absence of Detectable Basal and Agonist-Stimulated Prostaglandin E*2* Activity in HepG2 and Hep3B Cells

After determining lack of expression of cyclooxygenase by immunoblot, we sought to further confirm these findings by determining if there was detectable activity of COX as measured by PGE_2 levels in HepG2 and Hep3B cells. The COX-2–positive BxPC-3 control cells exhibited a PGE_2 level of 44 ± 3 pg/well, whereas HepG2 and Hep3B cells were at or below the lower limits of detectable range of the assay (Fig. 2). The substrate of cyclooxygenase, arachidonic acid (15 μmol/L), was used to enhance PGE_2 levels. BxPC-3 cells showed significant enhancement after a 3-hour treatment with arachidonic acid, achieving a PGE_2 level of 56 ± 2 pg/well, whereas there was no significant enhancement from baseline PGE_2 levels in HepG2 and Hep3B cells (see Fig. 2). PGE_2 levels were also assessed in the presence of the COX-2 specific inhibitor NS398. BxPC-3 cells treated with NS398 showed a significant decrease in PGE_2 levels (7 ± 2 pg/well). In the presence of arachidonic acid and NS398, this decrease in PGE_2 was blunted (22 ± 8 pg/well). Treatment with NS398 in HepG2 and Hep3B cells had no significant effect on PGE_2 levels, which remained at or below the lower limits of detectable range (see Fig. 2).

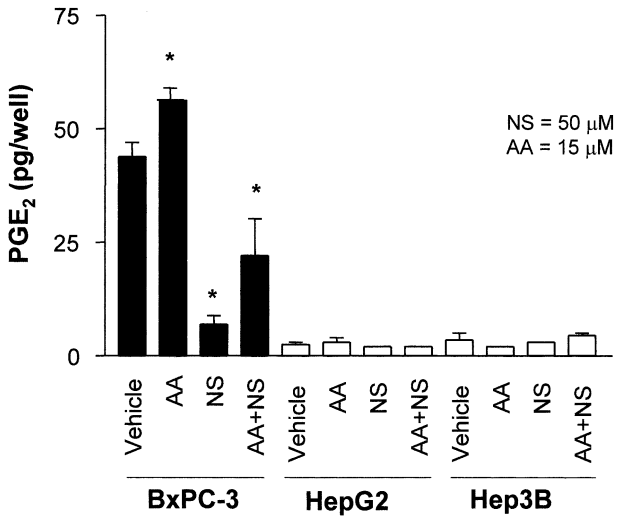

Fig. 2. Basal and agonist-stimulated prostaglandin E_2 (PEG_2) activity in HepG2 and Hep3B cells. PGE_2 levels were determined by a prostaglandin E_2 enzyme immunoassay (EIA) in a range 2.5 to 320 pg/well. PGE_2 levels were determined in whole-cell lysates of BxPC-3 (COX-2+), HepG2, and Hep3B cells treated with arachidonic acid (*AA*; 15 μmol/L), NS398 (*NS*; 50 μmol/L), or the combination. The results represent the mean ± SEM of four independent experiments performed in duplicate. * = $P < 0.05$ compared to vehicle control.

Increased MAPK Signaling in NS398-Treated HepG2 and Hep3B cells

The absence of COX-2 expression and activity in HepG2 and Hep3B cells suggested that any effects of NS398 might be mediated through a COX-2–independent pathway. Thus we examined the role of MAPK signaling in COX-2 inhibitor treatment of human hepatocellular carcinoma cells. We subjected HepG2 and Hep3B cells to treatment with the COX-2–specific inhibitor NS398, and determined expression levels of active (phosphorylated) ERK. The Western blots shown in Fig. 3 demonstrate a concentration response curve with NS398 in HepG2 (see Fig. 3, *left*) and Hep3B cells (see Fig. 3, *right*). In each cell line there is a concentration-dependent increase in phosphorylated ERK1/2. At the early time points of 30 minutes and 3 hours, this is most apparent. At the later time point of 24 hours, the Hep3B cells continue to exhibit an increase in the phosphorylation of ERK1/2 relative to control. HepG2 cells, however, no longer express elevated levels of phosphorylated ERK1/2 at 24 hours. We next sought to determine if we could inhibit this ERK1/2 phosphorylation with the MEK-specific inhibitor U0126. Indeed, U0126 effected a reduction in ERK1/2 phosphorylation to control levels or below (see Fig. 3). The combination effect of NS398 and U0126 on ERK1/2 phosphorylation appears, in some cases, to provide greater inhibition of ERK1/2 phosphorylation than would be expected from the relative ERK1/2 phosphorylation with each treatment alone. Equal loading of the samples was confirmed by stripping and reprobing the blots for actin.

Total ERK and COX-2 Expression Remain Unchanged With NS398 or U0126 Treatments

Although NS398 and U0126 have counteracting effects on MEK activity (i.e., the level of phosphorylated ERK), there was no effect of these agents alone

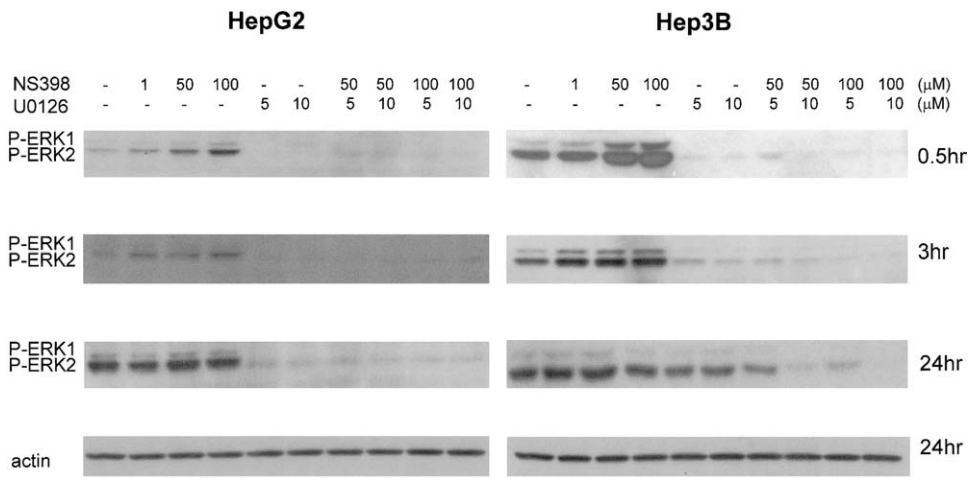

Fig. 3. Phosphorylated ERK 1/2 levels in NS398-treated HepG2 and Hep3B cells. The effect of NS398 (1 to 100 μmol/L), U0126 (5 to 10 μmol/L), or the combination on the expression of phophorylated ERK 1/2 in HepG2 (*left*) and Hep3B (*right*) is shown at 30 minutes, 3 hours, and 24 hours. Representative phosphorylated ERK 1/2 immunoblots are shown. Actin confirmed equal loading of the 24-hour immunoblot (30-minute and 3-hour actin gels not shown).

or in combination on total ERK expression (Fig. 4). Similarly, as expected, there was no change in COX-2 expression with these treatments (Fig. 5).

NS398 and U0126 Provide Complementary Growth Inhibition in Human Hepatocellular Carcinoma Cells

To determine the growth effects of treatment with COX-2 and MEK inhibitors, we subjected HepG2 and Hep3B cells to treatment with NS398, U0126, or the combination. Growth effects were determined with a colorimetric growth assay and cell counts. Cell counts are shown in Fig. 6. HepG2 cellular counts were decreased in a concentration-dependent fashion with NS398 treatment (see Fig. 6, *top*). Significant effects on growth were observed at the 50 μmol/L and 100 μmol/L concentrations. Similarly, U0126 exhibited a concentration-dependent inhibitory effect on growth in HepG2 cells. The combination of NS398 and U0126 resulted in an additive effect on growth. Hep3B cellular counts (see Fig. 6, *bottom*) were likewise significantly decreased in a concentration-dependent fashion with NS398 (10 μmol/L to 100 μmol/L) or U0126 (5 μmol/L to 10 μmol/L). The combination of NS398 and U0126 resulted in a synergistic effect on growth; namely, Hep3B cells

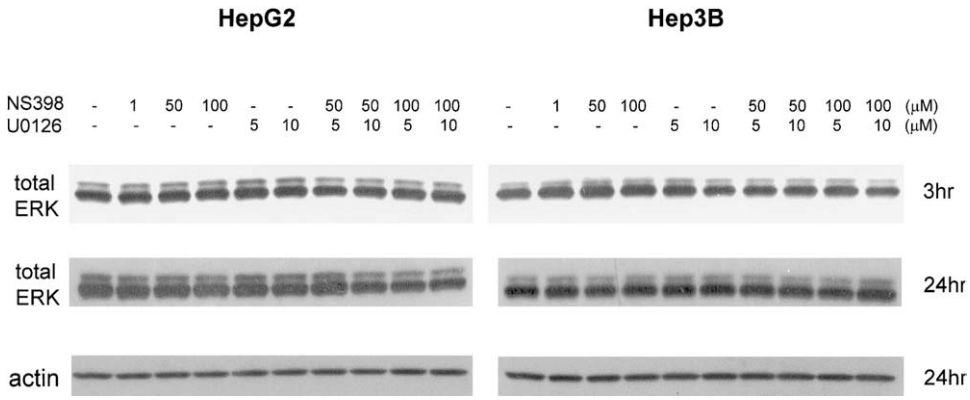

Fig. 4. Total ERK expression in HepG2 and Hep3B cells treated with NS398 and U0126. The effect of NS398 (1 to 100 μmol/L), U0126 (5 to 10 μmol/L), or the combination on the expression of total ERK in HepG2 (*left*) and Hep3B (*right*) is shown at 3 hours and 24 hours. Representative total ERK immunoblots are shown. Actin confirmed equal loading of the 24-hour immunoblot (3-hour actin gel not shown).

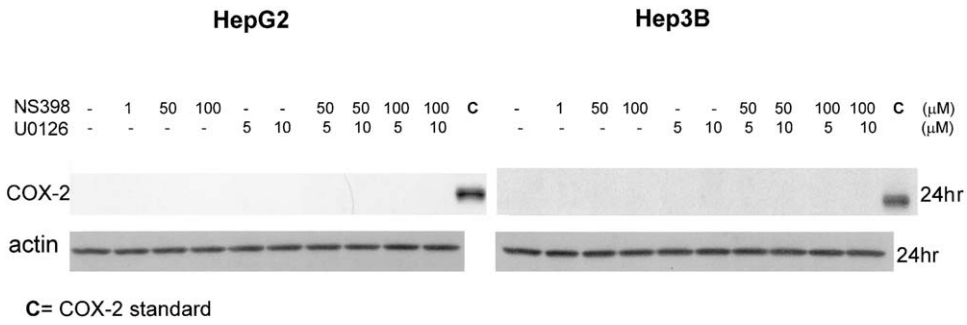

Fig. 5. COX-2 expression in HepG2 and Hep3B cells with NS398 and U0126 treatments. The effect of NS398 (1 to 100 μmol/L), U0126 (5 to 10 μmol/L), or the combination on the expression of phosphorylated COX-2 in HepG2 (*left*) and Hep3B (*right*) is shown at 24 hours. Representative COX-2 immunoblots are shown. Actin confirmed equal loading.

were inhibited more than the sum of the individual drugs alone when they were combined at the 50 μmol/L (NS398) and 5 μmol/L (U0126) doses. At higher doses of the drug, the combination appeared to be additive.

Combination of NS398 and U0126 Induced Synergistic Increases in Apoptosis in Human Hepatocellular Carcinoma Cells

To determine if the antiproliferative effects of NS398 and U0126 involve apoptosis as a mechanism, we employed a DNA fragmentation enzyme-linked immunosorbent assay (ELISA). Figs. 7 and 8 demonstrate the effects of NS398, U0126, or the combination on DNA fragmentation as determined by ELISA in HepG2 and Hep3B cells at 48 hours. In HepG2 cells, NS398 alone did not increase apoptosis at 48 hours. In contrast, it significantly decreased apoptosis at the highest concentration (100 μmol/L). HepG2 cells treated with U0126 alone showed a marked increase (10-fold) in apoptosis. The combination of NS398 and U0126 in HepG2 cells resulted in apoptosis at levels not significantly different from those with U0126 alone. Therefore the combination of an MEK inhibitor with NS398 effectively blocks the antiapoptotic effect of NS398. In Hep3B cells, NS398 did not effect an increase in baseline apoptosis, with the exception of the highest concentration employed (100 μmol/L). Hep3B cells treated with the MEK enzyme inhibitor U0126 alone also did not show a significant change in baseline apoptosis at the concentrations employed. The combination of NS398 and U0126, however, in Hep3B cells resulted in a synergistic increase in apoptosis at all concentrations employed, particularly at 50 μmol/L NS398 and 5 to 10 μmol/L U0126. Similar results were obtained for HepG2 and Hep3B cells at 24 hours (data not shown).

We also examined the effect of these treatments on the expression of specific apoptosis-related genes including Bcl-xL, phosphorylated ERK1/2, total ERK, CPP-32, survivin, and p53. The expression of the antiapoptotic protein Bcl-xL was highly negatively correlated with the degree of apoptosis in each of these cell lines in response to NS398, U0126, or the combination. Immunoblots of Bcl-xL are displayed above the apoptosis graphs in Figs. 7 and 8. Likewise, the expression of phosphorylated ERK 1/2 at 24 hours was highly negatively correlated with the degree of apoptosis in each of these cell lines (see Fig. 3). Recall that the combination of NS398 and U0126 on ERK1/2 phosphorylation in these cells is not additive but rather appears to provide greater inhibition of ERK1/2 phosphorylation. CPP-32, survivin, and p53 were also investigated and did not show any correlation with apoptosis at 48 hours (data not shown). Of note, wild-type p53 is expressed in HepG2 cells and was unchanged with treatments. p53 is not expressed in Hep3B cells.

Cell Cycle Arrest in HepG2 and Hep3B Cells With NS398, U0126, and the Combination

To determine whether the growth inhibitory effects of NS398 on hepatocellular carcinoma cells could be partly explained by changes in the cell cycle, we examined cell cycle phase distribution by flow cytometric analysis. Fig. 9 shows the cell cycle phase distribution of HepG2 cells after 24 hours of treatment with NS398, U0126, or the combination. The number of cells in the G_0/G_1 phase is increased by NS398 and U0126 treatment alone. The combination appears to be additive. Correspondingly, the number of cells in the S-phase decreases with either treatment alone and with the combination. Fig. 10 shows the cell cycle phase distribution of Hep3B cells. Similar

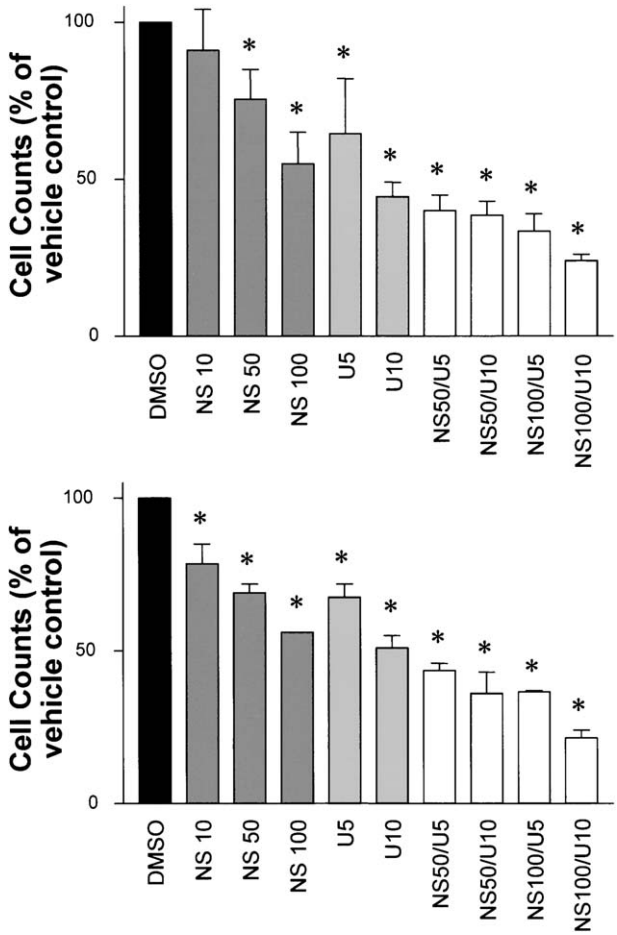

Fig. 6. Growth effects of NS398 and U0126 treatments on HepG2 and Hep3B cells. The effect of NS398 (1 to 100 μmol/L), U0126 (5 to 10 μmol/L), or the combination on the proliferation of HepG2 (*left*) and Hep3B (*right*) is shown at 48 hours. After treatment, cells were trypsinized, stained with trypan blue, and the cells that excluded trypan blue were counted with a hemacytometer. The data from at least two independent experiments performed in duplicate are shown and expressed as a percentage of vehicle control values (mean ± SEM). Our proliferation assay also confirmed these findings, n = 3 performed in triplicate (data not shown). * = $P < 0.05$ compared to vehicle control.

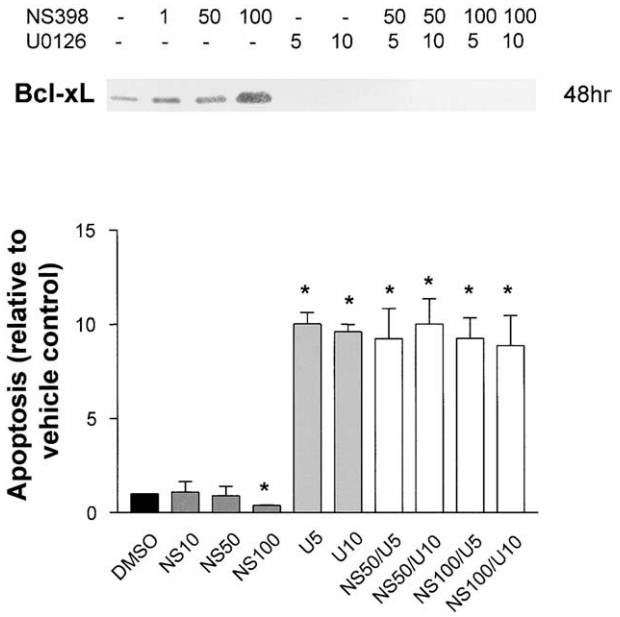

Fig. 7. Effect of NS398 and U0126 on apoptosis in HepG2 cells. The effect of NS398 (1 to 100 μmol/L), U0126 (5 to 10 μmol/L), or the combination on the expression of Bcl-xL (*top*) in HepG2 cells is shown at 48 hours. A representative Bcl-xL immunoblot is shown. Actin confirmed equal loading (data not shown). The effect of NS398 (1 to 100 μmol/L), U0126 (5 to 10 μmol/L), or the combination on apoptosis (*bottom*) in HepG2 cells is shown at 48 hours. Apoptosis was measured with a DNA fragmentation assay (Cell Death Detection ELISA Kit). Data from two independent experiments performed in duplicate are shown. Values are expressed as percentage of control values (mean ± SEM). * = $P < 0.05$ compared to vehicle control.

to HepG2 cells, the number of cells in the G_0/G_1 phase is increased by NS398 and U0126 treatment alone. In contrast, the combination appears to result in accumulation of cells in G_2/M.

Immunoblot analysis of several cell cycle regulatory proteins did not reveal a single protein that appeared to correlate with these cell cycle changes. It is likely that several proteins are involved with this regulation in HepG2 cells, as is shown above the cell cycle data in Fig. 9. The individual effects of NS398 and U0126 on G_0/G_1 arrest correspond to decreased survivin and perhaps for U0126, increased p21. The G_0/G_1 arrest caused by the combination of NS398 and U0126 corresponds to decreases in survivin and cyclin D1 (and perhaps cyclin A at low doses of the combination). In Hep3B cells, the effects of NS398 and U0126 alone on G_0/G_1 correspond to decreases in cyclin D1. The combination does not result in greater accumulation in G_0/G_1, which may result from an accumulation of cyclin B, which has been shown to arrest cells in G_2/M.[7]

DISCUSSION

Hepatocellular carcinoma remains a serious threat to our society. The incidence of hepatitis C is on the rise in the United States, and the incidence of hepatocellular carcinoma is expected to increase drastically in response to this epidemic.[1] Surgery remains the only possibility for cure. Promising treatments for patients who are not candidates for surgery are needed. Nonsteroidal anti-inflammatory drugs, and

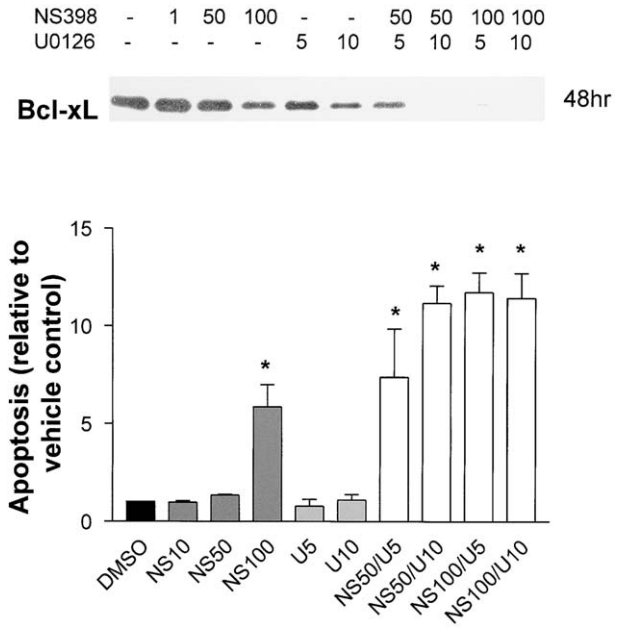

Fig. 8. Effect of NS398 and U0126 on apoptosis in Hep3B cells. The effect of NS398 (1 to 100 μmol/L), U0126 (5 to 10 μmol/L), or the combination on the expression of Bcl-xL (*top*) in Hep3B cells is shown at 48 hours. A representative Bcl-xL immunoblot is shown. Actin confirmed equal loading (data not shown). The effect of NS398 (1 to 100 μmol/L), U0126 (5 to 10 μmol/L), or the combination on apoptosis (*bottom*) in Hep3B cells is shown at 48 hours. Apoptosis was measured with a DNA fragmentation assay (Cell Death Detection ELISA Kit). Data from two independent experiments performed in duplicate are shown. Values are expressed as percentage of control values (mean ± SEM). * = designates $P < 0.05$ compared to vehicle control.

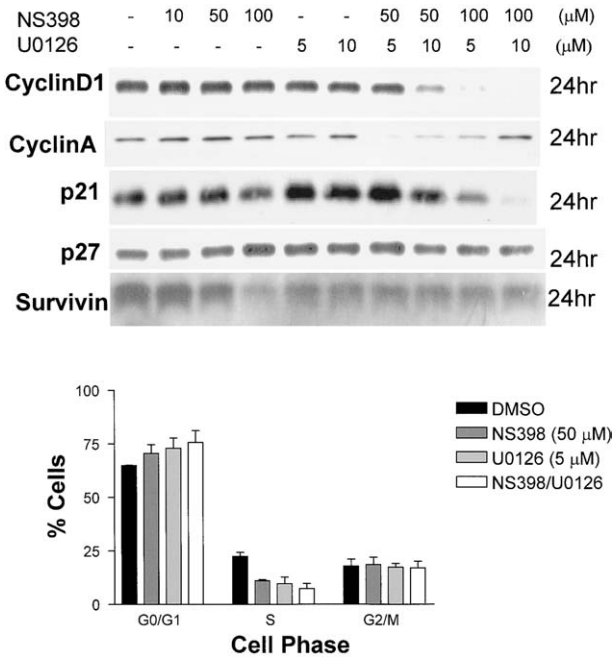

Fig. 9. Effect on cell cycle of NS398 and U0126 treatments in HepG2 cells. The effect of NS398 (1 to 100 μmol/L), U0126 (5 to 10 μmol/L), or the combination on the expression of cyclin D1, cyclin A, p21, p27, and survivin (*top*) in HepG2 cells is shown at 24 hours. Representative immunoblots are shown. Actin confirmed equal loading (data not shown). The effect of NS398 (1 to 100 μmol/L), U0126 (5 to 10 μmol/L), or the combination on the cell cycle as determined by flow cytometry (*bottom*) in HepG2 cells is shown at 24 hours. Data from two independent experiments are shown. Values are expressed as mean ± standard deviation.

in particular COX-2 inhibitors, have been found to have anticancer effects both in vitro and in vivo.

In this study we determined that the hepatocellular carcinoma cell lines HepG2 and Hep3B do not express COX-1 or COX-2 at detectable levels. In addition, PGE_2, one of the products of cyclooxygenase activity, is likewise at or below the minimum detectable level by the assay we employed. This result contradicts a recently published study, which has shown COX-2 RNA and protein in these hepatocellular carcinoma cell lines.[4] Unlike these investigators, we have employed a purified COX-2 standard, a control COX-2–positive cell line (BxPC-3), and two separate antibodies to detect COX-2 (including the one used by these investigators, N-20). Furthermore, as noted earlier, the activity of COX-2 as determined by PGE_2 levels in these cell lines is consistent with our findings.

Nonetheless, treatment with a COX-2 inhibitor results in significant growth inhibition in these hepatocellular carcinoma cell lines, and we postulated that this may be due to alterations in ERK-MAPK signaling. We therefore looked at the effects of the COX-2 inhibitor NS398 on the level of phosphorylated ERK1 and ERK2 in hepatocellular carcinoma cells. Unexpectedly, the COX-2 inhibitor caused an increase in phosphorylated ERK1/2. We postulated that this may result in the suboptimal efficacy of COX-2 inhibitor treatment in hepatocellular carcinoma, and that combining a COX-2 inhibitor with an MEK inhibitor may create treatment synergy.

We therefore looked at the effects of the COX-2 inhibitor NS398, in combination with an MEK inhibitor, U0126, in order to test this theory. Indeed, U0126 increased antiproliferative effects in hepatocellular carcinoma in the presence of NS398. In addition, this combination resulted in synergistic effects on apoptosis. In the case of HepG2 cells, MEK inhibition in effect blocked the antiapoptotic effect of NS398, causing a 10-fold increase in apoptosis over baseline levels. NS398 and U0126 were relatively ineffective at stimulating apoptosis in Hep3B cells but

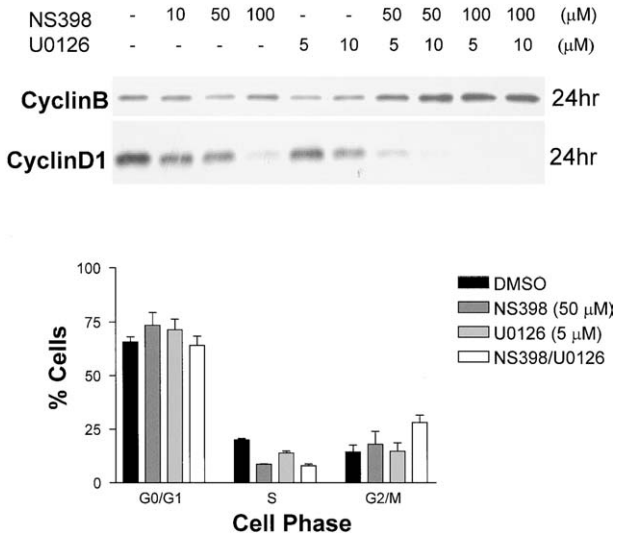

Fig. 10. Effect on cell cycle of NS398 and U0126 treatments in Hep3B cells. The effect of NS398 (1 to 100 μmol/L), U0126 (5 to 10 μmol/L), or the combination on the expression of cyclin B and cyclin D1 (*top*) in Hep3B cells is shown at 24 hours. Representative immunoblots are shown. Actin confirmed equal loading (data not shown). The effect of NS398 (1 to 100 μmol/L), U0126 (5 to 10 μmol/L), or the combination on the cell cycle as determined by flow cytometry (*bottom*) in Hep3B cells is shown at 24 hours. Data from two independent experiments are shown. Values are expressed as mean ± standard deviation.

in combination caused a 10-fold increase in apoptosis over baseline levels. These effects were mirrored in the long-term expression of the antiapoptotic protein Bcl-xL and phosphorylated ERK levels. Interestingly, Bcl-xL is overexpressed in human hepatocellular carcinoma, and endogenous Bcl-xL has been shown in vitro to inhibit apoptosis produced by various stress-inducing conditions.[8] Phosphorylated ERK has recently been implicated in Bcl-xL regulation, so these two may be causally related. Phosphorylation of Ser-112 of the proapoptotic protein BAD is regulated by the Ras-MAPK pathway.[9,10] Phosphorylation results in loss of the ability of BAD to heterodimerize with the survival protein Bcl-xL. Phosphorylated BAD binds to 14-3-3 and is sequestered in the cytoplasm allowing unregulated expression of Bcl-xL.[9,10]

Cell cycle mechanics were also altered by each drug alone and the combination. HepG2 cells showed G_0/G_1 arrest with NS398 or U0126 alone and additive effects on G_0/G_1 arrest with the combination. Survivin appeared to correlate with these findings and has recently been shown to regulate G_0/G_1.[11] Hep3B cells also showed G_0/G_1 arrest with each agent alone but the combination effected G_2/M arrest. Candidate mediators of these cell cycle changes are not entirely clear but appear to involve the interplay of several cell cycle regulatory proteins.

As demonstrated from these studies, COX-2 inhibitors do not necessarily inhibit MAPK signaling in all cellular systems. In HepG2 and Hep3B cells, ERK1/2 is activated in response to COX-2 inhibitor treatment. This may result in the suboptimal efficacy of COX-2 inhibitor treatment in hepatocellular carcinoma. By employing an MEK enzyme inhibitor in combination with a COX-2 inhibitor, we were able to produce complementary and synergistic effects on growth inhibition and apoptosis. COX-2 inhibitors do indeed provide antiproliferative effects in hepatocellular carcinoma cells and are worthy of further investigation in the treatment and prevention of hepatocellular carcinoma. Despite somewhat differential signaling in HepG2 and Hep3B cells, the sum effect of combining a COX-2 inhibitor and an MEK inhibitor results in enhanced antitumor actions. This novel combination may be useful for in vivo studies of hepatocellular carcinoma. Furthermore, these findings may also stimulate an interest in designing COX-2 inhibitors that do not promote MAPK, which may counteract the anticancer effects of these drugs in hepatocellular carcinoma.

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Discussion

Dr. A. Umar (Bethesda, MD): In regard to NS398, did you look at the activity of AKT phosphorylation or PDK1 inhibition as it has been reported for the other COX-2 selective inhibitors?

Dr. C. Schmidt: We have done some preliminary studies looking at the AKT/Pi3Kinase pathway, and it depends on which nonsteroidal drug you examine. With the NS398 compound, interestingly, AKT phosphorylation appears to be increased. To determine whether this represents another chemoresistance pathway, we will have to do further studies. To date, we have not looked at PDK1.

Dr. L. Rikkers (Madison, WI): With these new findings, are there going to be other ways to manipulate this pathway besides through the use of COX-2 inhibitors, such as ERK stimulation and so forth, that could be of benefit to the clinical treatment of hepatocellular cancer?

Dr. Schmidt: Although we have identified candidate mediators that correlate well with the changes we observed, we do not have confirmatory data to suggest that those are indeed the causes of these effects. We may ultimately employ Bcl-xL and survivin constructs to see if we can augment or block the effects that we are observing. Constructs, which stimulate ERK, may also help confirm our findings. We would like to know which molecule(s) is the primary mechanism for these changes. In theory, the further downstream we can get, the more specificity and less toxicity there might ultimately be with patient treatment.

Dr. T. Howard (Indianapolis, IN): How do you reconcile the differences that you found between the two cell lines in terms of a proposed mechanism of apoptosis?

Dr. Schmidt: Hep3B cell lines do not express p53, and HepG2 cell lines do express p53. There is some nice work that was recently published in *Hepatology* to suggest that if you do not have p53 then Bcl-xL–mediated apoptosis may actually not be as effective under certain conditions of stress. So this is why we need to use these constructs to see if this is the case in hepatocellular carcinoma under these conditions. Otherwise, I think the point of this study is that regardless of which hepatocellular carcinoma cell line we look at, it appears that the combination does provide benefits, although through apparently different mechanisms.

Impact of Steatosis on Perioperative Outcome Following Hepatic Resection

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Fatty liver disease may interfere with liver regeneration and is postulated to result in an adverse outcome for patients subjected to partial hepatectomy. This study examines the impact of steatosis on outcome following hepatic resection for neoplasms. All patients with fatty livers (n = 325) who underwent hepatectomy between December 1991 and September 2001 were identified from a prospective database. Slides were reviewed and steatosis was quantified as follows: <30% (mild) and ⩾30% (marked). Patient data were gathered and compared with results in 160 control patients with normal livers; subjects were matched for age, comorbidity, and extent of liver resection. There were 223 patients with mild and 102 with marked steatosis. Those with steatosis were more likely to be men (59% marked vs. 55% mild vs. 43% control; $P = 0.01$) with a higher body mass index (29.7 ± 5.5 marked vs. 28.2 ± 5.5 mild vs. 26.0 ± 5.4 control; $P < 0.01$), and treated preoperatively with chemotherapy (66% marked vs. 55% mild vs. 38% control; $P < 0.01$). Total (62%, 48%, and 35%; $P < 0.01$) and infective (43%, 24%, and 14%; $P < 0.01$) complications correlated with the degree of steatosis. No difference was observed in complications requiring major medical intervention, hospitalization, or admission to the intensive care unit between groups. On multivariate analysis, steatosis was an independent predictor of complications ($P < 0.01$, risk ratio = 3.04, 95% confidence interval = 1.7 to 5.54). There was a nonsignificant trend toward higher 60-day mortality in patients with marked steatosis who had lobe or more resections (9.4% marked vs. 5.0% mild vs. 5.0% control; $P = 0.30$). Marked steatosis is an independent predictor of complications following hepatic resection but does not have a significant impact on 60-day mortality. Steatosis alone should not preclude aggressive hepatic resection for neoplasms when indicated; however, patients with marked steatosis undergoing large resections should still be approached with due caution.
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KEY WORDS: Chemotherapy, surgical complications, nonalcoholic steatohepatitis, infection

Resection is the only potentially curative therapy for primary and metastatic tumors of the liver and biliary tract.[1–7] The past decade has seen dramatic advances in perioperative management of patients undergoing liver surgery.[8] As a result, the mortality rate for liver resection is less than 5% in most high-volume centers. Despite such improvements, underlying hepatic parenchymal disorders remain obstacles to an aggressive operative approach. Experimental models demonstrate that cirrhosis interferes with liver regeneration,[9,10] and the impact of this in humans following partial hepatectomy is evident from published reports.[11,12] As a result, cirrhotic patients with tumors are often referred for orthotopic liver transplantation or less aggressive ablative techniques, which may not be as effective as resection.

In Western countries, fatty liver disease (steatosis) is the most common hepatic parenchymal disorder, affecting 6% to 11% of individuals in the general population.[13,14] Steatosis is commonly associated with heavy alcohol ingestion, obesity, diabetes mellitus (type II), and other conditions promoting insulin resistance, lipase inhibition, and free fatty acid accumulation in the liver.[15–18] Hepatic macrophage (Kupffer

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cell) dysfunction has also been implicated in animal models of fatty liver disease, suggesting abnormalities in innate immunity in steatotic hosts.[19,20]

In contrast to what has been observed in patients with cirrhosis, the effects of steatosis on liver regeneration following hepatic resection are less obvious, as experimental models have provided conflicting results.[21–25] Transplant surgeons have demonstrated the potential pitfalls of using steatotic grafts for orthotopic liver transplantation,[26–29] but its effects on outcome following hepatic resection are not well characterized. The present study examines the clinical course of a large number of patients with histologically confirmed steatosis and compares results to a cohort of matched patients with normal livers to determine whether steatosis is a predictor of poor outcome following hepatic resection in humans.

METHODS

All patients undergoing liver resection for malignant or benign disease at Memorial Sloan-Kettering Cancer Center (MSKCC) during the 10-year period from December 1991 to September 2001 were identified from a prospectively maintained liver resection database. Patients subjected to only a diagnostic biopsy were excluded. Histologic findings were reviewed, and of the 1803 patients undergoing resections during this period, 331 (18%) had some degree of fatty change in the non–tumor-bearing liver. An additional control group of 160 patients with normal liver parenchyma were randomly selected through a cohort matching process. These patients were matched by age, comorbidity, and extent of resection. Sample size calculation demonstrated that 2:1 randomization would provide adequate power (80%) for this analysis.

Histologic Evaluation

Histologic evaluation of fatty change was performed in the Department of Pathology at MSKCC. Original slides, stained with hematoxylin and eosin, were reviewed under light microscopy by two gastrointestinal pathologists. Steatosis was quantified on the basis of the percentage of hepatocytes containing fat inclusions. Stratification was performed according to criteria described by D'Allessandro et al.[30] Specimens with fat inclusions in more than 30% of hepatocyes were labeled "mild steatosis"; if 30% to 60% of hepatocytes were affected, this was considered "moderate steatosis"; and those with more than 60% were considered "severe steatosis." For analysis, the moderate and severe groups were combined and termed "marked" steatosis (Fig. 1). No effort was made to differentiate between microvesicular and macrovesicular steatosis. Focal, peritumoral fatty infiltration was disregarded and only diffuse steatosis was included in this analysis.

Patient Data

Demographic data included patient age, sex, diagnosis, height, and weight at the time of the operation, presence of comorbid factors, history of heavy alcohol use, and history of preoperative chemotherapy. Diagnoses were divided into benign or malignant categories; malignant diagnoses were further stratifed as primary (hepatocellular carcinoma or biliary cancers) or metastatic (colorectal primary or other primary lesion). Height and weight were used to calculate body mass index (BMI) according to the following formula: $\text{BMI} = [\text{weight (kg)}/\text{height (cm)}^2] \times 10^4$, and obesity was defined as a $\text{BMI} \geq 30$.[31,32] Comorbid conditions were defined as the presence of (1) chronic obstructive pulmonary disease, (2) cardiac disease, or (3) diabetes mellitus, as obtained from the anesthesia preoperative evaluation, surgical consultation, and/or any perioperative medical consultations. Alcohol intake history was obtained by reviewing physician and nursing notes for evidence of long-term heavy alcohol use or history of frank alcoholism. Preoperative chemotherapy included any course of cytotoxic chemotherapy administered specifically for treatment of the resected tumor within the year preceding hepatic resection. The vast majority were treated with 5-fluorouracil–based therapy. More recently many patients received CPT-11 as well.

Operative data included the number of hepatic segments resected, operative time, portal triad clamp

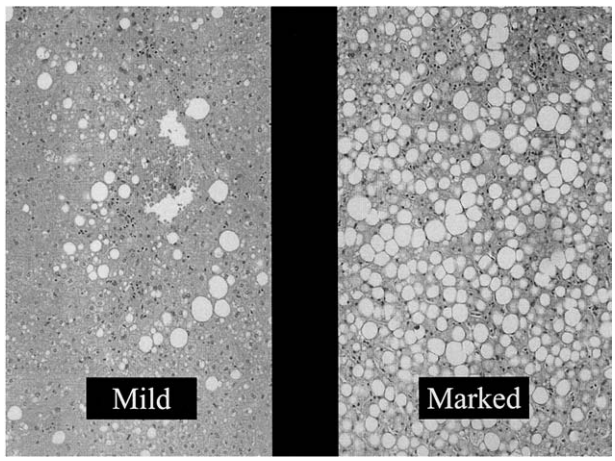

Fig. 1. Photomicrographs of steatotic livers (low power). Examples of "mild" and "marked" steatosis as observed under light microscopy. See text for additional details.

time (Pringle time), estimated blood loss, and perioperative transfusion history. Hepatic resections were coded as "less than a lobe" or "lobe or more" according to the definitions of Goldsmith and Woodburne.[33] Transfusion data were obtained from the computerized record system of the MSKCC blood bank. "Any transfusion" includes whole blood, packed red blood cells, fresh-frozen plasma, and/or platelets within 30 days of the operative procedure, and "RBC transfusion" includes only whole blood or packed red blood cells.

Outcome variables included length of hospital stay, postoperative complications (see below), 60-day or in-hospital mortality, change in biochemical profiles (total bilirubin and international normalized ratio [INR]), and overall survival. Change in biochemical profile was defined as the mean difference between the peak postoperative value and the immediate preoperative value.

Reporting of Complications

In an effort to standardize reporting, complications were graded on a scale of 1 to 5 according to a previously published grading system.[34] In this classification system grade 0 represents no complications. Grade 1 complications are those requiring no intervention or minor interventions such as oral antibiotics, bowel rest, or basic monitoring. Grade 2 complications are those requiring moderate interventions such as intravenous medications (e.g., antibiotics or antiarrhythmic drugs), total parenteral nutrition, prolonged tube feeding, or chest tube insertion. Grade 3 complications are those requiring hospital readmission, surgical intervention, or radiologic intervention. Grade 4 complications are those producing chronic disability, organ resection, or enteral diversion, and grade 5 complications are those resulting in death. In this system, grades 1 and 2 are grouped as minor and grades 3 to 5 are considered major complications.

Statistical Analysis

Outcome was evaluated for all patients (N = 485) and separately for the subset of patients who underwent lobe or more resections (N = 324). Total, major, and infective complication rates were determined for each group as was perioperative mortality. Univariate analysis was performed by Fisher's exact test, and the Cochran-Armitage trend test was used to assess strength of association between groups (control, mild steatosis, and marked steatosis) and various outcome variables. Multivariate analysis was performed using stepwise logistic regression for variables approaching significance on univariate analysis. All factors that were not inherently dichotomous were analyzed as continuous variables (age, operative time, BMI, and blood loss). Overall survival was a secondary end point and was estimated by the method of Kaplan and Meier. Survival curves were compared using the log-rank test. Results are expressed as mean ± standard deviation, except for survival, where standard error is reported.

RESULTS

Histologic Findings

A total of 331 patients with steatosis were identified from the database; however, original slides could not be recovered for six patients, leaving 325 available for review. Quantitative histologic evaluation of liver parenchyma was performed in all cases. The majority (N = 223) had mild steatosis, 64 had moderate steatosis, and the remaining 38 patients had severe fatty infiltration. For analysis, patients with moderate and severe steatosis were grouped together and labeled marked steatosis (N = 102).

Patient and Operative Characteristics

There were 485 patients evaluated, and their characteristics according to degree of steatosis are shown in Table 1. Mean age for control subjects and patients with steatotic livers were statistically similar at 61.0 ± 12.4 years and 61.6 ± 11.2 years, respectively. There were more women in the control group vs. the steatosis group (57% vs. 44%; $P = 0.01$). BMI was significantly different among the groups and correlated with the degree of fatty infiltration. Only 17% of control patients were obese, compared with 31% in the mild group and 36% in the marked group ($P < 0.01$). Comorbid factors (pulmonary, cardiac, and diabetes) were evenly distributed (control = 31%, mild = 31%, marked = 33%; $P = 0.67$) among the groups. Breakdown of specific comorbid conditions can be found in Table 1. Patients with steatotic livers were more likely to be those patients who received preoperative chemotherapy (control = 38%, mild = 55%, marked = 66%; $P < 0.01$). In this series a history of heavy alcohol use was the same in the control and steatotic groups (control = 5%, steatotic = 7%; P = NS). Patients with steatosis were more likely to be those with a diagnosis of malignant disease (98% steatosis vs. 91% control; $P = 0.02$) and those patients who were treated for metastatic liver disease (87% steatosis vs. 75% control; $P < 0.01$), echoing the fact that this group was also more likely to have received chemotherapy prior to surgery.

The operative variables for all patients according to the degree of fatty liver disease are shown in Table

Table 1. Patient characteristics stratified by degree of steatosis (N = 485)

Variable	Steatosis (all) (N = 325)	No steatosis (N = 160)	Mild steatosis (N = 223)	Marked steatosis (N = 102)	Trend
Age (mean ± SD)	61.6 ± 11.2	61.0 ± 12.4	62.4 ± 11.0	60.0 ± 11.6	0.58
Female	143 (44%)	91 (57%)	101 (45%)	42 (41%)	0.01
BMI (mean ± SD)	28.7 ± 5.5	26.0 ± 5.4	28.2 ± 5.5	29.7 ± 5.5	<0.01
BMI ⩾30	106 (33%)	27 (17%)	69 (31%)	37 (36%)	<0.01
Any comorbidity	103 (32%)	49 (31%)	69 (31%)	34 (33%)	0.67
Diabetes mellitus	37 (11%)	14 (9%)	24 (11%)	13 (13%)	0.29
Cardiac disease	41 (13%)	19 (12%)	27 (12%)	14 (14%)	0.68
Pulmonary disease	32 (10%)	15 (9%)	22 (10%)	10 (10%)	0.90
Preoperative chemotherapy	189 (58%)	60 (38%)	122 (55%)	67 (66%)	<0.01
Heavy ethanol use	23 (7%)	8 (5%)	15 (7%)	8 (8%)	0.34
Benign	7 (2%)	14 (9%)	3 (1%)	4 (4%)	
Malignant	318 (98%)	146 (91%)	220 (99%)	98 (96%)	0.02
Primary	41 (13%)	37 (25%)	29 (13%)	12 (12%)	
HCC	18 (44%)	18 (49%)	13 (45%)	5 (42%)	
Bilary	23 (56%)	19 (51%)	16 (55%)	7 (58%)	
Metastatic	277 (87%)	109 (75%)	191 (87%)	86 (88%)	<0.01
Colorectal	249 (90%)	89 (82%)	172 (90%)	77 (90%)	
Other	28 (10%)	20 (18%)	19 (10%)	9 (10%)	

BMI = body mass index; HCC = hepatocellular carcinoma.

2. "Lobe or more" resections were performed equally among control patients (63%) and patients with steatosis (69%; $P = 0.72$). Mean operative time (minutes; control = 254 ± 93, steatotic = 261 ± 89), portal triad clamp time (minutes; control = 32.7 ± 21.8, steatotic = 35 ± 21.3), and mean blood loss (ml; control = 922 ± 1137, steatotic = 845 ± 745) were also similar between groups. No difference was seen in transfusion requirements. The steatosis and controls groups were equally likely to receive "any transfusion" (52% vs. 52%, $P = 0.97$) or "red blood cell transfusion" (control = 46%, steatotic = 42%; $P = 0.50$).

COMPLICATIONS

Perioperative outcomes for all 485 patients and for the subset of patients (N = 324) who had lobe or more resections are shown in Tables 3 and 4, respectively. Average length of hospital stay (days) was similar for both groups (control = 11.0 ± 7.9, steatotic = 10.7 ± 7.1; $P = 0.74$). Thirty-five percent of control patients and 52% of patients with steatotic livers suffered at least one complication. There was a direct correlation between degree of steatosis and percentage of patients experiencing at least one complication (control = 35%, mild = 48%, and marked = 62%; $P < 0.01$). Similarly there were more steatotic patients with infective complications (control = 14%, mild = 24%, marked = 43%; $P < 0.01$). Major (grade 3 or higher) complications were similar among groups, with 24% of control patients and 27% of fatty liver patients experiencing at least one major complication ($P = 0.39$). The percentage of patients requiring admission to the intensive care unit at any point during their hospitalization was similar among groups (control = 8%, mild = 8%, marked = 9%;

Table 2. Operative variables stratified by degree of steatosis (N = 485)

Variable	Steatosis (all) (N = 325)	No steatosis (N = 160)	Mild steatosis (N = 223)	Marked steatosis (N = 102)	Trend
Lobe or more resection	224 (69%)	100 (63%)	160 (71%)	64 (63%)	0.72
Operative time (min)	261 ± 89	254 ± 93	263 ± 89	257 ± 90	0.71
Pringle time	35.0 ± 21.3	32.7 ± 21.8	36.1 ± 22.6	32.6 ± 17.8	0.51
Blood loss (ml)	845 ± 745	922 ± 1137	815 ± 640	911 ± 934	0.22
Any transfusion	168 (52%)	83 (52%)	119 (53%)	49 (48%)	0.62
Red blood cell transfusion	138 (42%)	73 (46%)	98 (44%)	40 (39%)	0.33

Table 3. Outcome—all resections (N = 485)

Variable	Steatosis (all) (N = 325)	No steatosis (N = 160)	Mild steatosis (N = 223)	Marked steatosis (N = 102)	Trend
Length of stay (days, mean ± SD)	10.7 ± 7.1	11.0 ± 7.9	10.5 ± 7.0	11.2 ± 10.1	0.92
Any complication	169 (52%)	56 (35%)	106 (48%)	63 (62%)	<0.01
Major complication	89 (27%)	38 (24%)	62 (28%)	27 (26%)	0.55
Infection	97 (30%)	23 (14%)	53 (24%)	44 (43%)	<0.01
ICU admission	26 (8%)	12 (8%)	17 (8%)	9 (9%)	0.72
Perioperative mortality	14 (4.3%)	5 (3.1%)	8 (3.6%)	6 (5.9%)	0.29
5-year survival	38% ± 6%	45% ± 5%	42% ± 4%	30% ± 6%	0.13*
Average change in total bilirubin	2.0 ± 3.9	1.8 ± 2.4	1.7 ± 2.8	2.7 ± 5.7	0.28
Average change in INR	0.5 ± 0.7	0.4 ± 0.3	0.4 ± 0.3	0.7 ± 1.2	0.98

SD = standard deviation; INR = international, normalized ratio.
*Log-rank test.

$P = 0.72$). These findings were the same for patients who underwent lobe or more resections (see Table 4).

Incidence of specific complications, grouped by category, is shown in Table 5. The steatotic group was significantly more likely to suffer wound-related (13% vs. 1%; $P < 0.01$), hepatobiliary (23% vs. 13%; $P = 0.01$), and gastrointestinal (11% vs. 5%; $P = 0.04$) adverse events in the postoperative period. No difference was observed in hemorrhagic/thrombotic, renal/urologic, cardiovascular, and pulmonary complications.

On univariate analysis, male gender ($P = 0.01$), prolonged operative time ($P = 0.04$), increased BMI ($P = 0.02$), presence of any comorbidity ($P = 0.02$), presence of mild or marked steatosis ($P < 0.01$), higher intraoperative blood loss ($P < 0.01$), and lobe or more resection ($P < 0.01$) were positive predictors of developing a complication (Table 6). On multivariate analysis, including these factors as well as age ($P = 0.08$), only the presence of comorbid factors (risk ratio [RR] = 1.65; $P = 0.02$), steatosis (mild, RR = 1.47; marked, RR = 3.04; $P < 0.01$), increased blood loss (RR = 1.42; $P < 0.01$), and lobe or more resection (RR = 2.16; $P < 0.01$) remained independent predictors of developing complications. The presence of marked steatosis increased the likelihood of suffering at least one complication following hepatic resection by a factor of three. The same findings were observed when the analysis was performed with infective complications in place of any complications (data not shown).

Perioperative Mortality

There were 19 postoperative deaths. Perioperative mortality for control patients was 3.1% for all resections and 5.0% for lobe or more resections (see Tables 3 and 4). The mild group had similar mortality rates of 3.6% (all resections) and 5.0% (lobe or more). Mortality was higher among the marked group (5.9% for all resections; 9.4% for lobe or more), but these differences were not statistically significant on trend analysis ($P = 0.92$ for all resections and $P = 0.68$ for lobe or more). Table 7 shows the results of univariate and multivariate analyses for perioperative mortality. Because all perioperative deaths occurred in patients who had "lobe or more" resections, this analysis was performed for this subset only (N = 324) and

Table 4. Outcome—⩾ lobe resection (N = 324)

Variable	Steatosis (all) (N = 224)	No steatosis (N = 100)	Mild steatosis (N = 160)	Marked steatosis (N = 64)	Trend
Length of stay (days, mean ± SD)	11.6 ± 8.3	12.5 ± 9.3	11.4 ± 7.7	12.0 ± 9.5	0.68
Any complication	128 (57%)	45 (45%)	86 (54%)	42 (66%)	0.01
Major complication	73 (33%)	31 (31%)	54 (34%)	19 (30%)	0.94
Infection	72 (32%)	19 (19%)	43 (27%)	29 (45%)	<0.01
ICU admission	25 (11%)	11 (11%)	16 (10%)	9 (14%)	0.62
Perioperative mortality	14 (6.3%)	5 (5.0%)	8 (5.0%)	6 (9.4%)	0.30
5-year survival	33% ± 4%	40% ± 6%	35% ± 5%	31% ± 7%	0.68*
Average change in total bilirubin	2.7 ± 4.7	2.5 ± 2.8	2.2 ± 3.1	4.2 ± 7.4	0.92
Average change in INR	0.6 ± 0.8	0.5 ± 0.3	0.5 ± 0.3	0.9 ± 1.5	0.55

*Log-rank test.

Table 5. Complications specified by category—all patients (N = 485)

Variable	Steatosis (all) (N = 325)	No steatosis (N = 160)	Mild steatosis (N = 223)	Marked steatosis (N = 102)	Trend
Wound*	43 (13%)	2 (1%)	20 (9%)	23 (23%)	<0.01
Hepatic/biliary†	76 (23%)	21 (13%)	50 (22%)	26 (25%)	0.01
Gastrointestinal‡	35 (11%)	8 (5%)	23 (10%)	12 (12%)	0.04
Hemorrhagic/thrombotic	15 (5%)	5 (3%)	7 (3%)	8 (8%)	0.09
Renal/urologic	26 (8%)	10 (6%)	17 (8%)	9 (9%)	0.32
Cardiovascular	20 (6%)	8 (5%)	12 (5%)	8 (8%)	0.37
Pulmonary	38 (12%)	19 (12%)	25 (11%)	13 (13%)	0.87
Other	16 (5%)	3 (2%)	11 (5%)	5 (5%)	0.17

*Includes seromas, infections, and hernias.
†Inlcudes cholangitis, biliary obstruction, liver failure, hepatic artery infusion pump failure, ascites, perihepatic fluid collection, and perihepatic abscess.
‡Includes gastrointestinal hemorrhage, bowel obstruction, paralytic ileus, infectious diarrhea, fistula, pancreatitis, and esophagtitis.

"lobe or more" was dropped as a covariate. On both univariate and multivariate analyses, only increased blood loss predicted mortality (RR = 1.89; $P < 0.01$). Steatosis did not independently predict postoperative death. Of note, in 38% (3 of 8) of patients with mild steatosis and 50% (3 of 6) of those with marked steatosis who died, death was related to postoperative liver insufficiency; in comparison, there were no deaths in the control group.

Overall Survival

Median follow-up time for all patients was 26 months (range 0 to 124 months) and for survivors was 32 months (range 0 to 124 months). Median survival was 48 months for control patients and 43 months for steatotic patients, and 5-year survival was 45% ± 5% and 38% ± 6%, respectively (see Table 4). Overall survival for all patients with steatosis was not different from survival in the control group ($P = 0.27$, data not shown). Survival for all patients who underwent "lobe or more" resections stratified by degree of steatosis is shown in Fig. 2 and correlating 5-year survival rates are provided in Table 4.

Biochemical Profile

Preoperative and peak postoperative total bilirubin and INR levels were obtained for the majority (80%) of patients in this study. Results, expressed as mean difference, are shown in Tables 3 (all resections) and 4 (lobe or more). No differences were observed among groups for preoperative bilirubin or INR levels. In patients who had lobe or more resections with marked steatosis, mean bilirubin (4.2 ± 7.4 mg/dl) and INR (0.9 ± 1.5) changes were greater than was seen among those with mild steatosis (2.2 ± 3.1 mg/dl, bilirubin; 0.5 ± 0.3, INR) or control patients (2.5 ± 2.8 mg/dl, bilirubin; 0.5 ± 0.3 INR), but these differences were not statistically significant ($P = 0.92$, bilirubin; $P = 0.55$ INR).

Table 6. Analysis of complications—all patients (N = 485)

Variable	Univariate	Multivariate	Risk ratio	Confidence interval
Age	0.08	0.11	1.15	0.97–1.38
Sex	0.01	0.29	0.81 (F vs. M)	0.54–1.20
Operative time	0.04	0.39	1.06	0.93–1.22
BMI	0.02	0.79	1.00	0.96–1.03
Preoperative chemotherapy	0.36	Excluded		
Diabetes	0.77	Excluded		
Any comorbidity	0.02	0.02	1.65	1.07–2.57
Steatosis (marked vs. mild vs. control)	<0.01	<0.01	3.04 (marked)	1.7–5.45
			1.47 (mild)	0.93–2.33
Blood loss	<0.01	<0.01	1.42	1.10–1.85
Lobe or more	<0.01	<0.01	2.16	1.38–3.38

Table 7. Analysis of perioperative mortality in patients having lobe or more resections (N = 324)

Variable	Univariate	Multivariate	Risk ratio	Confidence interval
Age	0.21	Excluded		
Sex	0.65	Excluded		
Operative time	0.25	Excluded		
BMI	0.31	Excluded		
Preoperative chemotherapy	0.39	Excluded		
Diabetes	0.32	Excluded		
Any comorbidity	0.13	0.54	1.20	0.42–3.40
Steatosis (marked vs. mild vs. control)	0.29	0.49	2.24 (marked)	0.57–8.83
			1.35 (mild)	0.36–5.03
Blood loss (L)	<0.01	<0.01	1.89	1.36–2.63

DISCUSSION

Autopsy reports demonstrate fatty liver disease to be present in 6% to 11% of the general population.[13,14] In a recent review of 1803 consecutive liver resections at MSKCC, steatosis was the most common parenchymal disorder encountered in the non–tumor-bearing liver, observed in 18% of patients in this select population.[8] Considerable information has been gathered in the transplant literature regarding impaired function of fatty liver donor grafts. In a multivariate analysis of 227 patients, Ploeg et al.[35] identified severe steatosis as an independent predictor of primary graft failure or subsequent dysfunction. This finding has been corroborated in other studies.[28,30] The impact of less than 30% steatosis on transplantation is uncertain, but primary graft dysfunction following the use of moderately steatotic grafts has been observed in some series.[26,36] For now, because of the paucity of donor organs, moderate steatosis remains only a relative contraindication for orthotopic liver transplantation.

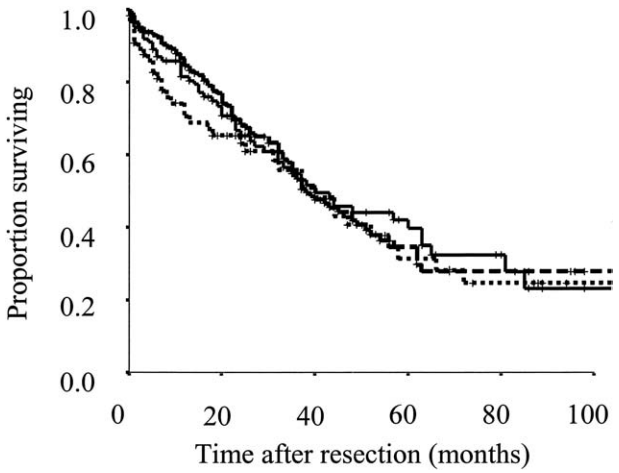

Fig. 2. Kaplan-Meier estimates of overall survival in patients with varying degrees of fatty liver disease who underwent lobe or more resections. Solid line = control subjects (N = 100); dashed line = "mild" steatosis (N = 160); and dotted line = "marked" steatosis (N = 64). No significant difference is observed on log-rank test ($P = 0.7$).

The effects of steatosis on outcome following hepatic resection for benign and malignant neoplasms have not been well defined. Few reports have examined this question, and it is the primary end point of only one study in the English language. Behrns et al.[37] evaluated 135 patients who had lobe or more resections, of whom 56 had mild steatosis and only seven had moderate to severe (marked) steatosis. As expected, the authors noted a direct correlation between steatosis and BMI. They observed longer operative times (355 ± 24 SEM vs. 290 ± 9 SEM, control) and a greater likelihood of transfusion among patients with marked steatosis (71% vs. 51%, control). They found that the group with marked steatosis suffered more complications (29%) than the group with mild steatosis (14%) or the control group (10%), without a difference in length of hospital stay. These investigators reported postoperative mortality rates of 3%, 7%, and 14% for control, mild, and marked steatosis, respectively, and concluded that marked steatosis is associated with a longer operative time, a greater likelihood of transfusion, increased morbidity, and higher postoperative mortality. Statistically meaningful conclusions from this study are limited, given the small number of patients with marked steatosis.

Belghiti et al.[38] reported on 747 consecutive liver resections and noted increased morbidity following resection when fatty infiltration was present (fatty = 22% vs. control = 8%; $P = 0.003$). Infective complications were higher in the group with steatosis, but no difference was seen in postoperative mortality rates (fatty = 0% vs. control = 1%; $P = 0.5$). Again, these conclusions are limited by the small sample size, as only 37 patients were in the steatosis group. Because steatosis was not the main objective of this

report, the degree of fatty change was not quantified, and other relevant parameters such as BMI and diabetes were not evaluated.

A previously published report from MSKCC examined the effects of diabetes mellitus on outcome following hepatic resection for colorectal metastases.[39] Although complication rates were similar among the groups, operative mortality was greater in the group with diabetes mellitus (diabetes mellitus = 8% vs. control = 2%; $P < 0.02$), especially those with underlying steatosis who had lobe or more resections. Although the conclusions regarding diabetes mellitus in this report are important, the small number of patients who had both diabetes mellitus and steatosis and underwent substantial resections (N = 22) did not permit multivariate analysis. Furthermore, the control group in this study was not matched for other comorbid factors, and specifics regarding intraoperative blood loss were not provided.

The present study pairs histologic assessment with chart review to evaluate the importance of underlying fatty liver disease in 325 patients who underwent partial hepatectomy for benign and malignant neoplasms. Observations are made regarding patient characteristics, operative variables, and short- and long-term outcome (morbidity, mortality, and overall survival). Comparison is performed against control patients (N = 160) matched for age, comorbidity, and extent of resection, and sample size is sufficient to provide meaningful conclusions from multivariate analysis. Regarding demographic factors, patients with steatosis were more likely to be men with an elevated BMI. No statistical association was seen between steatosis and diabetes mellitus, but this may be related to the matching process used to select control subjects for comparison. Likewise, heavy alcohol use was not a factor in this series. This may reflect the inherent bias involved in patient selection for liver resection, similar to the process used in transplant centers. In addition, retrospective gathering of alcohol intake history is limited by how carefully patients were screened at time of surgery.

An interesting observation was the strong association between steatosis and previous exposure to cytotoxic chemotherapy. It is well know that medicinal agents may promote subclinical hepatocellular damage resulting in fatty infiltration over time.[18,40] Hormone therapy for breast cancer has been the chemotherapeutic agent most often associated with steatosis.[41,42] Only a few small reports demonstrate an association between cytotoxic chemotherapeutic agents and fatty liver disease.[43–45] Peppercorn et al.[44] used CT findings to identify fatty change following therapy with 5-fluorouracil and folinic acid for advanced colorectal cancer, and noted that 47% of patients had CT findings suggestive of fatty change. No correlation was observed between laboratory findings, drug dose, and steatosis. A more recent case report from Zeiss et al.[43] demonstrated patchy fatty change documented histologically in a patient with unequal distribution of fluorodeoxyuridine via hepatic artery infusion pump. Although the present study made no effort to specify chemotherapeutic dose or drug choice, the patients all received one or several of the standard agents available (5-fluorouracil, fluorodeoxyuridine, leucovorin, and/or CPT-11). Patients with more profound fatty change were more likely to have received one or several of these agents preoperatively. This exposure to preoperative chemotherapy may explain why patients with steatosis were those more likely to have been treated for metastatic disease, and in particular, colorectal cancer metastases.

Contrary to what was observed by Behrns et al.,[37] we found no differences in operative time or transfusion requirements among control patients and those patients with fatty livers (see Table 2). Regarding laboratory values, changes in bilirubin and INR levels were greater in the group with marked steatosis, but these differences were not statistically significant because of the wide variation. Although conclusions from these results are limited, they suggest the possibilities of more profound hepatic dysfunction or impaired postoperative liver regeneration in patients with marked steatosis.

The higher complication rate observed among patients with steatosis reflects the higher incidence of postoperative infections. The frequency of major complications was not affected by the presence of steatosis, as reflected by similar lengths of hospital stay and frequency of admissions to the intensive care unit (Tables 3 and 4). Patients with steatosis were more likely to suffer wound infections, perihepatic abscesses, and postoperative bowel dysmotility. Multivariate analysis for complications, including important covariates such as BMI, preoperative chemotherapy, blood loss, and extent of resection confirmed this finding, as marked steatosis is a powerful predictor of overall and infective complications but not major complications.

In animal models, hepatic macrophages (Kupffer cells) in steatotic livers demonstrate altered cytokine production resulting in depletion of natural killer cells compared to control livers.[19,46] This has been shown to be a qualitative rather than a quantitative phenomenon[47] and is associated with decreased endogenous leptin activity. These macrophages also demonstrate reduced phagocytic activity in vitro.[48] Although immune disturbances specific to steatosis have not been identified in humans, similar mechanisms may be

at work and may help explain the higher rate of infective complications following hepatic resection.

Although this study demonstrates a relative doubling in postoperative mortality among patients with marked steatosis compared to control patients undergoing lobe or more resections (9.4% vs. 5%), no significant difference was found in a logistic regression model with pertinent covariates. It is noteworthy, however, that among patients who died in the perioperative period, death associated with liver insufficiency correlated with degree of steatosis (control = 0%, mild = 38%, marked = 50%). Thus, although steatosis is not statistically associated with increased mortality in this large retrospective series, those patients with significant fatty infiltration following hepatic resection may be at greater risk for hepatic failure. It is clear from the follow-up presented that overall survival is not affected by steatosis.

CONCLUSION

Steatosis is associated with increased overall and infective complications following hepatic resection for neoplasms. By contrast, complications requiring major therapeutic intervention, length of hospital stay, frequency of intensive care unit admission, and 60-day postoperative mortality are not significantly affected by steatosis alone, and intraoperative blood loss and extent of resection are the best predictors of perioperative outcome. Steatosis alone should not preclude hepatic resection in patients being considered for such operations, although caution must still be exercised in patients with marked steatosis who are undergoing large hepatic resections, especially if they suffer from additional comorbid factors.

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Discussion

Dr. L. Rikkers (Madison, WI): Were many of the infectious complications wound infectious in this obese population? I presume that you did obtain postoperative CT scans at various times in these patients. Did you notice any impact of steatosis on liver regeneration?

Dr. Kooby: In answer to your second question, because our study population dates over a decade, the scans were not always readily available, and since it is unlikely that we have serial scans for any given patient, the value of a single postoperative imaging study is limited. Furthermore, the patients requiring postoperative imaging usually have other factors in play, such as infection, which may interfere with liver regeneration. This question would have to be examined in an animal model or prospectively in humans. We did not look at regeneration, so I cannot answer that more specifically.

Regarding infections, most of the infectious complications in patients with steatosis were wound infections and perihepatic abscesses. Because obesity and body mass index were covariants in our multivariate model, and did not show independent associations with development of complications, although steatosis did, this supports our message.

Dr. H. Pitt (Milwaukee, WI): Congratulations on a very nice analysis. I was a little surprised that you only chose to have age as the factor that you used for your control group and not sex and underlying disease. If you had included sex and the type of underlying disease for your matched group, what would the analysis have shown? In addition, did you look at any subgroup with steatohepatitis?

Dr. Kooby: Actually the matching factors we used were age, extent of resection, and presence of comorbid

factors. We used these particular matching factors because we wanted to control for immediately obvious variables that could contribute to outcome following liver resection, that is, older, sicker people with larger resections seem more likely to suffer postoperative complications. Adding additional matching factors would further limit the control group too much. Sex was not used, as I am not aware of any data stating that men or women have more complications after liver resection.

As far as your second question, there was a subset of less than 20 patients who had some inflammation associated with the steatosis. We did not analyze this group separately because of its small size. The more interesting question was the relationship observed between chemotherapy and steatosis. Post-chemotherapy fatty change is another factor surgeons should take into consideration before sending patients for medical therapy prior to resection.

Dr. K. Behrns (Chapel Hill, NC): Just to pick up from Dr. Pitt, how about not only steatohepatitis but also fibrosis or cirrhosis related to a fatty liver?

Dr. Kooby: Those patients were definitely excluded from this analysis. The cohort I am showing are those who either had steatosis or steatohepatitis; the latter group included only a few patients. Patients with fibrosis or cirrhosis were excluded.

Dr. T. Howard (Indianapolis, IN): I enjoyed your presentation very much. I was surprised you did not have nutritional parameters in your covariants, particularly for patients with high body mass index who had received chemotherapy, and albumin has been shown to be a fairly strong correlate with postoperative complications.

Dr. Kooby: The albumin changes in the perihepatectomy phase probably reflect volume changes, and I do not know how relevant they are. We did look at preoperative and postoperative bilirubin and INR in terms of synthetic and functional status of the liver, and we did find slight differences in the group with marked steatosis compared with the control group, but the variation within each laboratory value was wide, and these results were not significant. These data are included in the manuscript.

Metabolic Acidosis Stimulates Intestinal Glutamine Absorption

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Glutamine is an essential nutrient for cell integrity during acidotic states such as shock, but the effect of extracellular pH on intestinal mucosal cell glutamine uptake is poorly understood. The purpose of this in vitro study was to investigate the intracellular signaling pathways involved in controlling intestinal glutamine transport during acidosis. Lowering the pH in the cell culture medium resulted in an increase in glutamine transport activity in a time- and pH-dependent fashion. Chronic acidosis (pH 6.6 for 48 hours) resulted in a twofold increase in glutamine transport activity (1.63 ± 0.25 nmole/mg protein/minute in acidosis vs. 0.78 ± 0.11 nmole/mg protein/minute in control) and a threefold increase in glutamine transport gene ATB^0 messenger RNA levels. This acidosis-induced increase in glutamine transport activity was due to a stimulation of transporter maximal transport capacity (V_{max} 13.6 ± 0.73 nmole/mg protein/minute in acidosis vs. 6.3 ± 0.46 nmole/mg protein/minute in control) rather than a change in transporter affinity (K_m = 0.23 ± 0.02 mmol/L glutamine in acidosis vs. 0.19 ± 0.02 mmol/L glutamine in control). This acidosis-stimulated glutamine transport activity was blocked by actinomycin-D or cycloheximide. Cellular mitogen-activated protein kinase (MAPK) MEK1/2 and p42/44 levels were elevated in acidotic cells, and the acidosis-induced glutamine transport activity was blocked by the MAPK MEK 1 inhibitor PD 98059. Acidosis stimulates glutamine transport in Caco-2 cells via signaling pathways that lead to transcription of the glutamine transporter gene and translation of functional transporters. Mitogen-activated protein kinases are key intracellular regulators involved in this signal transduction cascade. An increased availability of glutamine to cells subjected to redox stress may help in maintaining cellular integrity. (J GASTROINTEST SURG 2003;7:1045–1052)

KEY WORDS: Glutamine, intestine, epidermal growth factor, mitogen-activated protein kinase

Glutamine is the most abundant amino acid in the body and plays a central role in interorgan nitrogen transfer. Glutamine becomes an essential amino acid in stress states (i.e., severe surgical stress and sepsis) when the body synthesis cannot satisfy increased needs.[1] Glutamine has profound effects on gut-related immune functions and an anabolic effect on host protein synthesis. Luminal glutamine transport across the intestinal brush-border membrane into enterocytes is an essential step in maintaining host glutamine homeostasis and is regulated by various local and systemic factors.[1–4] The circulating concentration of glutamine is maintained at a relatively constant level and is dependent on the net rate of uptake and release by various organs; the small intestine is the predominant organ of glutamine uptake in the post-absorptive and basal state.[1]

Chronic metabolic acidosis, a common problem seen in sepsis, shock, and diabetes, is associated with many metabolic derangements.[5–10] The homeostatic response to metabolic acidosis involves alterations in interorgan glutamine flux leading to depletion of circulating glutamine.[11–17] Repletion of circulating glutamine, therefore, will require an increase in the amount of exogenous glutamine supplement via either the gastrointestinal tract or the parenteral

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route. Intestinal glutamine absorption may be a key point in exogenous glutamine entry into homeostasis. However, regulation of intestinal glutamine transport during acidosis is still unknown.

Previously we characterized the apical membrane glutamine sodium-dependent transport system B (90%) and the sodium-independent system L (10%) in a cultured Caco-2 cell line,[18] an in vitro model commonly used for intestinal epithelial nutrient and drug transport studies.[19,20] In this study we explored the intracellular signaling pathways involved in the activation of intestinal glutamine transport by acidosis in Caco-2 cells.

MATERIAL AND METHODS

Caco-2 Cell Cultures

The human intestinal epithelial Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD) at passage 6. Cells were routinely maintained in T-150 flasks in a 37C humidified incubator in 10% CO_2/90% O_2. Cells were routinely grown in Dulbecco's modified Eagle medium (DMEM) containing 25 mmol/L glucose and 0.4 mol/L sodium bicarbonate, supplemented with 10% fetal bovine serum, 4 mmol/L glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acids. The stock Caco-2 cells were passaged weekly after treatment with 0.05% trypsin and 0.02% EDTA. Cells were reseeded at a density of 4.5×10^6 cells per T-150 flask for future subculturing, seeded in six-well Costar tissue culture plates at a density of 10^5 cells per well for Northern blot or Western blot analysis, or seeded in 24-well Costar tissue culture plates at a density of 10^4 cells per well for transport experiments. Near-confluent cells (day 5, passages 15 to 40) were used for experiments. The day of seeding was designated as day 0. The growth medium was changed daily, and cultures were inspected daily using a phase-contrast microscope.

Cell Treatments

Prior to treatment, the cell monolayer was washed three times with serum-free medium and reincubated in serum-free medium (i.e., DMEM supplemented only with penicillin and streptomycin) for 6 hours at 37C. The cell monolayer was then washed three times with serum-free medium and exposed to each agent at various time points and concentrations described below. Treatment media (serum-free medium + agents) were replenished every 6 hours to ensure consistent agent exposure. Cells were treated individually at various extracellular pH levels (pH 6 to 9) for various periods of time (30 seconds to 72 hours). The pH of the treatment media was adjusted through the addition of appropriate amounts of 1 mol/L HCl or $NaHCO_3$.

Cells were also treated with individual inhibitors: mitogen-activated protein kinase (MAPK) MEK 1 inhibitor PD 98059 (0 to 100 μmol/L, dimethylsulfoxide [DMSO] as control), protein kinase C (PKC) inhibitor chelerythrine chloride (0 to 6.6 μmol/L, DMSO as control), as well as actinomycin (0 to 0.1 μmol/L, DMSO as control), and cycloheximide (0 to 10 μmol/L, DMSO as control). Caco-2 cells remained viable (viability >99% by dye exclusion) after at least 72 hours of exposure to serum-free medium.

L-Glutamine Uptake Measurements

L-glutamine transport activity was measured at 37C ± 1.0C. Following pretreatment of cells with various agents (described above), cells were rinsed three times with "uptake buffer" (37C) comprised of 137 mmol/L NaCl (or 137 mmol/L choline chloride), 10 mmol/L HEPES/Tris buffer (pH 7.4), 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, and 2.5 mmol/L $CaCl_2$. Transport was initiated by simultaneously adding 1 ml of this buffer containing also L-[^{3}H]glutamine (2 μCi/ml, 1 μmol/L to 10 mmol/L) into each transport plate (24 wells). Each transport plate contained both control and treatment groups. Cell culture plates were continuously shaken by an orbital shaker (1 Hz) during the uptake period. Uptake was arrested by discarding the uptake buffer and washing cells three times with ice-cold uptake buffer lacking substrate. The radioactivity of isotope extracted from the cells with 1 ml of 1N NaOH was neutralized with acetic acid and assayed by liquid scintillation spectrometry. Protein in the NaOH extract was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).[21] Initial rates of transport activity were determined during the linear uptake period (2 minutes), with zero time points serving as blanks.[21,22] Uptake rates are expressed as nanomoles of glutamine per minute per milligram of cell protein. Sodium-dependent system B glutamine transport was obtained by subtracting total glutamine transport measured in choline chloride buffer from that in NaCl buffer.

Northern Blot Analysis of System B ATB^0 mRNA

Following pretreatment of cells with various agents (described above), cells were rinsed three times with phosphate-buffered saline solution. Total RNA was isolated from control and treated Caco-2 cells using the "Totally RNA" isolation kit (Ambion, Austin,

TX). Briefly, total RNA (10 μg) was separated on a 1% formaldehyde gel and transferred to GeneScreen membrane (New England Nuclear, Boston, MA) in 20 × standard sodium citrate. The membrane was hybridized with an antisense oligonucleotide probe specific to human ATB^0 (5′-TTACATGACTGATT CCTTCTCAGAG-3′), and then stripped and rehybridized with an oligonucleotide probe specific for 18 S ribosomal RNA (5′-GTTATTGCTCAATCTCG GGTG-3′). Autoradiographs were scanned with a laser densitometer and the ATB^0 signal was normalized to 18S RNA. The ATB^0 probes were 3′ end-labeled using terminal transferase and ^{32}P-dATP, and the 18S probe was 5′ end-labeled using T_4 polynucleotide kinase and ^{32}P-ATP.

Western Blot Analysis of Phospho-Protein Kinase C and Mitogen-Activated Protein Kinases

Following pretreatment of cells with various agents (described above), cells were rinsed three times with phosphate-buffered saline. Caco-2 whole-cell lysate was obtained by incubated cells in lysis buffer (50 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1.0 mmol/L EGTA, 100 mmol/L NaF, 0.2 mmol/L Na_3VO_4, 1 mmol/L PMSF, and 10 μg/ml aprotinin) for 30 minutes. Equal amounts of protein from control and treated cells were separated on an SDS-PAGE gel and then transferred to Immobilon-P transfer membrane (Millipore, Medford, MA). The transfer membrane was then hybridized with phospho-PKC antibody or MAPK antibodies (Cell Signaling Technology, Beverly, MA) overnight at 4C and rehybridized with horseradish peroxidase–conjugated secondary antibody (1:2000). Protein signal was detected using the enhanced chemiluminescence system (Amersham Biosciences, Piscataway, NJ).

Statistical Analysis

All experiments were conducted at least in triplicate (including the zero-time blanks), and all experiments were confirmed using at least two independent generations of stock cells. Experimental means are reported ± standard deviation (SD). Comparisons of means were made by analysis of variance with pairwise multiple comparisons by the Newman-Keuls method at $P < 0.05$. Transport kinetic parameters were obtained by fitting data to the Michaelis-Menten equation by nonlinear regression analysis using the Enzfitter computer program (Biosoft, Cambridge, UK).

RESULTS

Effect of Extracellular pH on L-Glutamine Transport Activity

To test the effect of extracellular pH on glutamine transport activity, glutamine (50 μmol/L) transport was measured in a near-confluent Caco-2 cell monolayer after the cells had been treated at various extracellular pH levels (pH 6 to 8) for various periods of time (minutes to 72 hours) (Fig. 1). Acidotic pH stimulated glutamine uptake in a time-dependent manner. At least 24 hours of continuous incubation was required for glutamine transport activity to be stimulated by pH. A prolonged continuous incubation (48 hours) of pH 6.6 resulted in a twofold increase in glutamine uptake activity (see Fig. 1). Pulse pH changes, where cells were exposed to low pH for up to 6 hours and reincubated in neutral pH medium for the remaining incubation period (42 hours), did not affect the glutamine transport activity. Lower pH stimulated glutamine transport activity in a dose-dependent manner. Significant stimulation was observed at pH 6.6 (see Fig. 1). Therefore a 48-hour pH 6.6 treatment point was selected for the subsequent experiments in this study.

The effect of pH on glutamine transport kinetics was then measured, and uptake of glutamine of various concentrations (1 μmol/L to 10 mmol/L) was measured in control and acidotic (pH 6.6, 48 hours) cells. Fig. 2 represents the Eadie-Hofstee transformation of the Na^+-dependent system B glutamine transport kinetics. Acidosis stimulated the system B glutamine transport maximal velocity (V_{max} 6.3 ± 0.46 nmole/mg/min control vs. 13.6 ± 0.73 nmole/mg/min acidosis; $P < 0.01$). However, the transporter apparent affinity (K_m) was not affected by acidosis (K_m 190 ± 20 mu;mol/L glutamine control vs. 230 ± 20 mu;mol/L glutamine acidosis; P = NS) (see Fig. 2).

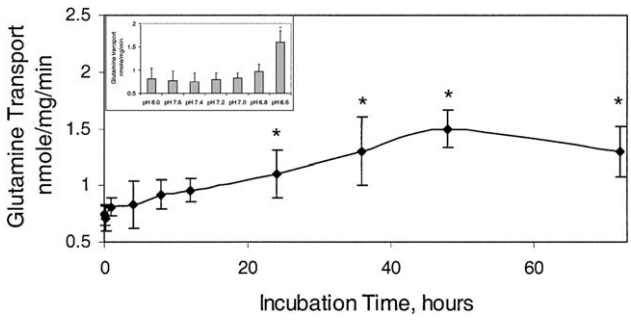

Fig. 1. Effect of pH on glutamine transport activity uptake of glutamine (50 μmol/L) was measured in cells incubated at various extracellular pH (6.6 to 8.0) for various periods of time (minutes to 72 hours). Transport values are means ± SD (n = 6, *$P < 0.05$).

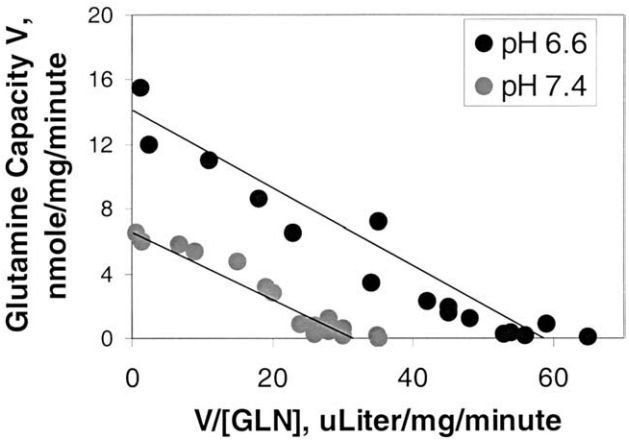

Fig. 2. Eadie-Hofstee transformation of system B glutamine transport kinetics. Uptake of glutamine (*GLN*) (1 μmol/L to 10 mmol/L) was measured in cells incubated in pH 7.4 and pH 6.6 media for 48 hours. Transport values are means ± SD (n = 9).

These data suggest that acidosis stimulates Caco-2 glutamine transport activity via a mechanism that involves an increase in functional transport units as indicated by the transport kinetic parameters.

Involvement of De Novo Transcription and Translation Processes in the Acidosis Stimulation of Glutamine Transport Activity

To test whether the acidosis stimulation of glutamine transport involves de novo transcription and protein synthesis, glutamine transport activity was measured in control and acidosis-treated cells ± actinomycin-D (Act-D; 0 to 0.1 μmol/L) or ± cycloheximide (CHX; 0 to 1 μmol/L) in the incubation medium. Act-D and CHX individually blocked the acidosis-induced system B glutamine uptake (Fig. 3). Baseline control cell transport activity was not affected by Act-D or CHX treatment. The concentration of Act-D and CHX was selected to minimize the nonspecific inhibition effect of Act-D and CHX. Protein content and cell numbers of the Act-D– or CHX-treated cells were comparable to the pretreatment levels. The viability (by dye exclusion) of both control and Act-D/CHX–treated cells was greater than 99%. Compared to the control group (with only DMSO treatment), the Act-D/CHX–treated cells had 20% less protein and 40% less cells. The inhibitory effects of Act-D and CHX on the system B glutamine uptake were likely due to inhibition of new protein synthesis rather than cytotoxic effect.

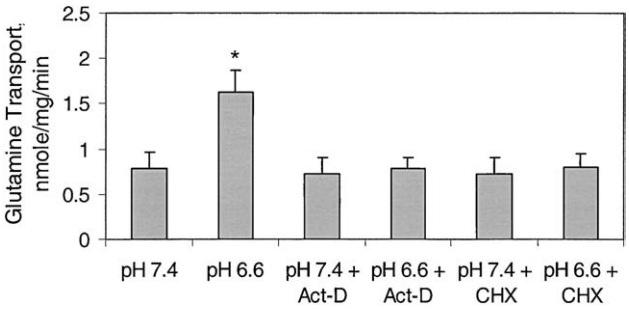

Fig. 3. Effect of actinomycin-D (*Act-D*) and cycloheximide (*CHX*) on the acidosis-stimulated system B glutamine transport activity. Uptake of glutamine (50 μmol/L) was measured in cells incubated in pH 7.4 and pH 6.6 media ± Act-D (0 to 0.5 μmol/L) and CHX (0 to 10 μmol/L). Transport values are means ± SD (n = 9, $^*P < 0.01$).

To assess the effect of acidosis on system B transporter gene ATB^0 expression, ATB^0 mRNA levels were measured in control and acidosis-treated cells. The ATB^0 mRNA level was increased almost threefold in the acidosis group (relative levels: 1.0 control vs. 2.9 ± 0.12 acidosis group; $P < 0.01$) (Fig. 4).

These data suggest that acidosis stimulates glutamine transport via a mechanism that involves de novo transcription and translation, and elevation of transporter mRNA levels.

Involvement of Protein Kinase C Activation in the Acidosis Stimulation of Glutamine Transport Activity

To assess the effect of acidosis on cellular PKC activity, total PKC and phospho-PKC levels were measured by Western blot analysis using commercially available total PKC and phospho-PKC antibodies in control and acidosis-treated cells. Phospho-PKC levels

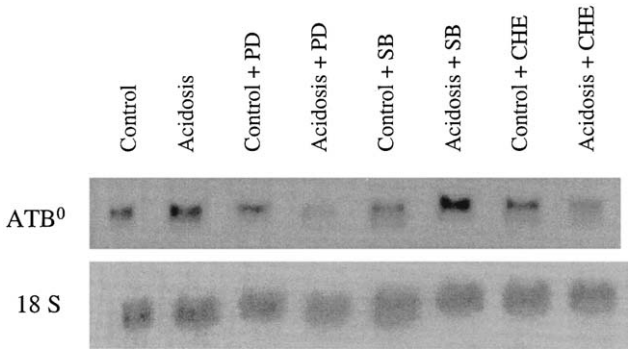

Fig. 4. Northern blot analysis of glutamine transporter system B mRNA (*ATB^0*). Glutamine transporter ATB^0 levels were measured in cells incubated in pH 7.4 and pH 6.6 media for 48 hours ± MAPK MEK 1 inhibitor PD 98059 (*PD*; 0 to 50 μmol/L), MAPK p38 inhibitor SB 203580 (*SB*; 0 to 10 μmol/L), and PKC inhibitor chelerythrine chloride (*CHE*; 0 to 6.6 μmol/L).

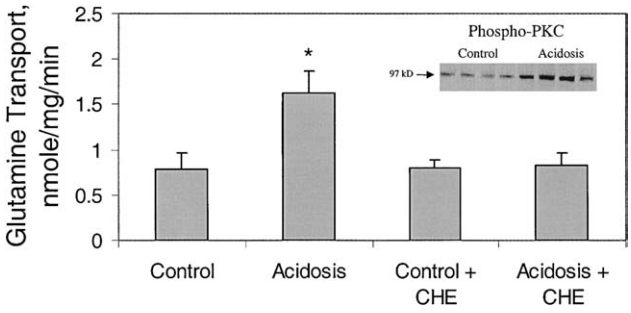

Fig. 5. Involvement of protein kinase C (*PKC*) in acidosis stimulation of system B glutamine transport. Uptake of glutamine (50 μmol/L) was measured in cells incubated in pH 7.4 and pH 6.6 media ± PKC inhibitor chelerythrine chloride (*CHE*; 0 to 6.6 μmol/L). Transport values are means ± SD (n = 9, $^*P < 0.01$). *Inset*, Western blot of phospho-PKC. Whole-cell phospho-PKC levels were measured using monoclonal phospho-PKC antibody in cells incubated in pH 7.4 and pH 6.6 media for 48 hours.

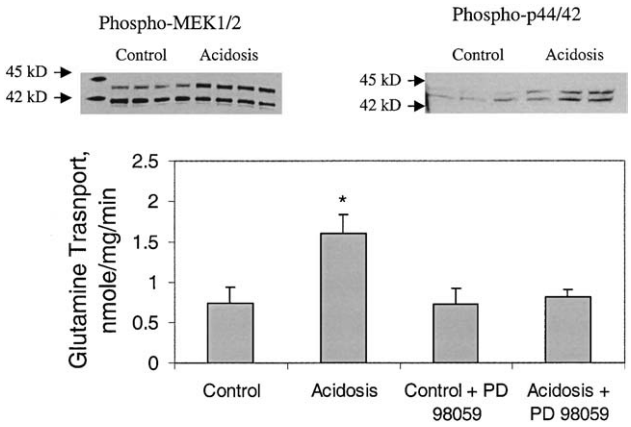

Fig. 6. Involvement of mitogen-activated protein kinase (MAPK) in acidosis stimulation of system B glutamine transport. Uptake of glutamine (50 μmol/L) was measured in cells incubated in pH 7.4 and pH 6.6 media ± MEK 1 inhibitor PD 98059 (0 to 50 μmol/L). Transport values are means ± SD (n = 9, $^*P < 0.01$). *Inset*, Western blot of MAPK phospho-MEK1/2 and MAPK phospho-p44/42. Whole-cell MAPK phospho-MEK1/2, and MAPK phospho-p44/42 levels were measured using monoclonal MAPK phospho-MEK1/2 and MAPK phospho-p44/42 antibodies in cells incubated in pH 7.4 and pH 6.6 media for 48 hours.

were elevated in acidosis-treated cells (Fig. 5), suggesting activation of PKC activity.

To further define the involvement of PKC activation in acidosis stimulation of system B glutamine transport, glutamine transport activity was measured in both control and acidosis-treated Caco-2 cells in the presence and absence of the specific PKC inhibitor chelerythrine chloride (0 to 6.6 μmol/L, DMSO as control). Chelerythrine chloride abolished the acidosis-stimulated system B glutamine transport activity (see Fig. 5) without affecting the baseline control transport level.

These data suggest that acidosis stimulates intracellular PKC activity, and the acidosis stimulation of system B glutamine transport activity is mediated by intracellular PKC activation.

Involvement of Mitogen-Activated Protein Kinases in the Acidosis Stimulation of Glutamine Transport Activity

To assess the effect of acidosis on MAPK activities in Caco-2 cells, MAPK p44/42, MAPK phospho-p44/42, and MAPK phospho-MEK1/2 activity were measured by Western blot analysis using commercially available MAPK p44/42, MAPK phospho-p44/42, and MAPK phospho-MEK1/2 antibodies in control cells and cells treated with acidosis for 48 hours. Acidosis stimulated the MAPK phospho-p44/42, total MAPK p44/42, and MAPK phospho-MEK1/2 levels (Fig. 6), suggesting activation of MAPK MEK1/2 cascade.

To further define the role of MAPKs in the acidosis activation of system B glutamine transport activity, Caco-2 cells were incubated in acidosis with or without coincubation of the MAPK MEK1 inhibitor PD 98059 (0 to 50 μmol/L, DMSO as control). PD 98059 blocked the acidosis-induced activation of glutamine transport without affecting the control cells (see Fig. 6). Similarly, PD 98059 blocked the glutamine transporter ATB^0 mRNA level induced by acidosis (see Fig. 4). However, the MAPK p38 inhibitor SB 230508 did not affect either glutamine transport activity or the transporter ATB^0 mRNA level induced by acidosis, suggesting that it is unlikely that MAPK is a p38 mediator.

These data demonstrate that acidosis stimulates the MAPK MEK1/2 cascade that mediates the acidosis stimulation of system B glutamine transport activity in Caco-2 cells.

DISCUSSION

In the present in vitro study, we investigated the effect of extracellular pH on intestinal glutamine transport in human epithelial Caco-2 cells and the possible associated intracellular pathways that involve activation of PKC and MAPKs.

As the most abundant amino acid, glutamine accounts for 60% of circulating amino acid and plays a pivotal role in interorgan nitrogen transfer, in addition to being the major fuel to support the increased

needs of enterocytes during the stress state. Luminal intestinal glutamine absorption provides a central point of entry for exogenous glutamine to replenish the decreased circulating glutamine levels during stress states.[1]

Chronic metabolic acidosis, a common problem seen in sepsis, shock, and diabetes, is associated with many metabolic derangements.[5–10] Metabolic acidosis causes changes in whole-body glutamine flux. The homeostatic response to metabolic acidosis involves alterations in interorgan glutamine flux leading to the depletion of circulating glutamine resulting in the following: (1) the kidney, which normally extracts little if any glutamine from the blood, becomes the major site of glutamine consumption; (2) the liver switches from an organ of net glutamine uptake to one of net glutamine release; and (3) glutamine release from skeletal muscle is doubled. Increased exogenous glutamine supplement, via either intestinal absorption or the parenteral route, is needed to replenish the circulating glutamine. However, the effect of regulation of metabolic acidosis on intestinal glutamine absorption is still unknown.

Human intestinal epithelial Caco-2 cells, derived from colon epithelial cells, undergo spontaneous differentiation in this cell culture environment. The differentiated cells display small intestinal epithelial characteristics, such as polarized cell membrane, with specific membrane marker enzymes, such as alkaline phosphatase, sucrase, and sodium-potassium ATPase.[19,20] Caco-2 cells have been widely used as the in vitro small intestinal epithelia model for nutrient transport and drug transport studies.[19,20] Intestinal glutamine absorption is mediated by discrete amino acid transport systems. In our previous studies we characterized L-glutamine transport systems in the Caco-2 cell brush-border membrane. Glutamine is predominantly transported by the sodium-dependent transport system B (90%) with minimal contribution from the sodium-independent transport system L and passive diffusion.[18]

As shown in Fig.1, prolonged continuous acidosis stimulated the glutamine transport activity in a time- and pH-dependent manner. More than 24 hours of continuous incubation was required for acidosis to exhibit the stimulatory effect while transient acidosis did not affect glutamine transport. Taken together, the data suggest that the acidosis stimulation of glutamine transport is a chronic process rather than an acute response.

Because of the chronic effect of acidosis on glutamine transport, it is anticipated that transcription transporter gene and translation of transporter protein may be involved in this acidosis stimulation. Act-D or CHX in the incubation medium each blocked the acidosis-induced glutamine uptake (see Fig. 3), indicating the involvement of transcription and de novo protein synthesis. Low concentrations of Act-D and CHX were selected to minimize the nonspecific inhibitory effect that Act-D and cycloheximide might have on cells. The elevation of transporter ATB^0 mRNA after acidosis treatment (see Fig. 4) further demonstrates that acidosis stimulates the system B glutamine transport activity by either specifically enhancing transcription of the system B transporter ATB^0 or stabilizing the transcribed mRNA. Transport activity can be altered by modulation of existing transporter and/or synthesis of new transporter units. Kinetic analyses of system B activity showed that acidosis stimulated the transport maximal capacity V_{max} without affecting the apparent K_m (see Fig. 2). These data suggest that acidosis stimulates glutamine uptake by increasing functional copies of system B transport units rather than modifying transport affinity. Kinetic analysis revealed that the passive diffusion coefficient was the same in control and acidosis-treated cells (data not shown). These data suggest that the acidosis-induced increase in glutamine transport is not due to an increase in cell membrane permeability commonly observed in cells in acidosis. Because a system B antibody is currently not available, it is unclear whether the observed increase in transport activity V_{max} reflects de novo protein synthesis of the transporter protein itself or another regulatory protein.

PKC is a family of intracellular enzymes that mediates diverse biological functions—intestinal mucosal functions that include amino acid transport.[23–26] As shown in Fig. 5, acidosis increases phospho-PKC level indicating increased PKC activity. By itself, increased phospho-PKC only indicates that acidosis activates PKC, it does not establish a linkage between acidosis and glutamine transport. Chelerythrine chloride, a specific PKC inhibitor that specifically inhibits the catalytic domain of PKC,[27,28] blocks the acidosis-induced glutamine uptake. These data demonstrate the involvement of PKC activation in the acidosis stimulation of system B glutamine transport in Caco-2 cells.

MAPKs are a family of kinases that mediate various biological activities and regulation of gene expression in response to various stimuli.[29,30] There are at least four distinctly regulated groups of MAPKs: extracellular signal-related kinases (ERK)-1/2, Jun amino–terminal kinases (JNK1/2/3), p38 protein (p38α/β/γ/δ), and ERK5. Each group is activated by a specific MAPK such as MEK1/2 for ERK1/2 or MKK3/6 for p38.[29–31] Acidosis initiates many of its biologic

activities via activation of intracellular MAPK pathways.[32,33] The intracellular signaling cascade for acidosis-induced system B glutamine transport in Caco-2 cells is unknown.

To assess the role of MAPKs in acidosis-induced system B glutamine transport, we first demonstrated that acidosis activates the MAPK kinase cascade. Acidosis increased the MAPK phospho-p44/42, MAPK P44/42, indicating that acidosis activates MAPK p44/42 activity (see Fig. 6). Furthermore, MAPK MEK1/2 levels were elevated by acidosis-exposure (see Fig. 6), suggesting that the acidosis activates MAPK MEK1/2 cascade. To delineate the relationship among acidosis, MAPK, and system B glutamine transport, we measured Caco-2 system B glutamine transport activity in the presence of individual MAPK inhibitors. 2′-amino-3′-methoxyflavone (PD 98059), a potent and selective inhibitor of MAPK/ERK kinase 1 (MEK1).[34] PD 98059 blocks the activation of MEK1, therefore, inhibiting the subsequent phosphorylation and activation of MAPKs such as ERK and biological responses. As shown in Fig. 6, PD 98059 blocked acidosis-stimulated glutamine transport activity without affecting the baseline activity, suggesting that acidosis-induced upregulation of system B glutamine transport activity involves MAPK ERK cascade. SB 230508, a specific MAPK p38 inhibitor,[35] did not inhibit the acidosis-induced glutamine transport, suggesting the unlikely involvement of MAPK in the p38 pathway.

In this study we explored the regulatory mechanisms of intestinal glutamine transport by acidosis in cultured intestinal epithelial cells. The major limitation of this in vitro study is the extreme chronic acidosis (pH 6.6) conditions employed. Even though the degree and duration of acidosis in this study may not represent an in vivo physiologic condition, the activation of glutamine transport activity in the present cultured Caco-2 cells studied is consistent with the increase in intestinal glutamine absorption in rats under more physiologic chronic metabolic acidosis conditions (pH 7.2 to pH7.3). Furthermore, this in vitro model enables us to study the intracellular signal pathways involved in a more controlled cell culture setting.

CONCLUSION

Chronic acidosis stimulates intestinal system B glutamine transport activity and transporter ATB^0 mRNA expression. This stimulation is the result of an increase in transporter units rather than a modifying transporter affinity, most likely because of a de novo synthesis of new transporters. Intracellular PKC and mitogen-activated protein kinase ERK are the likely pathways mediating this acidosis-induced system B glutamine transport.

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Convergence of the Thyroid Hormone and Gut-Enriched Krüppel-Like Factor Pathways in the Context of Enterocyte Differentiation

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The gut-enriched Krüppel-like factor (KLF4) and the ligand-bound thyroid hormone receptor (TR) have each been shown to play a critical role in mammalian gut development and differentiation. We investigated an interrelationship between these two presumably independent pathways using the differentiation marker gene, intestinal alkaline phosphatase (IAP). Transient transfections were performed in Cos-7 cells using luciferase reporter plasmids containing a 2.5 kb segment of the proximal human IAP 5′ regulatory region, as well as multiple deletions. Cells were cotransfected with TR and/or KLF4 expression vectors and treated ± 100 nmol/L thyroid hormone (T3). IAP reporter gene transactivation was increased independently by KLF4 (ninefold) and ligand-bound TRβ1 (sevenfold). Cells cotransfected with KLF4 and TRβ1 in the presence of T3 showed synergistic activation (70-fold). A similar pattern was seen with the other T3 receptor isoform, TRα1. The synergistic effect was lost with deletions of the T3 and KLF4 response elements in the IAP promoter and was completely or partially abolished in the case of mutant KLF4 expression vectors. The thyroid hormone receptor complex and KLF4 synergistically activate the enterocyte differentiation marker gene IAP, suggesting a previously unrecognized interrelationship between these two transcription factor pathways. (J GASTROINTEST SURG 2003;7:1053–1061)

KEY WORDS: Intestine, differentiation, transcription factors, thyroid hormone, gut-enriched Krüppel-like factor

The mammalian gut plays a vital role in maintaining the nutrition of the organism, and this is made possible by its unique morphologic characteristics. The epithelial layer lining the lumen of the intestine is composed primarily of enterocytes that facilitate digestion and absorption of dietary nutrients. This epithelium is arranged along a vertical axis composed of crypts and villi, and it continuously undergoes a process of maintenance and renewal. Stem cells located in the crypts proliferate to produce four cell lineages (enterocytes, mucus-secreting goblet cells, paneth cells, and enteroendocrine cells), which then migrate along this vertical axis, differentiate, and eventually undergo programmed cell death (apoptosis) and are extruded into the lumen of the intestine.[1,2] This ordered renewal of the epithelium is essential for the maintenance of normal intestinal function; alterations in these processes of growth and differentiation underlie various gut disease states such as inflammation and cancer. It is important, therefore, to unravel the molecular mechanisms responsible for gut epithelial development and homeostasis.

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The development of the phenotype of the mature enterocyte requires the controlled expression of cell-specific genes (e.g., sucrase, lactase) and is thought to occur through the interplay of specific transcription factors present in these cells. Several key gut transcription factors have been identified and include Cdx1, Cdx2, hepatocyte nuclear factor-1 alpha, and some of the GATA factors.[3–8] Previous work on gut gene expression has largely focused on a single transcription factor in the context of a particular target gene. It is clear, however, that the cellular environment contains many transcription factors that likely work in a cooperative fashion to ultimately control gene expression. In the present work, therefore, we have focused on the combined effects of thyroid hormone (T3) and the gut-enriched Krüppel-like factor (KLF4/GKLF), both of which are believed to play important roles in intestinal development and homeostasis.

At the time of weaning in mammals, increasing endogenous levels of thyroid hormone are partly responsible for the dramatic changes that occur in the histology and molecular phenotype of the intestine.[9,10] In addition to the critical role that thyroid hormone plays in gut development, it also exerts profound effects on the adult intestine. For example, hypothyroid rats exhibit decreased mucosal thickness, marked crypt-villus hypoplasia, and reduced enterocyte migration, as well as an altered pattern of expression of brush-border enzymes.[11–15] It is known that the cellular effects of T3 are generally mediated by thyroid hormone receptors (TRs), which bind to the promoters of target genes and then alter transcription. Three bona fide TRs (TRα1, TRβ1 and TRβ2), containing both DNA binding and ligand (T3) binding domains, have been described and are the products of two different genetic loci. TRα1 and TRβ1 are found in abundance in the intestine, and knockout studies have revealed that deletion of the TRα locus, in particular, leads to profound alterations in intestinal morphology, in the expression of gut transcription factors, and in the levels of digestive enzymes.[16,17]

The gut-enriched Krüppel-like factor is a zinc finger containing transcription factor that is expressed in a limited range of tissues and is especially enriched in the intestine.[18–20] Within the intestinal epithelium, KLF4 expression is markedly increased in the more differentiated cells.[18] These initial observations suggested a role for KLF4 in the vital cellular processes of growth and differentiation. KLF4 has now been identified as an important transcriptional regulator of a number of genes involved in cell cycle regulation including cyclin D1, p21, β-catenin, and ornithine decarboxylase.[21–26] KLF4 has also been implicated in the regulation of genes such as keratin 19, keratin 4, and rat laminin γ1 that have a role in tissue architecture and cellular differentiation.[27–29] A recent study using cDNA microarray technology has identified several gene clusters with similar functions that are regulated by KLF4.[30] In addition, although homozygous deletion of the KLF4 gene is fatal in early postnatal life in mice because of skin barrier dysfunction, examination of the colon in these mice revealed abnormalities in the mucus-secreting goblet cell population of the epithelium.[31,32]

Intestinal alkaline phosphatase (IAP) is a brush-border enzyme that is specific to the differentiated villus cells of the gut epithelium and is a well-known marker of enterocyte differentiation.[9,15,33] Previous work by our laboratory has established that both thyroid hormone[14,15,34,35] and KLF4 (manuscript submitted for publication) independently regulate IAP gene transcription. In the present work we have identified an interrelationship between these two important transcription factor pathways, demonstrating synergy in regard to activation of the IAP gene. This synergy requires the presence of the response elements of both T3 and KLF4, and may be mediated by protein-protein interactions involving specific domains within the KLF4 protein.

MATERIAL AND METHODS

Cell Culture

Cos-7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in standard Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Rockville, MD) with 10% fetal bovine serum, 2 mmol/L glutamine, and 100 U/ml penicillin-streptomycin (Bio-Whittaker, Walkersville, MD) at 37° C and 5% CO_2. Experiments were performed with cells at 70% confluence. Medium was changed every 3 days and just prior to each experiment.

Plasmids

The 2.5 kb *Sac* I-*Bam* HI fragment from $IAP_{2.4}CAT$ carrying the proximal human IAP promoter region (−2574 to −49, relative to translation initiation codon ATG) was subcloned into the pGL3-basic promoter-detection vector (Promega, Madison, WI) digested with *Sac* I and *Bgl* II, thus constructing the plasmid pIAP-2574/-49. Various 5′ deletion mutants were constructed by digesting the plasmid pIAP-2574/-49 with *Sac* I and another appropriate restriction enzyme followed by generation of blunt-ended fragments, agarose gel purification of the desired band, and recircularization. The internal

deletion plasmid pIAP-2574/Δ-224/-114 was generated by deleting a *Mlu* I-*Btr* I restriction fragment from pIAP-2574/-49. TRα1 and TRβ1 expression plasmids were obtained from A.N. Hollenberg. Wild-type and mutated KLF4 expression vectors were obtained from V.W. Yang.[36]

Transient Transfection Assay

Cells were seeded at a density of approximately 5×10^5 cells per well in six-well cluster plates (Sigma, St. Louis, MO). Prior to transfection, the cells were grown for 24 hours in DMEM containing fetal bovine serum "stripped" of thyroid hormone (T3) prepared by treating fetal bovine serum with ion-exchange resin and charcoal.[37] Transient transfections were accomplished using the SuperFect transfection kit (Qiagen, Valencia, CA) as per the manufacturer's protocol. The test plasmid DNA was cotransfected with control plasmid pRL-CMV DNA (1 μg/well) (Promega, Madison, WI), and the total amount of DNA was kept the same for each transfection by the addition of nonspecific plasmid TF12 DNA. After transfection, the cells were maintained in "stripped" media for 24 hours and then treated ± T3 (100 nmol/L) (Sigma). Another 24 hours later, Firefly and *Renilla* luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega) as per the manufacturer's instructions. The control *Renilla* luciferase activity (pRL-CMV) was used to determine transfection efficiency as well as to normalize the Firefly luciferase activity data.

Statistical Analysis

Statistical analyses were performed using a standard one-way analysis of variance (ANOVA) with Dunnet's post-test (InStat software; GraphPad Software, Inc., San Diego, CA). $P < 0.05$ was considered statistically significant.

RESULTS

Thyroid Hormone and KLF4 Synergistically Activate the 2.5 kb IAP Promoter

We subcloned the proximal 2.5 kb human IAP promoter into the pGL3-Basic promoter detection vector to construct pIAP-2574/-49. The IAP promoter contains a biologically functional thyroid hormone response element in between −632 and −612, and it also carries two KLF4 binding sites between −205 and −140 (unpublished data). The pIAP-2574/-49 plasmid was transfected into Cos-7 cells along with the plasmids expressing TRβ1 and KLF4, individually or both together. The basal activation was determined in the absence of T3 and any exogenous transcription factor. T3/TRβ1 caused sevenfold activation ($T3^+TR\beta1^+$/Basal; $P < 0.05$) of the IAP promoter, and KLF4 caused ninefold activation ($KLF4^+$/Basal; $P < 0.05$) (Fig. 1*A*). When the two pathways were tested together, they caused an activation of approximately 70-fold ($T3^+TR\beta1^+KLF4^+$/Basal; $P < 0.05$), a level higher than can be attributed to an additive effect between the two factors. It appears, therefore, that these two transcription factor pathways can function synergistically in activating the IAP gene.

The Synergy Is Dose Dependent

We sought to further define the nature of this interaction by increasing the amount of KLF4 in our transfections from 0 μg to 5 μg. Fig. 1, *B* demonstrates that the synergy between T3/TRβ1 and KLF4 follows a classic dose-response pattern with the degree of promoter activation mirroring the increase in transfected KLF4 plasmid.

TRα1 Also Confers Synergy

Three bona fide isoforms of the thyroid hormone receptor have been identified (TRα1, TRβ1, and TRβ2), two of which are known to be expressed in the intestine (TRα1 and TRβ1). We repeated the transfection experiments using a TRα1 expression plasmid and obtained results that were similar to those obtained with TRβ1 (Fig. 2). These data indicate that the interaction between thyroid hormone and KLF4 on the IAP promoter can be mediated by either of the two thyroid hormone receptor isoforms normally expressed in the intestine.

T3/KLF4 Synergy Is Dependent on Specific Sequences in the IAP Promoter

We constructed a number of 5′ deletions and an internal deletion of the IAP promoter, and used them to identify sequences in the IAP gene that function in mediating the T3/KLF4 synergy. In Fig. 3 the data have been expressed in terms of excess induction. With the use of this measure, a value of 1 corresponds to the degree of IAP reporter activation if T3/TRβ1 and KLF4 had an additive effect on the promoter. With this measure, the full-length 2.5 kb IAP reporter plasmid gives a value of approximately 4 ($P < 0.05$), which is indicative of synergistic activation. The thyroid hormone response element (TRE) in the IAP promoter has been localized between −632 and −612 (manuscript submitted, see also Fig. 3). Two KLF response elements (KLF4RE) have also been identified between −205 and −140 (manuscript in

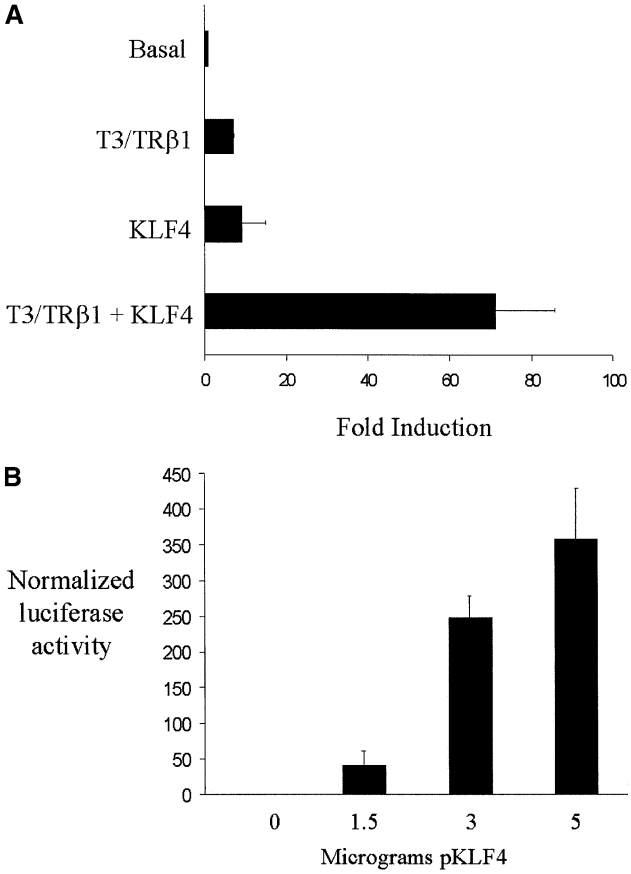

Fig. 1. A, Transient cotransfections of Cos-7 cells with the 2.5 kb IAP-luciferase reporter plasmid along with plasmids expressing TRβ1 and/or KLF4. The amount of each plasmid used in transfection was 1.5 μg. Nonspecific plasmid DNA was used to maintain equal amounts of transfected DNA in all cases. One μg of pRL-CMV, a *Renilla*-luciferase expression plasmid, was added to each sample to monitor transfection efficiency and to normalize the firefly luciferase data. T3 was added to the culture medium in a concentration of 100 nmol/L. Basal activity reflects the independent activity of the 2.5 kb IAP-luciferase reporter. Results are expressed in terms of the fold-induction of the firefly luciferase activity over basal after normalization with *Renilla* luciferase activity. The results were obtained from at least three independent experiments, and the values are expressed as mean ± standard deviation (SD) ($P < 0.05$). **B**, Transient cotransfections of Cos-7 cells with the 2.5 kb IAP-luciferase reporter plasmid (1.5 μg) along with the TRβ1 expression plasmid (1.5 μg) and increasing amounts of the KLF4 expression plasmid (0, 1.5, 3, and 5 μg). Nonspecific plasmid DNA was used to maintain equal amounts of transfected DNA in all cases. One μg of pRL-CMV was added to each sample to monitor transfection efficiency and to normalize the firefly luciferase data. All samples were treated with 100 nmol/L T3. Results are expressed in terms of firefly luciferase activity after normalization with *Renilla*-luciferase activity. The results were obtained from at least three independent experiments, and the values are expressed as mean ± SD ($P < 0.05$).

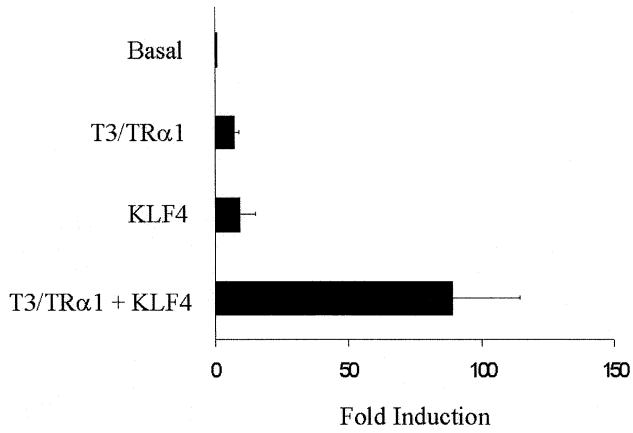

Fig. 2. Transient cotransfections of Cos-7 cells with the 2.5 kb IAP-luciferase reporter plasmid (1.5 μg) along with the TRα1 expression plasmid (1.5 μg) and the KLF4 expression plasmid (1.5 μg). Nonspecific plasmid DNA was used to maintain equal amounts of transfected DNA in all cases. One μg of pRL-CMV, a *Renilla*-luciferase expression plasmid, was added to each sample to monitor transfection efficiency and to normalize the firefly luciferase data. T3 was added to the culture medium at a concentration of 100 nmol/L. Basal activity reflects the independent activity of the 2.5 kb IAP-luciferase reporter. Results are expressed in terms of the fold-induction of the firefly luciferase activity over basal after normalization with *Renilla*-luciferase activity. The results were obtained from at least three independent experiments, and the values are expressed as mean ± SD ($P < 0.05$).

press, see also Fig. 3). Synergy is observed in the plasmids carrying both TRE and KLF4 response elements, for example, in pIAP-2574/-49 and pIAP-750/-49 (see Fig. 3). Deletion of TRE in plasmids pIAP-304/-49 and pIAP-228/-49 abolishes the synergy. These two plasmids showed no T3-mediated activation; however, they did display the expected KLF4-mediated activation (data not shown). The plasmid pIAP-113/-49 has lost both TRE and KLF4REs, and as expected shows no synergy. The IAP promoter in this plasmid cannot be activated either by T3 or by KLF4 (data not shown). To evaluate the effect of KLF4REs on synergy, we deleted the region between −224 and −114 carrying the identified two KLF4REs from the plasmid pIAP-2574/-49, thus constructing the plasmid pIAP-2574/Δ-224/-114 (see Fig. 3). Transfection experiments with this plasmid also show no synergy (see Fig. 3). These transient transfection experiments using various IAP luciferase reporter plasmids establish the requirement for both the identified TRE and KLF4REs in order for the T3/TR and KLF4 pathways to function in a synergistic fashion in the context of the IAP gene.

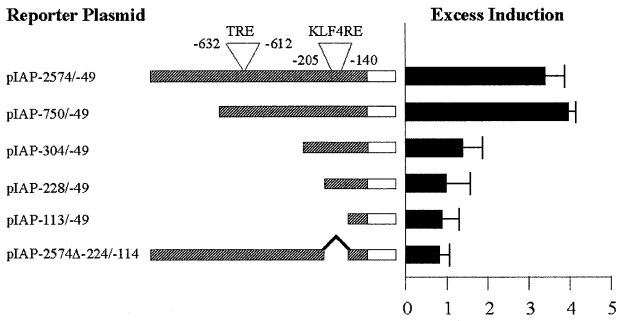

Fig. 3. Transient cotransfections of Cos-7 cells with various human IAP-luciferase reporter constructs (1.5 μg each). All samples were cotransfected with TRβ1 (1.5 μg) and KLF4 (1.5 μg), and treated with T3 (100 nmol/L). One μg of pRL-CMV, a *Renilla*-luciferase expression plasmid, was added to each sample to monitor transfection efficiency and to normalize the firefly luciferase data. Results are expressed in terms of excess induction over the predicted additive effect (a value of 1 corresponds to the degree of IAP reporter activation if T3/TRβ1 and KLF4 were to have an additive effect on the plasmid). The results were obtained from at least three independent experiments, and the values are expressed as mean ± SD ($P < 0.05$).

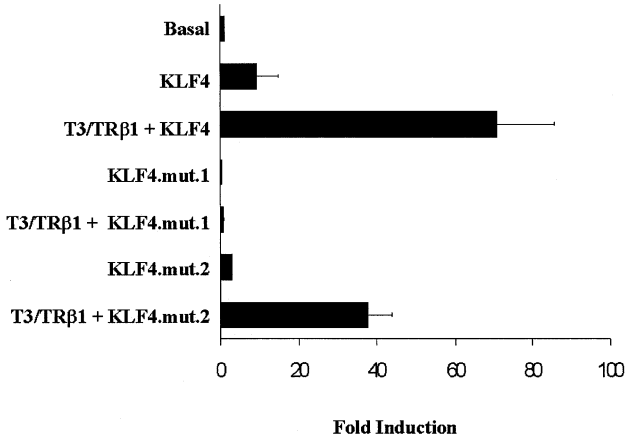

Fig. 4. Transient cotransfections of Cos-7 cells with the 2.5 kb IAP reporter plasmid, along with wild-type and mutant KLF4 expression vectors, independently and in conjunction with T3/TRβ1. The quantity of each plasmid mentioned above was 1.5 μg, Nonspecific plasmid DNA was used to maintain equal amounts of transfected DNA in all cases. The first mutant (*KLF4.mut.1*) contains the sequence for the DNA binding domain and a nuclear localization sequence. The second mutant (*KLF4.mut.2*) contains the sequence for the transactivation domain and a nuclear localization sequence. One μg of a CMV, *Renilla*-luciferase plasmid, was added to each sample to examine transfection efficiency and to normalize the firefly luciferase data. Basal activity reflects the independent activity of the 2.5 kb IAP-luciferase reporter. Results are expressed in terms of the fold-induction of the firefly luciferase activity over basal after normalization with *Renilla* luciferase activity. The results were obtained from at least three independent experiments, and the values are expressed as mean ± SD ($P < 0.05$).

Reduced Synergy With KLF4 Mutant Plasmids

The KLF4 protein contains a DNA binding domain, two nuclear localization sequences, and a transactivation domain.[36,38] We employed mutant KLF4 expression plasmids, in which some of these sequences had been deleted, to understand the mechanism of interaction between KLF4 and liganded TR. The first mutant (KLF4.mut.1) contains the sequences for the DNA binding domain and a nuclear localization sequence.[38,39] Fig. 4 illustrates the effect of this mutant on the full-length (2.5 kb) IAP promoter, on its own and in conjunction with T3/TRβ1. This mutant plasmid was unable to activate the IAP promoter on its own. In addition, not only was it unable to synergistically activate the IAP promoter in conjunction with T3/TRβ1, it also appeared to prevent T3/TRβ1 from exerting its own effect on the promoter.

The second mutant construct (KLF4.mut.2) contains the sequence for the transactivation domain and a nuclear localization sequence. The transactivation domain is also known to mediate interactions between KLF4 and other nuclear proteins.[36] As shown in Fig. 4, this mutant is unable to cause significant activation of the IAP promoter on its own. However, in the presence of T3/TRβ1 this construct activated the IAP promoter to an intermediate degree, more than the effect of T3/TRβ1 alone and less than the effect seen with T3/TRβ1 and wild-type KLF4. It appears, therefore, that this mutant KLF4 may activate the IAP promoter through a protein-protein interaction with T3/TRβ1.

DISCUSSION

Thyroid hormone (T3) is well known to play a critical role in the development and maturation of the mammalian intestine. In particular, T3 enables the maturation of the intestinal mucosa from the suckling to adult stage, with the corresponding changes in brush-border enzyme expression.[9,10] In addition, hypothyroid adult rats exhibit an intestinal phenotype that is more typical of the suckling stage.[15] Using both in vitro and in vivo model systems, we have previously identified one of the brush-border enzymes positively regulated by T3 as IAP, a well-characterized marker of differentiation.[14,15,34,35]

GKLF/KLF4 is a member of a family of transcription factors (Krüppel-like factors) that have been

shown to play key roles in the regulation of the cell cycle, differentiation, and the development of various cell lineages.[40,41] KLF4 is expressed in the terminally differentiated cells of the skin and intestine, and knockout studies have implicated it as vital for the development of the skin barrier and also for normal development of the colon.[18,31,32] Finally, recent works by us and by Chen et al.[30] have identified KLF4 as an important transcriptional regulator of the IAP gene.

Because both T3 and KLF4 appear to play critical roles in the attainment of a mature intestinal phenotype, it was of interest to examine a potential interaction between these two pathways using IAP as a target gene. With the use of a system of transient transfections, we have demonstrated synergistic activation of our target gene IAP by T3/TR and KLF4. This synergy is proportional to the amount of KLF4 protein and is seen with either of the thyroid hormone receptors present in the gut, TRα1 and TRβ1.

Recent knockout studies have implicated TRα1 as the key TR in regard to intestinal development. TRα knockout mice were noted to have profoundly altered intestinal development with decreases in cell numbers along the crypt-villus axis, decreases in proliferating crypt cells, and low levels of expression of digestive enzymes.[16,17,42] TRβ1 is likely able to partially, but not completely, substitute for TRα1 in the intestine.[16] Our data demonstrate that both TRβ1 and TRα1 are capable of synergy with KLF4 in the context of IAP gene activation; however, we cannot determine from these in vitro studies whether this synergy actually occurs in vivo and whether it is mediated by TRα1, TRβ1, or both.

Other isoforms of the thyroid hormone receptor have been identified as products of the TRα gene locus, including TRα2, TRΔα1, and TRΔα2.[43] The exact physiologic role and mechanism of action of these isoforms is unclear, but indirect evidence implicates them as playing an important role in intestinal development.[44–46] TRα2 has a DNA binding domain but no ligand binding domain, and it is thought to play a role in silencing thyroid hormone–responsive genes by blocking the TREs.[47,48] We have previously correlated the levels of TRα2 with unresponsiveness to T3 within the gut tissues in vivo.[44] Further study will be required to determine whether TRα2 plays any role in conjunction with KLF4. Similarly, the effects of the TRΔ isoforms on differentiation marker genes such as IAP, and the ability of these isoforms to interact with other transcription factors such as KLF4, is unknown.

In most thyroid hormone–responsive genes the thyroid hormone receptor binds to its TRE and regulates transcription when heterodimerized with the retinoid X receptor (RXR).[49] In addition, the thyroid hormone receptor is known to recruit and interact with a host of coactivators and corepressors.[49] To our knowledge this is the first report of an interaction between thyroid hormone and a member of the family of Krüppel-like transcription factors. Furthermore, although KLF4 is known to interact with the transcription factor Sp1 on a number of promoters and also with other Krüppel-like transcription factors (BTEB2/KLF5, Zf9/KLF6), this is the first report of an interaction between KLF4 and a nuclear receptor.[25–28,50–53] Interestingly, in all previous reports of interaction between KLF4 and Sp1, the two factors were found to bind the same DNA sequences and thus either compete for the same site or act cooperatively with each other.[25–28,50] However, in the case of the IAP promoter the transcription factors T3/TR and KLF4 appear to act through distinct and separate elements. In this work we have used 5′ and internal deletions of the IAP promoter in our transfection assays to demonstrate that the deletion of either of the response elements is sufficient to abrogate the synergy between these factors.

In the absence of T3/TR and KLF4, minimal IAP promoter activity was observed in the Cos-7 cell line, which is shown as the basal activation (Figs. 1*A*, 2, and 4). These results suggest that although it is probable that the region of the IAP promoter from −49 bp to −2.5 kb may contain other regulatory elements, these elements are not responsive to endogenous transcription factors expressed in the Cos-7 cell line. It is clear, therefore, that the observed IAP activation is the consequence of transactivation by T3/TR and KLF4.

Finally, in an effort to elucidate the mechanism by which KLF4 interacts with liganded TR, KLF4 expression constructs containing deletions of the sequences encoding its major domains were used in transient transfections with the longest IAP reporter construct and the TRβ1 expression plasmid. The KLF4 construct (KLF4.mut.1) containing the DNA binding domain and nuclear localization signal was unable to synergistically activate the IAP promoter with T3/TRβ1. Interestingly, this construct also prevented the activation normally seen with T3/TRβ1. It is possible that this mutant binds to the IAP promoter and hinders other transcriptional activity, even though it has been rendered incapable of transcriptional activation because of its lack of a transactivation domain.[36] Conversely, the other mutant (KLF4.mut.2) used in the transfections does not contain the sequence for the DNA binding domain.[36] As such, it is incapable of significantly activating the IAP promoter on its own. However, when cotransfected with T3/TRβ1, the activation noted was greater than that normally seen with T3/TRβ1 alone. It appears that

although this mutant KLF4 is unable to bind to the DNA itself, it is still able to interact with the thyroid hormone receptor complex through its nuclear localization and transactivation domains. Indeed, the transactivation domain is capable of mediating interactions between KLF4 and other nuclear proteins such as p300/CBP.[36] Taken together, our studies do not define the precise molecular mechanisms underlying the synergy between T3/TR and KLF4. Indeed, in the case of the IAP promoter, we believe that these two transcription factors might exert their synergistic effect either by a direct physical interaction, through shared coactivators such as p300/CBP, or possibly a combination of the two. Interestingly, TR knockout animals exhibit decreased levels of Cdx2 mRNA, and the KLF4 gene is a known target of Cdx2.[45,54] This raises the possibility of a further layer of complexity to this interaction.

For these investigations we have selected the IAP gene as our target gene. This gene encodes a brush-border enzyme that likely plays a role in fat digestion, although the exact nature of its function is unclear.[55] Its expression is specific to intestinal cells, indeed, to the differentiated enterocytes, and hence an increase in IAP expression is recognized as one of the end points to the process of crypt-villus differentiation.[9,33] These characteristics of the IAP gene give our findings greater significance in the overall context of enterocyte differentiation. It is clear that in the complex environment of the cell, a variety of transcription factors and pathways interact to produce a specific phenotype. Indeed, this complexity of interaction is likely responsible for the multitude of cellular phenotypes present in the mammalian organism. Although our work establishes a potentially important interaction between the T3 and KLF4 pathways, it is likely that a host of other factors (e.g., Cdx1, Cdx2, HNF-1 alpha, GATA-4, GATA-5, GATA-6) all interact to ensure the accurate development of the mature enterocyte phenotype.

CONCLUSION

We have demonstrated an interaction on the IAP promoter between the ligand (T3)-bound nuclear transcription factors TRβ1 and TRα1 and the zinc finger containing KLF4. To our knowledge, this is the first report of an interaction between these two transcription factor pathways. Our findings assume greater significance as the IAP gene is an important marker of intestinal maturation and differentiation, processes in which both thyroid hormone and KLF4 have been implicated. Further work is required to understand the precise mechanism of interaction between these two pathways and to determine the combined effects of these factors on the overall differentiation program.

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Discussion

Dr. S. Ashley (Boston, MA): Many studies now show that with very stimulated adaptation, such as occurs after massive small bowel resection, there is a change in the dynamics of how quickly the cells move up the villus, how rapidly they are proliferating, and how quickly they differentiate. It looks like now, at least early in the adaptive process, the enterocytes are proliferating very quickly and ending up at the top of the villus without being as differentiated as we would like them to be to increase absorption.

Do you have any evidence that, either by administering more thyroid hormone or doing something with this Kruppel factor, you could stimulate differentiation of an enterocyte that was proliferating and moving up the villus more quickly?

Dr. R. Hodin: That is an excellent question, and obviously it just points out how complex the gut epithelium is. The epithelium turns over every couple of days, and this process can be speeded up. A lot has to happen in a very short period of time and within a short amount of space.

The simple answer to your question is, "No." We know that thyroid hormone will affect the phenotype of these cells in vivo, but in the setting of a resection model we do not know its effects. We know thyroid hormone alters the differentiation process in the adult and during gut development. GKLF also seems to be an important factor in the differentiation process. But in the setting of adaptation, we do not know the effects of either thyroid hormone or GKLF.

Dr. A. Black (Buffalo, NY): Have you looked for any physical interaction between the thyroid hormone receptor and KLF4?

Dr. Hodin: Not yet. That is a good question, because, as you are obviously aware, the last experiment suggests that there may be a protein-protein interaction taking place. We are in the process of trying to figure that out.

Dr. Black: Do the two families interact and have other members of those families been found to interact?

Dr. Hodin: Yes.

Invited Discussion—Expert Commentator

James W. Fleshman, M.D. (St. Louis, MO): This project is an extremely complex combination of genetic circular DNA transfection technology and gene expression analysis to evaluate a small part of gut epithelial development in differentiation control mechanism. As a clinician, I must congratulate the authors for bringing the technology to the study of gastrointestinal physiology and also trust that this model does exactly what they say it does and that their controls in this experiment are correct and valid. In my ignorance, I have a few questions for the authors:

1. Is the in vitro model of the Cos-7 cell line applicable to the clinical setting of the gut in its natural milieu?
2. Do your results suggest that hypothyroidism affects gut function/development/differentiation in such a way that it can be related to the subsequent development of intestinal diseases such as irritable bowel disease and gut motility disorders?

As a clinician, I tend to look for the translational effect of any new basic physiologic discovery. How does this fit into our understanding of disease and where might we use this for treatment of disease? Can we potentially manipulate the in utero endocrine milieu to ensure adequate gut maturation and prevent disease? Can transfection of cDNA provide a means to produce epithelial regeneration after insult? The authors have raised many questions with some excellent work.

Hepatocyte Growth Factor Ameliorates Inflammatory Bowel Disease in a Rat Model

L.Grier Arthur, M.D., Keith A. Kuenzler, M.D., Marshall Z. Schwartz, M.D.

This study was designed to investigate the benefits of administration of hepatocyte growth factor in a rat model of inflammatory bowel disease. Transfection of the HLA-B27 gene into Fisher rats induces a phenotype similar to inflammatory bowel disease. Fisher rats and HLA-B27 rats were divided into six groups: (1) Fisher, intravenous saline; (2) HLA-B27, intravenous saline; (3) HLA-B27, intravenous hepatocyte growth factor; (4) Fisher, luminal saline; (5) HLA-B27, luminal saline; and (6) HLA-B27, luminal hepatocyte growth factor. Rats received a 14-day infusion through an osmotic pump attached to a catheter positioned in either the jugular vein or the terminal ileum. Rats were evaluated for stool character, and gross and microscopic bowel inflammation. Statistics were analyzed using analysis of variance or the Kruskal-Wallis nonparametric test. A value of $P < 0.05$ was significant. Compared to untreated HLA-B27 rats, intravenous administration of hepatocyte growth factor decreased diarrhea by 41% and microscopic inflammation by 54% ($P < 0.05$). Luminal hepatocyte growth factor exposure decreased total bowel lesions by 53% and microscopic inflammation by 40% compared to untreated HLA-B27 rats ($P < 0.05$), but it did not have an effect on diarrhea. Administration of hepatocyte growth factor ameliorates many of the features of bowel disease in this rat model and theoretically could have therapeutic applications in the management of inflammatory bowel disease in humans. (J GASTROINTEST SURG 2003;7:1062–1068)

KEY WORDS: Inflammatory bowel disease, hepatocyte growth factor, HLA-B27 rats

Crohn's disease and ulcerative colitis are two similar but distinct disease processes of the intestine that are collectively referred to as inflammatory bowel disease (IBD). Although the etiology of IBD is still unknown, the pathogenesis is thought to involve immunologic factors, environmental influences, and genetic susceptibility.

Advances in the understanding of IBD have been inhibited, in part, by a lack of an animal model similar to the human disease process. Previous animal studies have attempted to mimic the pathogenesis of IBD by administering irritating or toxic substances either orally or rectally.[1,2] Although these models induce an inflammatory process, they lack the genetic predisposition and the altered immunologic response thought to be associated with IBD.[3] Recently Hammer et al.[4] demonstrated that transfection of the HLA-B27 gene into normal Fisher rats induces a spontaneous chronic inflammation of the gastrointestinal tract. They reported that these gastrointestinal manifestations were present in 100% of the rats at 20 weeks of age, and were similar to lesions seen in patients with IBD.

Hepatocyte growth factor (HGF) is a pleiotropic growth factor with activity in the central nervous system, the lung, the kidney, and the intestine, in addition to the liver. HGF has been shown to enhance epithelial cell proliferation in both the lung[5] and intestine.[6] We hypothesized that HGF might play a role in repair of the damaged intestinal mucosa in HLA-B27 rats. This study was designed to investigate the potential benefits of intravenous and intraluminal administration of HGF on a genetically altered rat model of IBD.

MATERIAL AND METHODS

Animal Model

All animal protocols were approved by the Institutional Animal Care and Use Committee of the Nemours Research Foundation, Wilmington, DE. Eleven

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adult female Fisher rats (F344; Harlan Sprague-Dawley, Indianapolis, IN) and 23 adult female Fisher-HLA-B27 rats (Taconic, Germantown, NY) were housed in cages in groups of three and allowed to acclimate to the environment for a period of several weeks. Rats were fed standard rat chow and given water ad libitum. Previous studies have shown that increasing the bacterial concentration has been shown to enhance gastrointestinal inflammation in this rat model of IBD.[7,8] Therefore cage beddings were changed once a week to maximize intestinal bacterial colonization. Animals were divided into two separate treatment arms: an intravenous treatment group and an intraluminal treatment group.

Intravenous Group. Six normal Fisher rats and 12 HLA-B27 rats underwent placement of a polyethylene (PE-60) catheter in the internal jugular vein. The catheter was connected to a subcutaneously placed osmotic pump (model 2002; Alza Corporation, Cupertino, CA), which was designed to deliver its contents at a rate of 0.5 μl/hr for a period of 14 days. Animals were then divided into the following three groups: group 1, normal Fisher rats receiving saline solution (n = 6); group 2, HLA-B27 rats receiving saline solution (n = 6); and group 3, HLA-B27 rats receiving HGF (Genentech, San Francisco, CA) at a rate of 150 μg/kg/day reconstituted in 20 mmol/L Tris-HCl (pH 7.5) and 500 mmol/L NaCl (n = 6). All rats were allowed free access to rat chow and water during the infusion period.

Intraluminal Group. Five normal Fisher rats and 11 HLA-B27 rats underwent placement of a terminal ileal PE-60 catheter, which was connected to a subcutaneously placed osmotic pump designed to deliver its contents at a rate of 0.5 μl/hr for a period of 14 days. Animals were then divided into the following three groups: group 4, normal Fisher rats receiving saline solution (n = 5); group 5, HLA-B27 rats receiving saline solution (n = 5); and group 6, HLA-B27 rats receiving HGF at a rate of 150 μg/kg/day (n = 6). All rats were allowed free access to rat chow and water during the infusion period.

Stool Evaluation

Stool from each rat was assessed on a weekly basis in a blinded fashion both before and after treatment. Stool was scored based on the following scale: 1 = normal stool; 2 = loose pellet-shaped stool; 3 = loose stool, no pellets; 4 = severe diarrhea with mucous, and 5 = bloody diarrhea.

Gross Analysis of Bowel Mucosa

After 14 days of treatment, all animals were killed and the gastrointestinal tract from the ligament of Treitz to the rectum was harvested. The gastrointestinal tract was opened longitudinally along its antimesenteric border, and the mucosa was rinsed of luminal contents. The bowel was then placed mucosal side down on a flatbed scanner, and an image of the mucosa was scanned into a computer. Gross analysis of focal areas of hyperemia was scored as bowel lesions by two blinded reviewers and expressed as mean ± standard error of the mean (SEM).

Microscopic Analysis of the Small and Large Intestine

Samples measuring 0.5 to 1.0 cm in length were taken from the jejunum (10 cm distal to the ligament of Treitz), the cecum, and the descending colon (10 cm distal to the cecum) and fixed in formalin. The tissue samples were then embedded in paraffin, and 5 μ thick sections of each sample were then processed for hematoxylin and eosin staining. Microscopic analysis of each section was performed by a blinded pathologist, and each section was scored as follows: 0 = no inflammation; 1 = mild inflammation limited to the mucosa; 2 = moderate inflammation extending into submucosa or focal crypt abscess; or 3 = severe inflammation with extension into muscularis and crypt abscesses. Results of all of the sections from the three locations were then tabulated with a maximum score of 9 and expressed as the median with a range of scores.

Statistical Analysis

Statistical analysis of stool character and histologic scoring were determined using a Kruskal-Wallis nonparametric analysis of variance test. Significant results were analyzed by two group comparisons using the Mann-Whitney test. Gross intestinal lesions were expressed as mean ± SEM, and statistical significance between groups was determined using analysis of variance. A P value ≤ 0.05 was considered significant.

RESULTS

Stool Evaluation

Intravenous Treatment Group. As expected, Fisher rats (group 1) had normal stool (score = 1) throughout the experiment. The untreated HLA-B27 rats (group 2) had a median pre- and post-saline exposure score of 4.0. The HGF-treated rats (group 3) had a median pretreatment stool score of 3, and a range of 3 to 4 and a post-treatment stool score of 2 with a range of 1 to 3 ($P < 0.05$). HGF-treated rats demonstrated a 41% improvement in their mean final stool

scores compared to the untreated rats (Fig. 1). When comparing the change in stool score (post-treatment stool score minus pretreatment stool score), using the Wilcoxon signed-rank test, the HGF-treated rats also demonstrated significant improvement compared to the untreated HLA-B27 rats ($P < 0.05$).

Intraluminal Treatment Group. The Fisher rats (group 4) had normal stool throughout the experiment. The untreated HLA-B27 rats (group 5) had a median presaline exposure score of 4 with a range of 3 to 4 and a median post-saline exposure score of 4 with a range of 3 to 4. No improvement in diarrhea was seen in the luminal HGF-treated rats (group 6), which had a median pretreatment stool score of 4.0 with a range of 3 to 5 and a median post-treatment stool score of 4 with a range of 3 to 4.

Gross Analysis of Bowel Mucosa

Intravenous Treatment Group. Normal Fisher rats demonstrated very few total bowel lesions (1.67) and small bowel lesions (1.33). The lesions identified likely represent trauma secondary to bowel manipulation. Untreated HLA-B27 rats had more than a fourfold increase in the number of lesions compared to normal rats (8.17). HGF-treated rats had fewer lesions (5.67) than the untreated HLA-B27 rats, but the difference was not statistically significant (Fig. 2). The mean number of small bowel lesions was also less in the HGF-treated rats (4.2) compared to the untreated rats (5.0), but again it was not statistically significant (Fig. 3).

Intraluminal Treatment Group. Normal Fisher rats had the lowest number of overall bowel lesions (1.0) and small bowel lesions (1.0). The total number

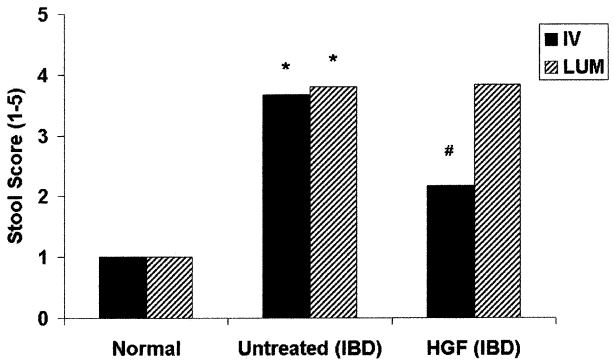

Fig. 1. Mean final stool score for intravenously (*IV*) and intraluminally (*LUM*) treated groups. $^{*}P < 0.05$: indicates statistical significance of the *untreated IBD group* compared to matched *normal Fisher rats*. $^{\#}P < 0.05$: indicates statistical significance of *the IV HGF–treated group* compared to the *IV untreated IBD group*.

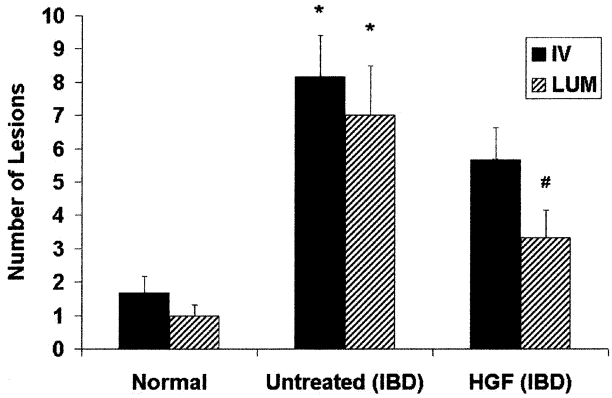

Fig. 2. Mean number of total lesions for intravenously (*IV*) and intraluminally (*LUM*) treated groups. $^{*}P < 0.05$: indicates statistical significance of the *untreated IBD group* compared to matched *normal Fisher rats*. $^{\#}P < 0.05$: indicates statistical significance of the *LUM HGF–treated group* compared to the *LUM untreated IBD group*.

of bowel lesions in HGF-treated rats (3.3) was significantly reduced compared to untreated HLA-B27 rats (7.0; $P < 0.05$; see Fig. 2). Similarly, when only the number of small bowel lesions was assessed, the HGF-treated rats had significantly fewer lesions (1.33) compared to the untreated HLA-B27 rats (5.2, $P < 0.05$; see Fig 3).

Microscopic Analysis of the Small and Large Intestine

Intravenous Treatment Group. There was no microscopic inflammation in the normal Fisher rats, whereas untreated HLA-B27 rats demonstrated mild inflammation with a median score of 4 with a range of 2 to 5. Treatment with HGF significantly decreased the inflammation score to a median of 1 with a range of 1 to 3 ($P < 0.05$; Fig. 4).

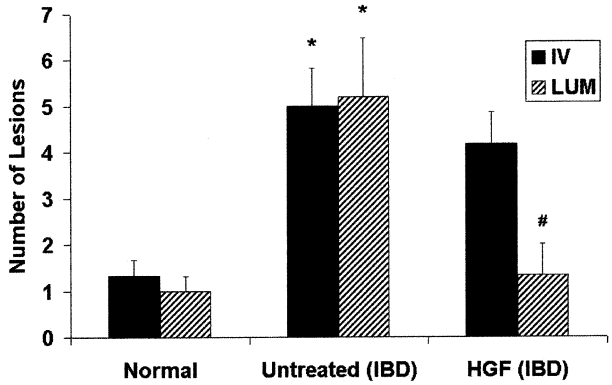

Fig. 3. Mean number of small bowel lesions for intravenously (*IV*) and intraluminally (*LUM*) treated groups. $^{*}P < 0.05$: indicates statistical significance of the *untreated IBD group* compared to matched *normal Fisher rats*. $^{\#}P < 0.05$: indicates statistical significance of the *LUM HGF–treated group* compared to the *LUM untreated IBD group*.

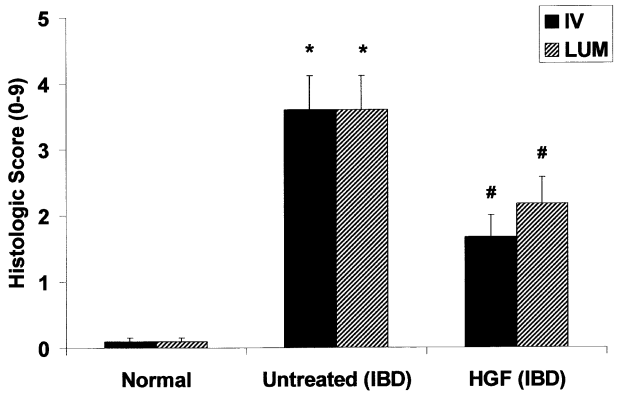

Fig. 4. Mean microscopic inflammation score for intravenously (*IV*) and intraluminally (*LUM*) treated groups. $^{*}P < 0.05$: indicates statistical significance of the *untreated IBD group* compared to matched *normal Fisher rats*. $^{\#}P < 0.05$: indicates statistical significance of the *HGF-treated group* compared to the matched *untreated IBD group*.

Intraluminal Treatment Group. The normal Fisher rats did not show signs of microscopic inflammation. Moderate inflammation was present in untreated HLA-B27 rats with a median score of 4 and a range of 2 to 5. Exposure to HGF decreased inflammation to a median of 2 with a range of 1 to 4 ($P < 0.05$; see Fig. 4).

DISCUSSION

The pathogenesis of IBD is complex and likely involves altered immunologic responses, environmental influences, and a genetic predisposition. Current pharmacologic therapies for IBD are aimed at suppressing the inflammatory response with the use of corticosteriods, immunomodulators such as azathioprine and mercaptopurine, and monoclonal antibodies such as tumor necrosis factor-α antibody. Despite a wide variety of pharmacologic options for patients with IBD, a consistent cure or a prolonged remission has yet to be achieved.

Further scientific investigation of IBD has been hampered by the lack of an animal model that simulates the human disease process. Many of the animal models induce a direct mucosal injury using toxins, which is substantially different from the spontaneously occurring disease process that occurs in humans. Hammer et al.[4] developed a unique model of IBD using a transgenic rat with the HLA-B27 gene transfected into its genome and induced a spontaneous, immunologically mediated gastrointestinal inflammation similar to what is seen in humans with IBD. In addition to its association with IBD in humans, the HLA-B27 gene is associated with the spondyloarthropathies and arthritis, which these rats also develop. Finally, the bowel disease in this rat model is influenced by environmental factors, such as increasing concentrations of bacteria in the bowel lumen.[7,8] Because this model appeared to have a similar multifactorial etiology, as well as comparable disease manifestations, it seemed appropriate to use it as a tool to investigate new treatment modalities for IBD.

Over the past two decades, several peptides and cytokines have been found to enhance intestinal growth. Our laboratory and other investigators have demonstrated that peptides, such as HGF, growth hormone, epidermal growth factor, interleukin-11, and glucagon-like peptide-2, increase enterocyte proliferation after massive small bowel resection in a model of short bowel syndrome.[6,9–13] Other studies have demonstrated that growth factors enhance nutrient absorption in the normal intestine and in short bowel syndrome.[6,9,12–14] Finally, our laboratory has demonstrated that HGF and interleukin-11 enhance enterocyte survival in an ischemia-reperfusion model, another form of intestinal injury.[15–17]

In addition to being one of the most potent intestinal mitogens, HGF has been found to stimulate epithelial proliferation in the lung,[5] the stomach,[18] and the liver.[19] In the majority of tissues, HGF is secreted from mesenchymal cells (i.e., fibroblasts), and its receptor, c-Met, is typically found on epithelial cells. HGF has recently been found to bind to heparin sulfate proteoglycans found in connective tissues following an injury, suggesting that it might play a role in stimulating epithelial proliferation following an insult.[20,21] Further evidence for the possible involvement of HGF in tissue regeneration is that HGF and c-Met have been found to be elevated in rats following injuries to the lungs,[22] the stomach,[18,20] the small intestine,[23] and the colon.[24] Furthermore, HGF levels have been found to be elevated in humans following both peritonitis[25] and IBD.[26] Therefore it is believed by some investigators, including ourselves, that HGF is part of the body's own defense mechanism to respond to injuries. Both Schmassmann et al.[18] and Kinoshita et al.[20] have separately demonstrated that HGF levels were elevated during the time period of gastric ulcer healing in rats. Similarly, Adamson and Bakowska[22] showed elevated levels of HGF within 3 to 14 days of a bleomycin-induced injury to the lung epithelium. Finally, both Goke et al.[27] and Nishimura et al.[28] have demonstrated increased proliferation of intestinal epithelial cells in response to administration of HGF in vitro, suggesting that HGF accelerates repair of damaged mucosa in the intestine. Although none of these studies definitively prove that HGF induces tissue regeneration following an injury, the

fact that it is upregulated soon after an injury suggests a causal relationship between HGF and tissue repair.

Because of its apparent success in inducing epithelial regeneration following injury in several other organ systems, it seemed worthwhile to explore whether HGF would positively influence the inflammatory process in this rat model of IBD. To our knowledge, our laboratory was the first to study the role of HGF in the treatment of IBD, although several investigators have demonstrated an improvement in bowel disease in animal models using other growth factors, such as platelet-derived growth factor,[29] epidermal growth factor,[30] insulin-like growth factor-1,[31] keratinocyte growth factor,[2] interleukin-11,[32] and glucagon-like peptide-2.[33] Thus the aim of this study was twofold: (1) to determine the effects of HGF on the disease manifestations of IBD, namely, diarrhea and bowel inflammation, and (2) to determine which route of administration was more effective.

Analysis of stool character revealed a significant decrease in diarrhea following intravenous administration of HGF. By contrast, intraluminal HGF had no effect on the treatment of diarrhea. At this point in time it is not known why intravenous administration is so much more effective than intraluminal administration of HGF, but the higher systemic levels of HGF could potentially explain this. This improvement in stool character elicited by HGF has not been reported before, but similar findings have been seen with the administration of interleukin-11[32] and glucagon-like peptide-2 (unpublished data from our laboratory).

HGF administration decreased the amount of inflammation appreciated on gross analysis in both treatment groups, but only intraluminal administration of HGF achieved statistical significance with regard to the total number of bowel lesions and small bowel lesions. Microscopic analysis revealed mild to moderate inflammation in all of the HLA-B27 rats and both HGF treatment groups had lower histologic scores than the untreated HLA-B27 rats.

The precise mechanism of improvement by HGF in this animal model is unknown. Earlier investigations in our laboratory, using semiquantitative reverse transcription–polymerase chain reaction, by Alavi et al.[34] demonstrated that intravenous HGF decreased the RNA expression of two inflammatory cytokines, tumor necrosis factor-α and interferon-γ. These findings suggest that HGF may have a direct or indirect effect on these inflammatory mediators. Thus this may be the basis for the reduction in bowel inflammation rather than its role as a growth factor. That is, HGF may block the effects on the mucosa of inflammatory mediators rather than accelerate the proliferation of intestinal mucosa and replace the damaged and inflamed intestine. Further research focusing on gene and protein regulation after HGF administration is necessary to identify its mechanism(s) of action.

HGF ameliorates many of the signs of IBD in HLA-B27 rats. Although intravenous HGF seems to be more effective in reducing diarrhea, intraluminal HGF seems to be more effective in ameliorating inflammation. Because of the small size of this initial pilot study, further investigation is necessary to identify potential mechanisms of action. However, the results of this study, along with this model's similarities to human IBD, makes us speculate that HGF might have therapeutic impact on the disease manifestions of IBD in humans.

We thank Dr. Ruth Birbe for her work in reviewing the histologic specimens in this study.

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Discussion

Dr. J. Fleshman (St. Louis, MO): Was there a difference in the histologic scores and inflammatory appearance between the small intestine and the large intestine, and was there a difference in the response to therapy between these two areas?

Does HGF have an effect on T-cell populations that we know of, because we now know that administration of GM-CSF actually can reduce inflammation by improving the normal T-cell population and thereby act as a non–anti-inflammatory method? Is HGF broken down in the lumen of the bowel, and can you show us evidence that the HGF is indeed reaching the mucosal layer that was inflamed when it was treated?

Dr. L. Arthur: In our model the majority of the gross lesions involved the small bowel, but we have not examined quite enough histologic samples to know if there is any statistical difference between the small bowel and the large bowel microscopically.

As far as the effect of HGF on T-cell populations, I am not aware of any evidence to that effect. We have not measured the levels of HGF in the bowel lumen. However, we have delivered HGF intraluminally in other models and have seen improvements in bowel function in a short bowel model and in normal bowel, so we believe that it is effective when given intraluminally.

Dr. S. Ashley (Boston, MA): My concern with the idea of using growth factors to treat inflammatory bowel disease relates to the issue of stimulating a potentially premalignant epithelium. So if you were going to use

this clinically, you would want some evidence that it was doing something anti-inflammatory as opposed to just stimulating growth. How then are the effects of HGF compared to some of the other gut growth factors? Would epidermal growth factor or GLP-2 be as effective in this model?

Dr. Arthur: HGF has been shown to increase intestinal proliferation in several studies, including previous studies from our laboratory. There is an obvious concern about inducing tumors, but to date there is no evidence of this phenomenon in our treatment protocol.

Second, we have looked at interleukin-11 and GLP-2. Both seemed to have similar effects in terms of treatment of inflammation. Interleukin-11 intravenously seems to be the best; HGF intraluminally seems to be the best.

Dr. M. Dayton (Salt Lake City, UT): Did you have a chance to observe any of the animals after administration of HGF to see how long lasting the effect of the HGF was—that is, if you gave it to the animals and did not kill them, did they get better clinically, and how long did that clinical improvement last? Epidermal growth factors have been very expensive. Do you foresee a time when they might be used clinically, or is the high cost going to prohibit the use of this as a truly mainstream clinical agent?

Dr. Arthur: Because we killed all of the animals after a 2-week course of HGF, we do not know if it would have resulted in a sustained effect.

Regarding the cost of HGF, you are absolutely correct; it is very expensive. However, HGH can be synthesized, and thus if there was a large demand for HGF, the cost would likely decrease.

Invited Discussion—Expert Commentator

James W. Fleshman, M.D. (St. Louis, MO): I am particularly pleased to review this article because the research was funded in part by the American Society of Colon and Rectal Surgeons Research Foundation. The authors have begun the search for potential therapeutic agents for IBD in a new model of IBD that uses HLA-B27–transfected mice in a well-designed, controlled small animal study. The parameters of mucosal involvement and histologic evidence of inflammation are able to be evaluated in an objective blinded fashion. Diarrhea is not as easily converted to an objective measure (especially when part of the protocol calls for intraluminal infusion of the therapeutic agent). The questions I have for the authors are as follows:

1. Was there a difference in the histologic scores in inflammatory appearance between the small intestine and the large intestine? Was there a difference in the response to therapy between these areas?
2. Does HGF have an effect on T-cell populations now that we know that the administration of granulocyte macrophage–colonony-stimulating factor (GM-CSF) can normalize the bone marrow production of T-cells and potentially reduce the effect of the abnormal responding T-cells in Crohn's disease?
3. Is HGF broken down in the lumen of the bowel, and can you provide us with evidence that HGF is indeed reaching the mucosal layer that is inflamed?

It is important that we continue to look for novel therapies in IBD. Infliximab has been modified to reduce the antimouse response, but it is still not 100% effective. GM-CSF may offer some new response. Immunosuppression can only ameliorate, not cure. A combination of approaches such as restoration of mucosal integrity, normalization of T-cell function, and suppression of abnormal cell populations may allow a complete resolution similar to multimodality therapy in the treatment of neoplasms and HIV.

Short Bowel Syndrome and Crohn's Disease

Jon S. Thompson, M.D., Kishore R. Iyer, M.D., John K. DiBaise, M.D., Renee L. Young, M.D., Cindy R. Brown, R.N., Alan N. Langnas, M.D.

Patients with Crohn's disease are at high risk for recurrent disease and often undergo multiple operations. Our aims were to evaluate surgical management and outcome of patients with Crohn's disease who develop short bowel syndrome (SBS) and to identify factors leading to this complication. We reviewed the records of 170 adult patients with SBS evaluated over a 20-year period. Thirty (18%) had Crohn's disease. SBS was defined as an intestinal remnant less than 180 cm with associated malabsorption. There were 20 women and 10 men ranging in age from 18 to 62 years. Eighteen (60%) presented initially with ileocolonic disease, seven (23%) with colonic disease, and five (17%) with small intestinal disease. The interval from initial diagnosis to development of SBS ranged from 2 to 32 years, with 21 patients (71%) having an interval greater than 15 years. The number of resections leading to SBS varied from 2 to 12 with 24 patients (80%) having four or fewer resections. Nineteen patients (63%) had an ostomy. Small intestinal remnant length was less than 60 cm in 10 patients, 60 to 120 cm in six patients, and greater than 120 cm in 14 patients. Only one patient underwent stricturoplasty before developing SBS. Five patients were initially diagnosed as having ulcerative colitis and underwent a pouch procedure, which was subsequently resected. Twenty patients (67%) required parenteral nutrition. Three patients have undergone reversed intestinal segment to slow intestinal transit. Two patients underwent intestinal transplantation. Two patients have died: one from parenteral nutrition–related liver failure and the other after intestinal transplantation. Crohn's disease remains a common cause of SBS. Aggressive resectional therapy, surgical complications, and errors in initial diagnosis contribute to development of SBS in these patients. Selected patients are candidates for surgical therapy for SBS. (J GASTROINTEST SURG 2003;7:1069–1072)

KEY WORDS: Short bowel syndrome, Crohn's disease

Patients with Crohn's disease frequently require surgical intervention and are at risk for recurrent disease and further resection.[1] The need for resection of recurrent Crohn's disease may be related to several aspects of the primary disease including age at diagnosis, site of initial disease, and nature of disease (e.g., perforative).[1–6] Further resection may also become necessary for complications of surgical treatment.[7] Current medical and surgical management aims at preventing recurrence, minimizing the extent of resection, and preventing further resection.[2] Surgical strategies to achieve this include minimal resection of gross disease, stricturoplasty, and segmental colon resection.[3,5,8] Despite these efforts, however, patients with Crohn's disease remain at risk for short bowel syndrome (SBS).[5,7,9] Our aims were to evaluate surgical management and outcome of patients with Crohn's disease who develop SBS and to identify factors leading to this complication.

METHODS

We reviewed the records of 170 adult patients with SBS evaluated at the University of Nebraska Medical Center between January 1982 and December 2002. SBS was defined as an intestinal remnant less than 180 cm with associated malabsorption. Crohn's disease was diagnosed by standard clinical, radiographic, endoscopic, and pathologic criteria. The causes of SBS in this population are presented in Table 1.

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Table 1. Causes of short bowel syndrome

Irradiation/cancer	45 (27%)
Mesenteric vascular disease	38 (22%)
Postoperative	33 (19%)
Crohn's disease	30 (18%)
Other benign causes	24 (14%)
Total	170

Thirty patients (18%) had Crohn's disease. There were 20 women and 10 men ranging in age from 18 to 62 years. Only six patients (20%) had their index operation performed at this institution. Follow-up ranged from 6 to 252 months. Sixteen patients (53%) have been followed for more than 5 years. Records were reviewed to determine the initial presentation, time to development of SBS, surgical treatment, and nutritional support. Statistical comparisons were made using chi-square analysis with $P < 0.05$ signifying statistical significance.

RESULTS

Initial Presentation

Age at initial diagnosis of Crohn's disease ranged from 13 to 54 years. Twenty-eight patients (93%) were less than 40 years of age at diagnosis. The interval from initial diagnosis to development of SBS ranged from 2 to 32 years, with 21 patients (70%) having an interval of more than 15 years.

Eighteen patients (60%) presented initially with ileocolonic disease, seven (23%) with colonic disease, and five (17%) with small intestinal disease. Outcome by presentation is given in Table 2. Patients presenting initially with ileocolonic or small intestinal disease only were more likely to have an intestinal remnant less than 120 cm compared to patients with colonic disease. Patients with colonic disease were more likely to have an ostomy.

Operative Management

Small intestine remnant length was less than 60 cm in 10 patients, 60 to 120 cm in six patients, and greater than 120 cm in 14 patients. Nineteen patients (63%) had an ostomy. Sixteen (53%) had a colonic remnant. The number of resections leading to SBS varied from 2 to 12 with 24 patients (80%) having four or fewer resections. Patients with ileocolonic disease were more likely to have four or more resections compared to colonic or small intestine disease (see Table 2).

Twenty patients (67%) had repeated resection for recurrent disease. Repeat operations for complications of surgical treatment were required in 10 patients (33%). Only one patient underwent stricturoplasty before developing SBS. Five patients were initially diagnosed as having ulcerative colitis and underwent either an ileoanal procedure (n = 4) or a continent ileostomy (n = 1), both of which were subsequently excised in all five.

Outcome

Overall, 20 patients (67%) initially required home parenteral nutrition (PN). PN was needed in nine patients (90%) with a small intestine remnant less than 60 cm, six (100%) with a remnant 60 to 120 cm, and five (36%) with a remnant greater than 120 cm. Five of these patients were eventually weaned to enteral

Table 2. Site of initial disease and outcome

	Ileocolonic	Colonic	Small intestine	Total
No. of patients	18	7	5	30
Small intestine remnant length (cm)				
<60	7 (39%)	1 (14%)	2 (40%)	9 (30%)
60–120	5 (28%)	0 (0%)	1 (20%)	6 (20%)
>120	6 (33%)	6 (86%)*	2 (40%)	15 (50%)
Colon remnant				
Right	2 (11%)	0 (0%)	0 (0%)	2 (7%)
Left	10 (56%)	1 (14%)	3 (60%)	14 (47%)
None	6 (33%)	6 (86%)	2 (40%)	14 (47%)
Ostomy	10 (56%)	7 (100%)*	2 (40%)	19 (63%)
Resections				
≤4	12 (67%)	7 (100%)	5 (100%)	24 (80%)
>4	6 (33%)*	0	0	6 (20%)
TPN at home	12 (67%)	4 (57%)	4 (80%)	20 (67%)

TPN = total parenteral nutrition.
*$P < 0.05$ vs. others.

nutrition, four of whom had remnants greater than 120 cm. Overall, two patients have died, one after intestinal transplantation and the other of PN-induced liver failure while on long-term PN.

Surgical Therapy

Sixteen patients have undergone 19 further intestinal operations after developing the SBS (Table 3). Three patients have undergone reversed intestinal segments to slow intestinal transit. One was revised 9 months later because of persistent vomiting. One patient had the reversed segment created at the time of ostomy closure. She was able to be weaned off of PN within 3 months and is doing well 22 months later. The third patient initially experienced decreased ileostomy output and reduced PN but within 2 years developed recurrent disease and continued need for PN. Two patients underwent intestinal transplantation. One had the transplanted intestine removed 1 month later for acute rejection and died. The other is alive and well 6 months after transplantation with no signs of recurrent Crohn's disease.

DISCUSSION

Crohn's disease remains a common cause of SBS, accounting for 18% of our adult patients with this condition. This finding is similar to other reports.[10] The risk of developing SBS in patients with Crohn's disease is approximately 5% to 10% in reported series.[3,5] This risk will vary depending on the underlying disease process and its medical and surgical management.[3,7] Nightingale and Lennard-Jones[11] suggested that patients with Crohn's disease have shorter intestinal length before resection, which would predispose them to this condition. However, this is not a consistent finding.[12]

Patients with ileocolonic disease at initial presentation accounted for 60% of patients with SBS in the present study. This group accounts for approximately 70% of patients undergoing resection for Crohn's disease.[1] Furthermore, patients with ileocolonic disease or small intestinal disease are at increased risk for resection compared to those with colorectal disease, and they have a higher risk of postoperative recurrence. All of our patients having more than four resections had ileocolonic disease. Dietz et al.[3] found that SBS occurred more frequently in patients with diffuse jejunoileitis compared to segmental small intestine disease (15% vs. 10%).

Patients developing SBS had undergone multiple resections over a long period of time. The number of resections and interval until the development of SBS in the present study were similar to other reports.[7] The majority of resections in our experience were due to recurrent disease. We found that one third of our patients required resection related to complications of surgical treatment, including obstruction and fistula formation. In contrast, Agwunobi et al.[7] reported that almost three fourths of patients developing SBS after resections for Crohn's disease had surgical complications leading to unplanned procedures rather than resection for recurrent disease. They also noted that these patients had more resections in a shorter period of time compared to elective resection for recurrent disease.

Several surgical strategies are important in minimizing resection in patients with Crohn's disease. Resection should be performed only when necessary to manage specific complications. Segmental colectomy should be performed when possible.[8] Only grossly normal, not microscopically negative, margins are necessary to minimize recurrence.[13] Stricturoplasty should be employed preferentially for stenosis.[3,5] This technique is applicable to fairly long segments and multiple stenoses. Stricturoplasty was used infrequently in the patients in the present study. The majority of patients in the present study were referred for treatment when they already had SBS. This complication might have been avoided or delayed in many patients employing these techniques. Recent advances in medical therapy may also reduce the need for surgical procedures.[14]

Another important factor in the development of SBS is error in initial diagnosis in patients who originally present with colitis. Five patients underwent ileal pouch construction for presumed ulcerative colitis and subsequently had their pouches excised. Further resections then led to SBS. The recognized failure rate of the pouch procedures for patients with Crohn's disease varies between 10% and 50%.[14–16] Furthermore, these patients frequently have postoperative complications, which can lead to further resection.[17] Approximately 20% of patients with initial Crohn's colitis will have small bowel extension of disease.[18] Although the development of SBS has been mentioned as a potential long-term consequence of this procedure, it is rarely reported.[15] However, our

Table 3. Intestinal procedures after development of short bowel syndrome

Intestinal resection	6
Repair fistula	3
Ostomy/ostomy takedown	3
Reversed segment	3
Stricturoplasty	2
Intestinal transplantation	2

experience would suggest that patients who require pouch excision for Crohn's disease are at risk for recurrent disease or complications that lead to further resection and SBS.

We found previously that SBS patients with Crohn's disease have a nutritional prognosis similar to that in patients with other causes of SBS.[9] Two thirds of the patients in the present study required home PN initially and 50% permanently. Similarly, Stokes and Irving[19] found that 75% of patients with Crohn's disease with SBS required PN initially and 50% permanently.

Quality of life and nutritional prognosis in patients with SBS are also influenced by the presence of an ostomy and a colonic remnant. Approximately half of patients with SBS had an ostomy. Heimann et al.[20] also found that the frequency of ostomy was 47% in patients who had undergone more than two resections for Crohn's disease. Approximately two thirds of our patients had a colonic remnant, most frequently the left colon.

Surgical therapy for SBS has been applied cautiously to patients with Crohn's disease because of concerns about recurrent disease and surgical complications. We performed reversed intestinal segments in three patients to slow transit and improve absorption. Although there were no perioperative complications, this procedure resulted in definite clinical improvement in only one patient. Two of our patients underwent isolated intestinal transplantation but the follow-up period is short. Several other patients with Crohn's disease have also had transplantation at other centers.[10,21] There had been at least one report of recurrent Crohn's disease in the transplanted segment.[22] This occurred within 6 months despite the immunosuppressive therapy and raises concern about the outcome of intestinal transplantation in this population.

In summary, Crohn's disease remains a common cause of SBS. SBS develops over a long period of time after multiple resections. Aggressive resectional therapy for recurrent disease, surgical complications, and errors in initial diagnoses contribute to the development of SBS in these patients. Selected patients are candidates for surgical therapy for SBS.

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Postoperative Colonic Motility in Patients Following Laparoscopic-Assisted and Open Sigmoid Colectomy

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Clinical reports on laparoscopic-assisted sigmoid colectomy (LASC) suggest that the period of postoperative inhibition of gastrointestinal motility is shortened as compared to open sigmoid colectomy (OSC). We aimed to specifically investigate whether colonic motility increases more rapidly following LASC compared to OSC. LASC was performed in 11 patients and OSC in nine patients for recurrent diverticulitis or carcinoma. During surgery a manometry catheter was inserted into the colon via the anus, and the tip was placed in the splenic flexure. Continuous manometric recordings were performed from the day of surgery until postoperative day 3 with a four-channel microtransducer manometry system combined with a portable data logger. The postoperative colonic motility index was 101 ± 18, 199 ± 30, and 163 ± 27 mm Hg/min on days 1, 2, and 3 after LASC, respectively, which was increased compared to indexes of 53 ± 15, 71 ± 18, and 76 ± 23 following OSC (mean ± standard error of the mean; $P < 0.05$). The amplitude but not the frequency of contractions was higher following LASC compared to OSC. Following LASC, patients requested a similar amount of pain medication but resumed oral food more rapidly on postoperative days 2 and 3 ($P < 0.05$), and they were discharged from the hospital earlier ($P < 0.05$). Colonic motility in particular and the patient's condition in general seem to improve more rapidly following LASC compared to the open procedure. (J GASTROINTEST SURG 2003;7:1073–1081)

KEY WORDS: Postoperative ileus, laparoscopic colectomy, colonic motility, postoperative oral nutrition

The aging population in Western countries means that surgeons are facing a rising number of patients suffering from diseases that typically occur in the elderly, such as recurrent diverticulitis or cancer of the sigmoid colon. Consequently sigmoid colectomy is a frequent procedure in many surgical departments today. Several studies have shown that sigmoid colectomy is technically feasible as a laparoscopic-assisted procedure and that it can be performed with low complication rates.[1] Besides the obvious cosmetic benefit, which is a consequence of small abdominal incisions, further advantages concerning the postoperative course, length of hospital stay, and hospital costs appear to exist.[1–5] Furthermore, results of some animal studies have suggested that laparoscopic colectomy may lessen postoperative immunosuppression compared with the open operation, which potentially has a positive influence on wound healing and recurrence after colectomy for colon cancer.[6–9]

Rapid recovery of postoperative gastrointestinal motility after surgery is advantageous because this allows earlier oral food intake, which usually heralds a marked improvement in the patient's general condition and early discharge from the hospital. Although postoperative motility of the stomach and the small intestine normally recurs within 1 to 2 days, recovery of colonic motility may take 3 to 4 days.[10] Thus full recovery of postoperative gastrointestinal motility seems to be limited by the normalization of colonic motility.

It has been shown in animal experiments that after laparoscopic operations gastrointestinal motility increases more rapidly when compared to open procedures.[11] This was supported by the observation that laparoscopic-assisted sigmoid colectomy (LASC) in patients is usually followed by earlier passage of gas and bowel movements compared to open sigmoid colectomy (OSC).[4,5] However, differences in

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postoperative colonic motility between patients undergoing LASC and OSC have never been explored in detail. The aim of this study, therefore, was to determine by state-of-the-art motility recordings whether postoperative colonic motility is increased after LASC compared to OSC in these patients.

PATIENTS AND METHODS

Twenty patients (median age 63 years, range 38 to 91 years) undergoing elective sigmoid colectomy for recurrent diverticulitis (n = 12) or cancer of the sigmoid colon (n = 8) were investigated. All patients operated on for recurrent diverticulitis had an elective operation and did not show any signs of ongoing inflammation at the time of the operation. LASC (n = 11) was performed when the patients were referred to a staff surgeon trained in minimally invasive surgery, whereas OSC (n = 9) was performed by all other staff surgeons in the department. All patients gave written informed consent before surgery. The study protocol was approved by the local ethics committee at the University of Tübingen. One day before the operation, patients ingested 4 liters of a colonic lavage solution composed of polyethylene glycol and electrolytes (Klean-Prep; Norgine Ltd, Marburg, Germany). General anesthesia was administered according to standards established by the University of Tübingen as published previously.[12] The opioid piritramide (Dipidolor; Janssen-Cilag GmbH, Neuss, Germany), which is a selective μ-receptor agonist, was administered intravenously on demand for postoperative pain relief.[13] All patients were offered clear liquids on the day of surgery. If this was well tolerated, soup was offered on postoperative day 1, and if soup was eaten and well tolerated, oral food intake was advanced to a semisolid diet. During the time of motility recordings, no laxatives were administered.

For LASC four-trocar ports were inserted after a pneumoperitoneum had been established with the aid of a Veress needle. One trocar was placed 3 cm above the umbilicus, two in the right and one in the left lower abdomen. The patient was placed in a steep Trendelenburg position with a right lateral tilt. The sigmoid colon, the left colon and, if necessary, the splenic flexure were mobilized. Clips and ligatures were used to divide the mesenteric vessels. The colon was cut with a linear cutting and stapling device at the rectosigmoid junction (Linear-Cutter ETS flex 45 mm, Ethicon-Endosurgery, Inc., Norderstedt, Germany) and then the distal end was exteriorized through a small oblique incision (4 to 6 cm) in the left lower quadrant of the abdomen. Following sigmoid colectomy in front of the abdominal wall, the anvil of a circular stapler (Proximate intraluminal stapler, 29 mm or 33 mm; Ethicon-Endosurgery, Inc.) was inserted in the proximal colon and tied before it was replaced in the abdominal cavity. Subsequently the laparotomy was closed, the pneumoperitoneum was reestablished, and colorectal end-to-end anastomosis was performed with the above-mentioned circular stapler that was introduced via the anus. The integrity of the anastomosis was checked by filling the rectum with a povidone iodine solution.

OSC was performed according to standard surgical technique. After a midline laparotomy, the sigmoid colon, left colon, and if necessary the splenic flexure were mobilized, the mesocolon was divided, and a sigmoid colectomy was performed from the descending colon down to the rectosigmoid junction. The anvil of the circular stapler was inserted and tied into the proximal colon, the anastomosis was completed, and its integrity was tested as for LASC.

In both patient groups (LASC and OSC) a four-channel microtransducer manometry catheter (Standard Instruments, Karlsruhe, Germany) was inserted via the anus into the lumen of the left colon after the anastomosis had been completed (Fig. 1). Thus the tip of the catheter was located in the splenic flexure in the left upper quadrant. Accidental dislocation of the catheter was prohibited by a suture to the perineum. The recording points were 10 cm apart from each other, located 2.5, 12.5, 22.5, and 32.5 cm from the tip of the catheter.

A portable data logger (Superlogger SI; Standard Instruments, Karlsruhe, Germany) was connected to the catheter at 6 PM on the day of the operation, and gastrointestinal motility was recorded until the evening of the third postoperative day. Recordings were interrupted every 24 hours from 6 to 7 PM in order to download the recorded data to the hard disc of a stationary personal computer. Analysis of manometric recordings was performed with dedicated software (Intestinal Data Acquisition and Analysis, version 3.40.15; Standard Instruments). Motility recordings were divided into three sequential recording periods with a duration of 24 hours. Each recording period was evaluated blind in terms of the performed operation and the postoperative day. Motility index (area under the curve per minute), contraction frequency, and contraction amplitude were

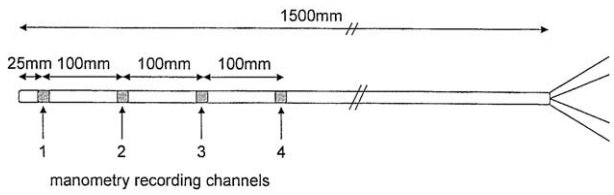

Fig. 1. Four-channel microtransducer manometry catheter.

calculated each time for the whole 24-hour period after computerized elimination of artifacts. Furthermore, high-amplitude propagated contractions were identified visually. According to Bassotti et al.,[14] these contractions were defined by their sequential presence in at least three manometry channels within 30 seconds and a minimum pressure amplitude of 50 mm Hg in one or more manometry channels.

Data related to patients were recorded regarding operative risk using the American Society of Anesthesiologists (ASA) classification, body mass index, operating time, intra- and postoperative complications, tumor stage using the International Union Against Cancer (IUCC) classification if applicable, postoperative administration of analgesics, tolerated oral food, nausea, first postoperative bowel movement, and length of hospital stay. Patients were examined for abdominal pain and bowel sounds three times per day, and findings were recorded as a score that is given in Table 1 together with the data.

Table 1. Postoperative course

	LASC (n = 11)	OSC (n = 9)
Incidence of nausea		
Postoperative day 1	2 (18%)	2 (22%)
Postoperative day 2	1 (9%)	3 (33%)
Postoperative day 3	0 (0%)	3 (33%)
Postoperative abdominal pain (1 = slight; 2 = moderate; 3 = severe)		
Postoperative day 1	1 (range 1–1.75)	1 (range 1–1.75)
Postoperative day 2	1 (range 1–1)	1 (range 1–1)
Postoperative day 3	1 (range 1–1)	1 (range 1–2)
Bowel sounds (0 = absent, 1 = few, 2 = normal, 3 = agile)		
Postoperative day 1	1 (range 1–1)	1 (range 0.25–1)
Postoperative day 2	2.5 (range 2–3)	2 (range 1–2)
Postoperative day 3	3 (range 2.25–3)	2.5 (range 2–3)
First postoperative bowel movement (days)	3 (range 2.25–4)	3 (range 2.75–4)
Postoperative hospital stay (days)	7 (range 6–8.5)*	10 (range 9–13.5)

Data are median with interquantile ranges in parentheses.
*$P < 0.05$.

Statistical analysis was performed using two-way analysis of variance, chi-square analysis, or Student's *t* test where applicable. Data on postoperative abdominal pain, bowel sounds, and postoperative food intake were analyzed for each postoperative day by the Mann-Whitney rank-sum test and a subsequent Bonferroni correction for multiple testing. Motility data are given as mean ± standard error of the mean and clinical data as median and interquartile ranges. A *P* value of less than 0.05 was considered as statistically significant.

RESULTS

Patients undergoing LASC or OSC did not differ with regard to age, sex, body mass index, or preoperative ASA status. Furthermore, there was no difference in operating time between the two groups (Table 2). In the LASC group three patients who underwent surgery for carcinoma of the sigmoid colon were classified as UICC stage I, whereas in the OSC group

Table 2. Patient characteristics and operating time

	LASC (n = 11)	OSC (n = 9)
Age (yr)	63 (range 56–68)	64 (range 57–75)
Sex (male: female)	6:5	3:6
BMI (kg/m^2)	26.2 (range 24.1–28.3)	23.1 (range 21.5–31.9)
ASA	2 (range 2–2)	2 (range 2–3)
Preoperative comorbidities		
Previous myocardial infarction	1	1
Coronary heart disease	1	2
Arterial hypertension	5	4
Asthma	1	0
Diabetes	0	2
History of prior abdominal operation	5	4
Indication for sigmoid colectomy		
- Recurrent diverticulitis	8	4
- Carcinoma	3	5
Operating time (min)	150 (range 136–169)	130 (range 102–161)

Data are median with interquantile ranges in parentheses.

two patients had stage I and three patients had stage III carcinoma.

Intraoperative complications did not occur in either group, and no conversions to an open procedure were necessary in the LASC group. Postoperative complications included one urinary tract infection in each group that was successfully treated with antibiotics. No further complications occurred in the LASC group. In the OSC group one patient suffered from repeated episodes of urinary retention requiring a temporary suprapubic catheter. One patient developed a prolonged postoperative ileus after OSC requiring reinsertion of a nasogastric tube on postoperative day 3 that was kept in place for 5 days because of high output. Another patient in the OSC group developed a wound infection. No anastomotic leakage occurred in either group.

The request for pain medication did not differ between the two groups. Patients undergoing LASC tolerated a more rapid advancement of the diet on days 2 and 3 after surgery compared to OSC (both $P < 0.05$; Fig. 2). There were no differences with regard to nausea, bowel sounds, and postoperative abdominal pain. Most patients had their first bowel movement on postoperative day 3 or 4, which was no different after LASC or OSC. However, the postoperative hospital stay was shorter in the LASC group (see Table 1).

Representative motility recordings in one patient each after LASC and OSC from postoperative day 1 to day 3 are shown in Fig. 3 including exemplary snapshots of 60-minute periods in channel 1 for each day. Two-way analysis of variance revealed that the surgical procedure that was evaluated as one variable had a significant influence on the postoperative colonic motility index and mean amplitude of contractions. Colonic motility index ($P = 0.0008$) and mean amplitude of contractions ($P = 0.0002$) were higher in the LASC group, whereas the mean frequency of contractions did not differ between the two groups (not significant [NS]; Fig. 4, *A* to *C*). The postoperative day, which was evaluated as the second variable by two-way analysis of variance, did not have any influence on the colonic motility index, mean amplitude, or mean frequency of contractions within the first 3 postoperative days (NS). Three high-amplitude propagated contractions occurred in one patient after LASC on postoperative day 3. No high-amplitude propagated contractions were observed in patients undergoing OSC.

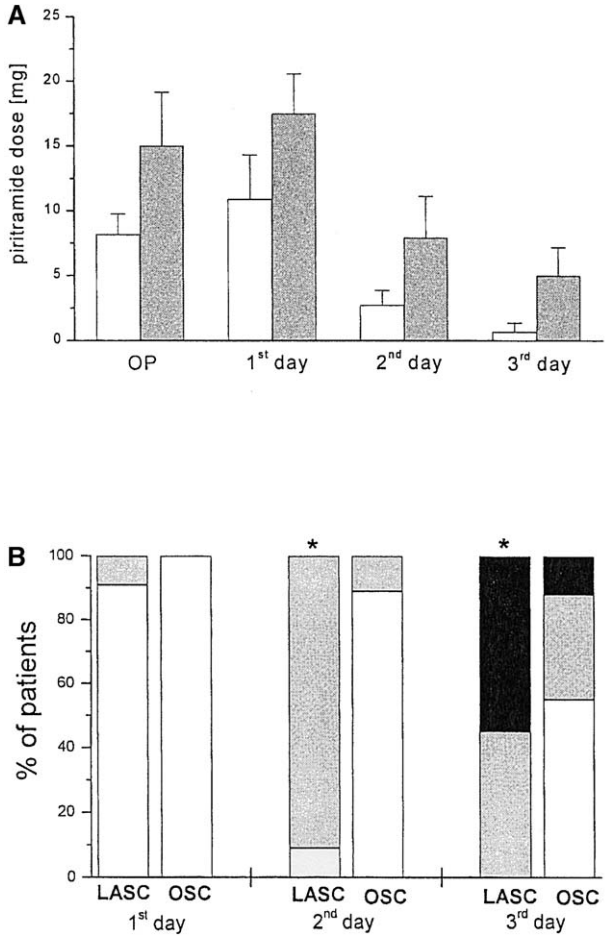

Fig. 2. A, Dose of pain medication (intravenous piritramide) during the postoperative period (mean ± SEM). The need for piritramide following LASC (*white columns*) was not different compared to OSC (*gray columns*). **B**, Comparison of the tolerated oral food intake following surgery. The diet could be advanced more rapidly in patients undergoing LASC compared to OSC on days 2 and 3 after surgery (*$P < 0.05$; data analysis for each postoperative day by Mann-Whitney rank-sum test and Bonferroni correction; liquids = white columns; soup = gray columns; semisolid diet = black columns).

DISCUSSION

Colonic motility index and amplitude of contractions but not the frequency of contractions were increased after LASC compared to OSC. The maximum difference occurred on the second day after surgery. There was no difference in terms of nausea, bowel sounds, postoperative abdominal pain, and the request for pain medication between the LASC and OSC groups. In most patients the first bowel movement occurred between postoperative days 3 and 4 regardless of the technique of sigmoid colectomy, except in one patient who developed prolonged postoperative ileus after OSC. The postoperative hospital stay was shorter and patients tolerated oral food intake more rapidly after LASC. Operation times were similar for both procedures with no intraoperative complications and a low rate of minor postoperative complications that occurred more often in the OSC group.

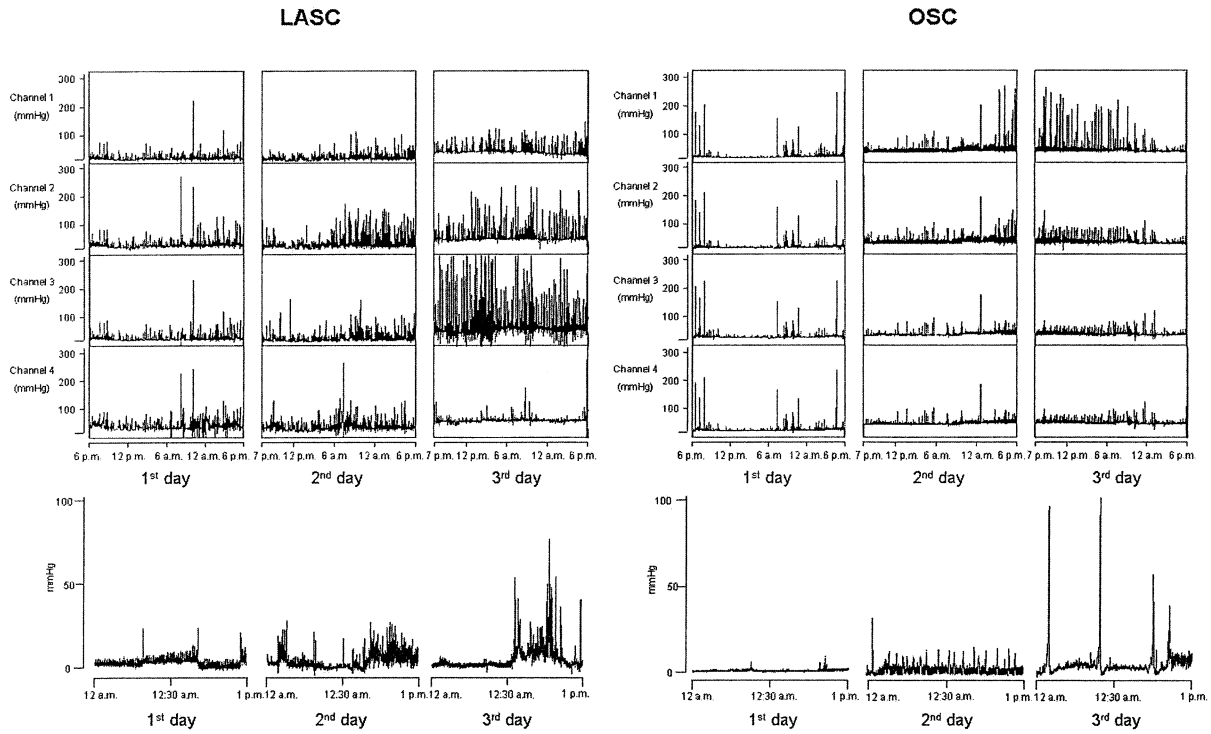

Fig. 3. Complete raw data tracing of one representative patient each after laparoscopic-assisted sigmoid colectomy (*LASC*) or open sigmoid colectomy (*OSC*). Snapshots from 12 A.M. to 1 P.M. of channel 1 of the same patient are shown in the *lower panels*.

Several parameters obtained during manometric recordings of colonic motility, including the observation that one patient after LASC developed high-amplitude propagated contractions, suggest that colonic motility increased more rapidly following LASC compared to OSC in the present study. It is of note that intraluminal pressure recordings show only limited correlation with the shifts in luminal contents (i.e., colonic transit, which is clinically relevant).[15,16] In patients, transit studies with intraluminal contrast or radioactive materials, however, are problematic postoperatively because an empty colon is generally considered to be advantageous for the outcome of sigmoid colectomy. Thus, despite its methodologic limitations, manometry was chosen for postoperative colonic motility studies. Various factors may have contributed to the differences between recordings following LASC and OSC that need to be considered.

The ideal design for a study comparing two treatments certainly is to conduct a randomized, double-blind controlled study. When comparing two surgical procedures, this design is difficult to comply with because it is difficult to motivate patients to undergo randomization. Furthermore, successful blinding for the procedure is almost impossible in most surgical departments inasmuch as information about the operation performed is likely to spread. We managed to conduct a controlled trial taking advantage of the fact that only a small number of the surgeons in our department perform LASC, whereas the others prefer OSC. Indeed, this resulted in two quite comparable groups of patients who did not differ with regard to age, sex, ASA status, or body mass index. Nevertheless, an obvious referral bias was present concerning the indication for surgery, as more patients who were operated on for sigmoid cancer underwent OSC. Physicians may have preferentially referred to surgeons who perform OSC, because LASC continues to be a topic for debate when performed for sigmoid cancer, although recent studies suggest that results with regard to survival and control of cancer are comparable.[17,18]

The intra-abdominal operative trauma during sigmoid colectomy for sigmoid cancer may be slightly more severe as compared to surgery for recurrent diverticulitis because in cancer patients the mesentery is taken together with the lymph nodes, which is not necessary in sigmoid colectomy for diverticulitis. Although some studies claim that the postoperative inhibition of gastrointestinal motility is virtually independent of the extent of surgery in the abdomen

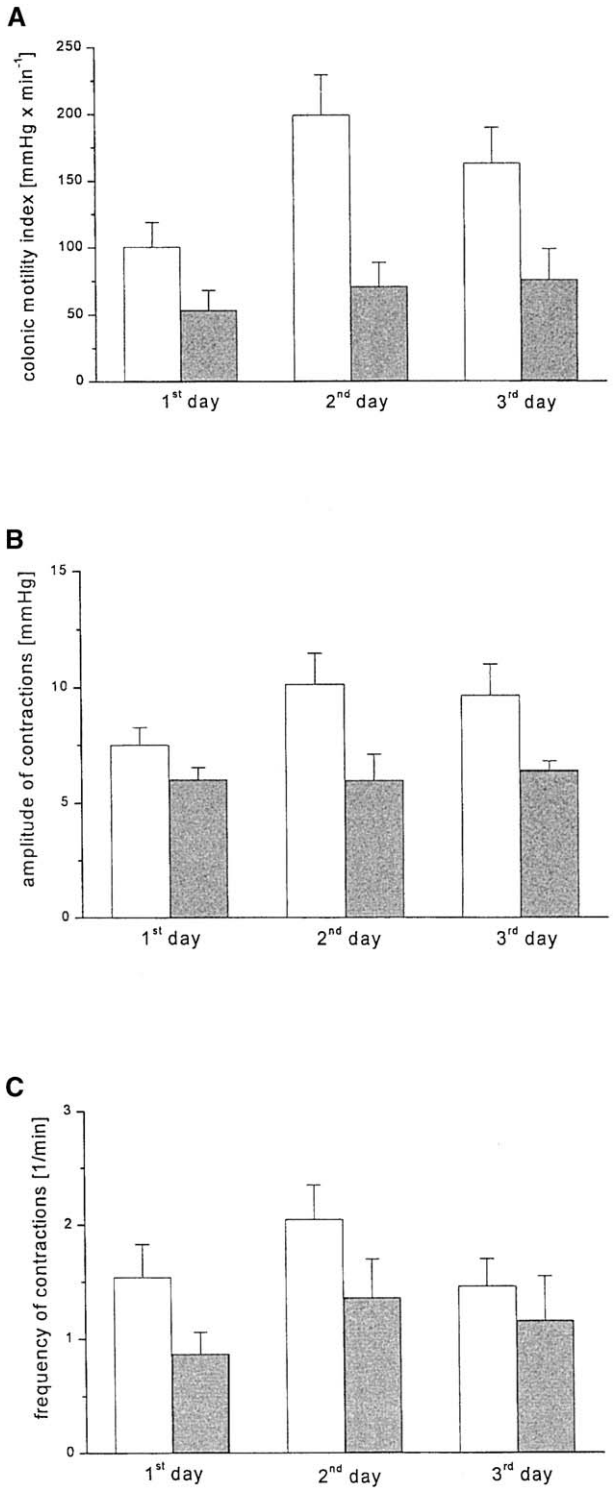

Fig. 4. Colonic motility index (**A**), amplitude (**B**), and frequency of contractions (**C**) after LASC (*white columns*) or OSC (*gray columns*). It was determined by two-way analysis of variance that colonic motility and amplitude of contractions but not frequency of contractions were increased after LASC compared to OSC.

once the peritoneum is incised,[19,20] other investigators found that the extent of surgical dissection has an influence.[21] We believe that it is very questionable whether the minor difference in operative trauma between sigmoid colectomy for diverticulitis or cancer would have any influence on postoperative motility in the present study. Thus it appears unlikely that the greater number of patients operated on for sigmoid cancer in the OSC group may have biased postoperative colonic motility recordings. In contrast, the length of the incision, which is naturally greater in OSC, has been shown to correlate with the duration of impaired postoperative gastrointestinal motility.[22] The greater extent of incisions in the abdominal wall, therefore, may have contributed to a more severe inhibition of colonic motility after OSC compared to LASC.

During LASC, manipulation of the intestines in the abdominal cavity is likely to occur to a lesser degree compared to OSC. This may influence the inhibition of postoperative gastrointestinal motility by several mechanisms. First, handling of the bowel activates afferent nerve fibers innervating the gut, which results in a reflex activation of sympathetic nerves that subsequently mediates an inhibition of gastrointestinal motility.[23] Second, it has been described in recent animal studies that the inhibition of postoperative gastrointestinal motility depends largely on a local inflammatory response in the gut wall, which appears to involve predominantly the activation of cyclooxygenase-2 and the subsequent release of prostanoids.[24] The magnitude of this inflammatory response seems to correlate with the extent of intestinal manipulation.[25] Thus a reduced activation of these two mechanisms secondary to little manipulation during LASC may explain why colonic motility was impaired to a lesser degree after LASC compared to OSC.

In both patient groups, the food offered was changed each day depending on whether the diet given on the previous day was well tolerated. This was more often successful in patients after laparoscopic surgery compared to OSC. Possibly patients after LASC were able to resume oral food intake more rapidly because their gastrointestinal and—in particular—their colonic motility recovered more rapidly. However, oral food intake may also have stimulated colonic motility[26,27] so that the increased postoperative colonic motility in the LASC group may as well have been a consequence rather than a cause of a more rapid increase in oral nutrition compared to the OSC group. Furthermore, differences in oral food intake may be substantially influenced by the surgeon, nurses, and even patients themselves, as they may expect a priori that oral food intake is possible more

rapid after a laparoscopic procedure than after open surgery.

Opioids that are used for postoperative pain relief are well known to inhibit gastrointestinal motility.[22,28] In the present study there was no difference in the need for pain medication between the LASC and the OSC groups, possibly because the number of patients investigated was too small. Small patient numbers are also most likely responsible for a missing statistically significant difference as regards the usually observed increase in colonic motility that is present with increasing numbers of postoperative days. Indeed, in an investigation of a larger group of patients, we have shown previously that colonic motility increases from day 1 to day 3 after colorectal surgery.[29]

Although the present study describes differences in postoperative colonic motility between LASC and OSC, it is worth noting that clinical parameters such as nausea, abdominal pain, bowel sounds, and time to the first postoperative bowel movement were not statistically different with the exception of the postoperative food intake, which was tolerated earlier after LASC. Nevertheless, patients undergoing LASC were generally discharged from the hospital earlier compared to patients in the OSC group. Patients in our country usually leave the hospital after surgery, when they feel well enough to manage on their own at home. Although earlier advancement to a full oral diet may have been helpful to reach this condition, the expectations of the staff surgeon, the nurses, and the patients themselves may also have biased the decision to leave toward a shorter stay in the laparoscopic group.

As has been reported by others, a shortened duration of hospital stay after laparoscopic procedures and a more rapid overall recovery help to reduce the costs of surgery even if expensive laparoscopic instruments and the use of large quantities of disposable products are considered.[1–5,30] It is questionable, however, whether a shortened hospital stay and the involved reduction in treatment costs can only be achieved by moving from open to laparoscopic surgery. Kehlet et al.[31] and others[32–34] have shown that with a multimodal rehabilitation program it is feasible to accelerate patients' recovery after open colectomy such that most patients could be discharged on postoperative day 2. Their treatment protocol includes the general use of thoracic epidural analgesia, early mobilization, and early oral food intake.

We conclude from our study that colonic motility is increased after LASC as compared to OSC. Possibly this is secondary to smaller abdominal incisions, less manipulation during surgery, and/or a more rapid oral food intake after the laparoscopic operation.

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Discussion

Dr. D. Rattner (Boston, MA): I congratulate you on a very nice presentation. I am sure there are some cultural differences that may account for this, but if the open group and the laparoscopic group were having their first bowel movement on the same day, how do you explain the difference in length of stay in the hospital? Was there a defined clinical pathway that was used for both the open and laparoscopic groups or did the surgeons themselves decide when to introduce different types of food?

Dr. M. Kreis: I will answer your second question first. Patients on the day of surgery are offered clear liquids, and when they are able to tolerate them, then they advance to the next step, which is soup, and then they advance to semisolids. It is a day-by-day process. Patients who do not tolerate one of these steps are kept at the same level as the day before. This is the algorithm that is administered in these patients.

With regard to the difference in terms of discharge from the hospital, you can see from the data that patients' oral food intake was advanced to a full diet obviously later after the conventional operation. So I think this may be a contributing factor, and although this was only a trend, these patients requested more postoperative pain medication. So I think these factors may have contributed to the fact that after the conventional operation, patients stayed in the hospital longer.

Dr. M. Sarr (Rochester, MN): This is a nicely carried out study, but it suffers, as you are well aware, from using only contractions (amplitude and number of contractions) as a surrogate marker of motility or transit. Shouldn't you be more interested in transit than in the actual number of contractions? Do you consider some type of transit study, because that is really the key, isn't it?

Dr. Kreis: I think it is a fairly elegant way of approaching gastrointestinal motility, and particularly colonic motility, to obtain recordings with a manometric device. I think that it is a different approach, to try transit studies. I do not really have a particular model in mind to measure transit that could be easily administered in postoperative patients, but I think this is certainly an aspect that needs to be considered; we also need to be careful in concluding from differences in motility data that we really had differences in transit. I totally agree with that point.

Dr. J. Fleshman (St. Louis, MO): I have several questions. Do you think there may have been some influence on the rapidity of recovery of bowel function based on the differences in the number of patients with diverticulitis in each group?

Do you think opioid blockade would have had any effect on the recovery rate and is there something that you are planning for studying that response in the future?

Do you think you can explain sympathetic discharge resulting from bowel handling and incisional pain as the source of the ileus, and is there something that we can possibly do to improve that in open colectomy?

Finally, do you think there may have been any difference in your outcome had you performed a right colectomy on these patients rather than a left colectomy?

Dr. Kreis: With regard to the indication for surgery or the reason why surgery was performed, all of these patients who underwent surgery for recurrent diverticulitis did not have active disease during the time of surgery. When we do the procedure for a carcinoma, we dissect the inferior mesenteric artery and the mesentery a little bit higher than we usually do for a patient that we operate on for diverticulitis. I personally think this is such a minor difference in the operative trauma that I do not believe it should influence the data as they were presented here. However, there is certainly a potential that this may have affected it, but, again, I do not believe so.

As to the question of what would happen if we were to do a right hemicolectomy, basically I do not think there would be a major difference. We do know that after a right-sided anastomosis, motility recovers slightly more quickly as compared to a left-sided anastomosis. This has been shown in studies, but basically I do not think that the data between a laparoscopic-assisted right hemicolectomy and a conventional right hemicolectomy would be completely different.

With regard to the opioids, it is very difficult to study them in postoperative patients because we cannot really withdraw the pain medication from these patients. So I cannot really comment on this. This is a potential factor, and there have been many studies done on opioids and their effect on colonic motility; I dare say that it is not even clear whether this really reduces transit. There have been some studies showing with certain doses of opioids that contractility is even increased. So I think the issue is unsettled, and it is not clear whether this is a contributing factor. We have to consider it, but I do not have a really good idea of how we could reasonably study this. We cannot really take away the pain medication from our patients.

Now, in regard to the manipulation and handling of the skin incision that you were mentioning, there have been studies, and it was also shown during this meeting in a poster presentation that with an increasing length of skin incision there is a greater inhibition of postoperative motility, which is probably due to a reflex activation most likely by sympathetic nerves that get activated when the peritoneum is incised.

In terms of the bowel handling, there have been studies by Dr. Anthony J. Bauer, beginning in 1998, the results of which have been published in *Annals of Surgery*, and later in *Gastroenterology*, as well, showing that following a severe manipulation of these intestines, infiltrates with leukocytes occur. If you block that leukocyte infiltration, the motility will recover much more quickly postoperatively. So I think as we reduce manipulation of the intestines, we should reduce this effect with leukocyte infiltration, and therefore we should also have a positive influence on postoperative motility.

Dr. H. Freund (Jerusalem, Israel): A major consideration in a study such as this is the length of stay. Who made the decision about discharging of these patients?

Dr. Kreis: There are many factors involved when you look at this. Basically the patient is making the decision whether or not he or she feels fit to leave the hospital, and the surgeon who is in charge will certainly recommend that the patient stay if they are still having problems. Alternatively, the surgeon will allow the patient to leave only a few days after surgery if there are no problems. So basically I think in Germany it is still based on the subjective feelings and subjective comfort of the patients as to whether they feel well enough to go home and to manage on their own.

Invited Discussion—Expert Commentator

James W. Fleshman, M.D. (St. Louis, MO): The measurement of postoperative ileus has been very difficult to accomplish. The group in Tübingen has been able to show a colonic component of postoperative ileus in a way that is objective. I am not convinced that it is a complete picture, but it does give us a measurement of function in the rate-limiting area of the gastrointestinal tract. Unfortunately, it does not take into account individual variations in the colon preoperatively and thus may be influenced by slow or rapid transit, which occurs in the patient before intervention. Even so, this is an excellent prospective, controlled, comparative group physiologic study, which may not be able to be performed in the United States. I have the following questions for the authors:

1. Could the increased number of patients with diverticulitis in the laparoscopic group influence the rapidity of recovery of the colonic motility?
2. How would opioid blockade have affected the outcomes and do you plan to study this in the future?
3. Can sympathetic discharge due to bowel handling and incisional pain be countered by any current method to reduce ileus after open colectomy?
4. Would there have been a greater difference after right colectomy in your model?

The small difference between the two groups is somewhat surprising. Indeed, it is almost surprising to find that laparoscopy is an independent factor in colonic motility recovery after a colon resection in this study, given the minimal differences between the two groups. The fact that frequency of contraction is unaffected by surgical manipulation of the colon suggests a local phenomenon at the neuromuscular junction rather than a proximal nerve effect. Thus the lessons we learn from laparoscopy—that is, minimal tissue handling and avoidance of dehydration—could potentially be useful in the open setting to reduce postoperative ileus. Unfortunately, we still cannot explain the more rapid recovery of the patients in the laparoscopic group by anything other than a smaller incision.

Perioperative Complications in Patients Undergoing Major Liver Resection With or Without Neoadjuvant Chemotherapy

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Systemic chemotherapy is used increasingly prior to resection of hepatic colorectal metastases. Previous reports have indicated an increased risk of perioperative complications associated with the use of systemic chemotherapy prior to resection. The purpose of this study was to investigate perioperative complications in patients receiving neoadjuvant systemic chemotherapy consisting of 5-fluorouracil (5-FU) and leucovorin (LV) with or without CPT-11 within 6 months of major liver resection. A retrospective review of 108 patients undergoing major liver resection for colorectal metastases with curative intent from 1997 to 2002 was performed. Patient and tumor characteristics, perioperative parameters, and morbidity and mortality were measured. Forty-seven patients (44%) received no chemotherapy, 27 patients (25%) received systemic 5-FU/LV, and 34 (31%) received systemic 5-FU/LV/CPT-11. A significantly higher number of patients in the group treated with preoperative 5-FU/LV plus CPT-11 had multiple tumors. Patients in this group also tended to have smaller tumors, fewer complications, and a higher R0 margin resection rate, but these findings were not statistically significant. Median blood loss and length of hospital stay were also not significantly different. There were no perioperative deaths. We conclude that the use of fluoropyrimidine-based chemotherapy and CPT-11 prior to major liver resection is not associated with increased morbidity or mortality. It may therefore provide a better therapeutic option, particularly in patients with multiple colorectal metastases. (J GASTROINTEST SURG 2003;7:1082–1088)

KEY WORDS: Liver resection, preoperative chemotherapy, complications

Of the more than 150,000 Americans diagnosed annually with colorectal cancer, half develop a recurrence at some point during their lifetime, with the liver being the most common site.[1] Left untreated, patients with liver metastases from colorectal carcinoma seldom survive longer than 1 year. Surgical resection of hepatic metastases has become the standard of care for resectable disease with 5-year survival rates after resection ranging from 25% to 45%.[2–4] Even after successful hepatic resection, however, the majority of patients will develop recurrent disease, either in the liver and/or extrahepatic sites.[4,5] Systemic chemotherapy, primarily fluoropyrimidine based, has therefore been used both for recurrent disease and for adjuvant therapy following resection as part of a multimodality approach.

Although traditionally given postoperatively, systemic chemotherapy has increasingly been used in the neoadjuvant setting, prior to liver resection, with several theoretical advantages. These include the ability to potentially downsize a tumor preoperatively and increase curative resection rates and/or to use more conservative surgical approaches; to identify patients who are responders to a particular chemotherapeutic regimen and consequently select more

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effective agents or discontinue therapy in "nonresponders" and potentially spare these patients the morbidity of nontherapeutic surgery; to simultaneously treat hepatic and systemic metastases early; and to better allow patients to tolerate systemic chemotherapy rather than after a major liver resection.

Whereas proponents of neoadjuvant therapy argue that its efficacy and safety are evident in other types of cancers including colorectal, gastric, and pancreatic, opponents argue that preoperative chemotherapy is hepatotoxic and leads to increased fatty degeneration, decreased hepatic regeneration, and an increased incidence of postoperative liver failure.[6–9] This has led some to advocate preoperative portal vein embolization prior to major liver resection after neoadjuvant therapy.[7] The purpose of our study was to investigate the effects and perioperative complications in patients receiving systemic chemotherapy consisting of 5-fluorouracil (5-FU) and leucovorin (LV) with or without CPT-11 (irinotecan) prior to major liver resection for colorectal metastases.

METHODS

Patients

A retrospective review of patients undergoing major hepatic resection for colorectal metastases with curative intent at the University of Texas M.D. Anderson Cancer Center between June 1997 and June 2002 was undertaken. Major hepatic resection was defined as a formal hepatectomy ($\geq$3 segments) or extended hepatectomy ($\geq$5 segments); segmental and lesser resections were excluded from this study. Patients were divided into three groups based on their preoperative chemotherapy regimen. Group I consisted of patients who had not received chemotherapy within 6 months of resection, group II included patients who received preoperative 5-FU plus leucovorin within 6 months of resection, and group III included patients who received preoperative 5-FU plus leucovorin plus CPT-11 (irinotecan) within 6 months of liver resection. Patients who received other systemic regimens or who received preoperative regional therapy were excluded.

Standard demographic data were collected on all patients. Perioperative parameters measured included the type and duration of preoperative chemotherapy, type of resection, estimated blood loss, characteristics of resected tumors, margin status, and 30-day mortality and morbidity. For those patients who received preoperative chemotherapy, radiographic response to therapy was defined as an objective decrease in the size or number of hepatic metastases as shown by preoperative imaging. The presence or absence as well as the degree of steatosis in the adjacent nonmalignant liver parenchyma in all resected specimens was determined retrospectively after review by an attending pathologist (T.-T.W.) with hepatobiliary expertise at the University of Texas M.D. Anderson Cancer Center. The degree of steatosis was graded as none, mild (<25%), moderate (25% to 50%), or severe (>50%).

Operations

For patients who received preoperative chemotherapy, a 4-week "rest" period after the last dose of chemotherapy was observed prior to hepatic resection. The extent of hepatectomy was defined according to the functional anatomy of the liver. Hepatectomy was defined as resection of three or more hepatic segments of the right or left liver; extended hepatectomy was defined as resection of five or more segments.[10] Operations were undertaken with the aid of intraoperative ultrasound imaging to achieve greater than 1 cm margins and to determine the presence of additional lesions not seen on preoperative imaging studies. An operation was considered to be an R0 resection if the entire tumor was removed with greater than 1 mm microscopic negative margins, an R1 resection if positive or $\leq$1 mm microscopic margins were present and an R2 resection if margins were grossly positive.

Statistical Analysis

Summary statistics were obtained by means of established methods. Fisher's exact tests were used for comparing the categorical variables, and the Kruskal-Wallis test was used to compare continuous variables among the treatment groups.

RESULTS

A total of 108 patients undergoing major hepatic resections from June 1997 to June 2002 were used in this analysis. Patients who underwent minor resections, R2 resections, resections after regional therapy or systemic therapy outside of the study parameters, or resections for tumors other than colorectal metastases were excluded from the study. Forty-seven patients (44%) had not received any chemotherapy within 6 months of resection (group I), 27 patients (25%) received a median of 20 weeks of systemic 5-FU and LV within 6 months prior to resection (group II), and 34 patients (31%) received a median of 12 weeks of preoperative 5-FU, LV, and CPT-11 (irinotecan) during the 6 months prior to resection (group III). The demographics of the patients in each group are shown

Table 1. Demographic characteristics of 108 patients undergoing major liver resection for colorectal metastases

	Group I (no preoperative chemotherapy)	Group II (preoperative 5-FU/LV)	Group III (preoperative 5 FU/LV/CPT 11)	*P* value
Patients	47	27	34	—
Median age (yr)	64 (range 37–83)	62 (range 41–79)	54 (26–73)	0.01
M/F	1.35:1	0.8:1	1.8:1	NS
Median disease-free interval (mo)	13 (range 0–84)	4 (range 0–96)	0.5 (range 0–70)	0.0005
Synchronous disease	11 (23%)	11 (41%)	17 (50%)	0.01
Median cycles of chemotherapy (wk)	—	20 (range 5–70)	12 (range 4–40)	—
Radiographic response		4/16 (25%)	21/29 (72%)	—
Primary tumor				NS
Rectal	12 (25%)	6 (22%)	8 (23%)	
Right colon	13 (28%)	7 (26%)	6 (18%)	
Left colon	5 (11%)	3 (11%)	2 (6%)	
Sigmoid	12 (25%)	9 (33%)	13 (38%)	
Other	5 (11%)	2 (7%)	5 (15%)	
Primary tumor stage				0.005
I	9 (19%)	1 (4%)	0 (0%)	
II	8 (17%)	2 (7%)	1 (3%)	
III	17 (36%)	13 (48%)	12 (35%)	
IV	10 (21%)	11 (41%)	17 (50%)	
Unknown	3 (6%)	0 (0%)	4 (12%)	

in Table 1. Patients in group III were younger, but the groups did not differ in terms of sex or location of the primary tumor. Patients in both groups II and III had a significantly shorter disease-free interval (4 and 0.5 months, respectively, vs. 13 months for group I), defined as the time between treatment of the primary tumor and discovery of liver metastases. These groups also had a significantly higher number of patients with synchronous disease. Although most of the patients in all three groups had node-positive colorectal primary lesions, the stage of the primary lesion was significantly higher in groups II and III, particularly stage IV, consistent with a higher number of patients with synchronous disease.

Of the 16 patients in group II who underwent restaging CT after receiving preoperative chemotherapy, 25% were judged to have a radiographic response to chemotherapy. Of the 29 patients in group III who underwent restaging CT, 72% were judged to have a radiographic response.

Perioperative parameters for all three groups are shown in Table 2. The majority of patients underwent a right hepatic lobectomy, and this was no different among the three groups. Median blood loss ranged

Table 2. Perioperative parameters of patients undergoing major liver resection for colorectal metastases

	Group I (no preoperative chemotherapy)	Group II (preoperative 5-FU/LV)	Group III (preoperative 5-FU/LV/CPT-11)	*P* value
Procedures				NS
Right lobectomy	28 (59%)	21 (78%)	17 (50%)	
Extended right lobectomy	7 (15%)	1 (4%)	7 (21%)	
Left lobectomy	7 (15%)	2 (7%)	6 (18%)	
Extended left lobectomy	5 (11%)	3 (11%)	4 (12%)	
Median EBL (ml)	425	400	500	NS
Tumors				0.003
Single	28 (60%)	17 (63%)	9 (27%)	
Multiple	19 (40%)	10 (37%)	25 (73%)	
Median maximum diameter (cm)	4.3 (range 0.9–10.3)	4.0 (range 1.4–10.5)	2.0 (range 0.8–6.5)	0.9
R0 resection	42 (89%)	26 (96%)	33 (97%)	0.4

EBL = estimated blood loss.

from 400 to 500 ml and was similar in all three groups. A significantly higher percentage of patients in group III (73%) had multiple tumors removed as compared to the other groups (40% in group I and 37% in group II). Patients in group III also tended to have smaller tumors, with a median maximum diameter of 2.0 cm, but this was not statistically significant. Patients in both groups II and III also tended to have a higher R0 resection rate (96% and 97% vs. 89%), but again this did not reach statistical significance.

Patient outcomes are presented in Table 3. There were no preoperative or in-hospital deaths in any of the groups. The complications rates were 49% in group I, 37% in group II, and 29% in group III ($P = 0.15$). The most common complications in all three groups were infectious in origin and included pneumonia, wound infections, and intra-abdominal abscess. Hepatic complications including biliary leaks/bilomas, and hepatic insufficiency (defined as total serum bilirubin $\geq$10 mg/dl)[11] were uncommon in all three groups. Hepatic insufficiency was present in only one patient in both groups II and III, and in three patients in group I. Median length of hospital stay was 7 to 8 days and did not differ among the three groups.

Pathologic examination of the adjacent nonmalignant liver parenchyma revealed the presence of steatosis in 36% of examined specimens in group I, 43% of examined specimens in group II, and 65% of examined specimens in group III ($P = 0.03$). In the majority of patients mild (<25%) steatosis was present, and severe steatosis was only seen in one patient in group II and three patients in group III (see Table 3).

DISCUSSION

Surgical resection for isolated hepatic colorectal metastases has become increasingly popular, as the safety and effectiveness of this treatment modality have been documented in several series. Mortality rates for major resection are often less than 5%, and 5-year survival has been reported to range from 25% to 45%.[3,4,12] Unfortunately, only a few patients have resectable disease and even after successful resection, recurrences are observed in approximately two thirds of patients, but with only 40% occurring within the liver.[13,14] More often, therefore, relapses occur outside the liver and this has fueled an interest in developing effective adjuvant treatment modalities. Until recently, standard systemic therapy consisted of bolus 5-FU and LV, but this has led to response rates of only 10% to 20% in patients with nonresectable metastases. Biomodulation and the use of infusional 5-FU have resulted in only modest increases in responses and no significant increases in survival.[15–17]

Recently irinotecan or CPT-11, a topoisomerase I inhibitor,[18,19] has produced response rates of 15% to 32% in chemotherapy-naïve patients with metastatic colorectal cancer[20–22]; it has also shown promise in patients who previously progressed while receiving 5-FU–based regimens. Large randomized trials have also recently shown that CPT-11 combined with

Table 3. Postoperative outcomes and characteristics of patients undergoing major liver resection for colorectal metastases

	Group I (no preoperative chemotherapy)	Group II (preoperative 5-FU/LV)	Group III (preoperative 5-FU/LV/CPT 11)	*P* value
Mortality	0	0	0	NS
Total complications	23 (49%)	10 (37%)	10 (29%)	0.15
Types				
Pneumonia	7	2	3	
Wound infection	5	3	3	
Intra-abdominal abscess/biloma	4	2	3	
Hepatic insufficiency*	3	1	1	
Median LOS (days)	8 (range 5–32)	7 (range 5–35)	7 (range 5–26)	NS
Steatosis				0.03
None	26 (55%)	13 (48%)	10 (29%)	
Mild (<25%)	11 (23%)	6 (22%)	15 (44%)	
Moderate (25–50%)	4 (8%)	3 (11%)	1 (3%)	
Severe (>50%)	0	1 (4%)	3 (9%)	
Unknown	6 (13%)	4 (15%)	5 (15%)	

LOS = length of stay.
*Defined as total serum bilirubin $\geq$10 mg/dl.

either bolus 5-FU/LV[23,24] or continuous infusion 5-FU/LV[25–29] can result in a significantly higher objective response rate as compared to 5-FU/LV alone. Specific studies aimed at investigating the treatment of hepatic metastases have not been done, however.

Chemotherapy for hepatic and systemic metastases can be given either in the adjuvant or neoadjuvant (preoperative) setting. Although traditionally given in the adjuvant setting, preoperative therapy has been used with increasing frequency in the treatment of many solid tumors including rectal, esophageal, breast, and pancreatic tumors. Neoadjuvant systemic chemotherapy for the treatment of colorectal liver metastases offers several advantages. It has been reported by several groups that patients with a short disease-free interval and those with synchronous hepatic metastases are at risk for increased recurrence, both local and distant, as well as decreased survival after hepatic resection.[30–34] In these high-risk patients, preoperative systemic chemotherapy allows one to treat systemic micrometastases and hepatic metastases simultaneously and immediately. A recent report by Allen et al.[35] showed that the response to preoperative chemotherapy in patients with synchronous colorectal hepatic metastases is a prognostic indicator of survival. The response to neoadjuvant chemotherapy may therefore assist in the selection of patients who would be candidates for hepatic resection versus those who may qualify for other therapeutic options.

Preoperative therapy may also allow one to downstage previously unresectable metastases. Bismuth et al.[36] reported on the effect of a chronomodulated regimen of systemic 5-FU/LV/oxaliplatin in 330 patients with unresectable colorectal metastases. Fifty-three of these patients (16%) became resectable and of these resected patients, 5-year survival was reported as 40%, which is comparable to that of initially resected patients. A recent update of these results by Adam et al.[37] reported on 95 (13.5%) of 701 patients with initially unresectable metastases who underwent hepatic resection after 5-FU/LV/oxaliplatin. There were no deaths and overall 5-year survival was 39%. In a similar study, 77 (51%) of 151 initially unresectable patients underwent hepatic resection after treatment with a similar regimen of 5-FU/LV/oxaliplatin. Overall median survival was reported as 48 months and had not been reached in the 48 patients who underwent R0 resection. Although our study consisted only of patients who were surgically resectable and was not designed to measure survival, recent data suggest that newer 5-FU/LV/CPT-11 regimens have equal response rates for metastatic colorectal cancer when compared to 5-FU/LV/oxaliplatin regimens.[38] In light of our data, therefore, the use of 5-FU/LV/CPT-11 for patients with colorectal metastases who are initially deemed nonresectable is an attractive option and warrants further study.

Although preoperative chemotherapy appears to be well tolerated and often effective, some studies have suggested that certain types of chemotherapeutic agents may cause significant hepatotoxicity and resultant hepatic failure. Pocard et al.[6] reported on patients undergoing hepatic resection for metastatic breast cancer after receiving various combinations of doxorubicin, navelbine, taxoid, and epirubicin. When compared to patients who did not receive preoperative chemotherapy, these patients were noted to have alterations in postoperative prothrombin times and γ-GGT levels, which is thought to be indicative of delayed hepatic regeneration. A majority of patients were also noted to have increased fatty degeneration as compared to control subjects, although overall morbidity and mortality did not differ.[6] Although this study involved chemotherapeutic agents that are not used in the treatment of colorectal metastases, a few studies have suggested that the fluoropyrimidines may also have an adverse effect on the liver. In animal studies, 5-FU has been shown to adversely affect liver regeneration after resection.[39] A report by Didolkar et al.[8] suggested that patients who received preoperative systemic fluoropyrimidine-based chemotherapy had a significant delay in hepatic regeneration, as shown by radiologic and laboratory evaluations, but only a trend toward an increase in mortality ($P = 0.29$). Similarly, Elias et al.[9] reported significantly increased (57%) postoperative complications in patients who had undergone intra-arterial chemotherapy compared to those who did not (18%). Although 5-FU was primarily used, other agents including cisplatin, mitomycin-c, and pirarubicin were also employed.

Largely because of this fear of hepatic toxicity, it has been recommended by some investigators that the threshold for preoperative portal vein embolization to induce ipsilateral atrophy and compensatory hypertrophy be adjusted for those patients receiving neoadjuvant chemotherapy. Azoulay et al.[7] reported on 30 patients who received an average of 11 courses of preoperative 5-FU/LV/oxaliplatin prior to major liver resection. Preoperative portal vein embolization was used when the estimated rate of remnant functional liver parenchyma was 40% or less, as compared to the standard 30% or less in their standard protocol.

Our study suggests that the use of preoperative chemotherapy using a combination of 5-FU LV/CPT-11 prior to major liver resection is safe, and allows one to perform major liver resections with no increase in morbidity or mortality. As shown, overall complication rates, particularly the risk of hepatic

insufficiency, was not increased in patients receiving this three-drug regimen compared to patients receiving 5-FU/LV alone or no preoperative chemotherapy. Although the presence of moderate to severe steatosis is thought to be a potential risk factor for liver failure after resection,[40] the majority of patients who received 5-FU/LV/CPT-11 in this study had only mild (<25%) steatosis noted. Only four patients (12%) were noted to have moderate to severe steatosis, of which one patient suffered hepatic insufficiency.

Furthermore, although specific volumetric measurements were not performed in our study, 11 patients (33%) who received preoperative 5-FU/LV/CPT-11 underwent extended right or extended left resections, of which only one patient suffered hepatic insufficiency. In comparison, three patients suffered postoperative hepatic insufficiency (one who had undergone an extended right hepatic resection) in the group not receiving preoperative therapy. This suggests that the indications for preoperative portal vein embolization should not differ solely because of the administration of neoadjuvant chemotherapy.

Preoperative therapy may also offer additional advantages. In this study, a significantly higher percentage of patients who received neoadjuvant 5-FU/LV/CPT-11 had multiple hepatic lesions resected, an independent predictor of recurrence and poor long-term outcome.[31] Certainly in these high-risk patients, providing preoperative systemic chemotherapy would allow one to treat systemic and hepatic micrometastases simultaneously and early, in hopes of decreasing the recurrence rates as well as selecting out those patients who progress and therefore would have undergone a major liver resection with no benefit in survival. Patients who received neoadjuvant therapy also tended to have an improved R0 resection rate, a factor that has been associated with improved outcome in several studies.[30,31,41,42] Although small numbers may have precluded a statistically significant difference, the ability of preoperative chemotherapy to provide a better resection margin combined with the potential ability to downstage a percentage of patients, as discussed previously, provides further support for the role of neoadjuvant therapy in the treatment of hepatic metastases.

In summary, the use of systemic neoadjuvant fluoropyrimidine-based chemotherapy and CPT-11 (irinotecan) for the treatment of hepatic colorectal metastases prior to major liver resection was not associated with increased morbidity or mortality and does not require an alteration in preoperative planning. Studies investigating pathologic and clinical response rates in addition to resectability rates and overall survival are warranted in order to ultimately define the role of preoperative 5-FU/LV and CPT-11 in the treatment of colorectal metastases to the liver.

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Efficacy of Venous Reconstruction in Patients With Adenocarcinoma of the Pancreatic Head

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Pancreaticoduodenectomy is often avoided in patients with portal or superior mesenteric venous involvement due to the perception that venous resection is complex, morbid, and carries a poor long-term survival. Our recent experience using state-of-the-art imaging and strict resection criteria show that venous reconstruction increases operative time, transfusion requirements, intensive care unit stay, and total hospital length of stay, but has no significant impact on operative morbidity rates, mortality rates, or the incidence of positive histologic margins. Kalpan-Meier life table analysis shows similar survival curves when compared to a contemporary cohort of patients who do not undergo venous reconstruction. (J GASTROINTEST SURG 2003;7:1089–1095)

KEY WORDS: Pancreaticoduodenectomy, pancreatic adenocarcinoma, venous reconstruction, endoscopic ultrasound, helical computer tomography

Pancreatic carcinoma is the second most common malignancy of the gastrointestinal tract with an annual death rate roughly equivalent to the rate of incidence.[1] The only potentially curative treatment is surgical resection, which is limited to those patients without metastatic disease and with a tumor that is anatomically situated to ensure complete tumor removal with negative surgical margins.[2,3] Patients who undergo pancreaticoduodenectomy (PD) resulting in a positive surgical margin have a survival rate similar to patients with locally advanced disease treated nonoperatively with 5-fluorouracil–based chemoradiotherapy.[4] Of all patients with pancreatic cancer, a small percentage have localized tumors where only portal vein (PV) or superior mesenteric vein (SMV) involvement is the sole obstacle to a complete, margin negative resection. PD is often avoided in this select patient population because of the perception that venous resection is complex, morbid, and has poor long-term survival.[5] With the recent decline in morbidity and mortality rates for standard PD, more aggressive resections involving segmental resection and reconstruction of the portal/mesenteric venous systems have been achieved.[3] Even with the technologic advancements in both the PD procedure and perioperative care, patient selection remains critical to achieving a complete resection of all tumor tissue with negative histologic margins.[6,7]

Since 1998, our group has staged patients with pancreatic malignancies using both dual phase helical CT scanning and endoscopic ultrasonography (EUS).[8] Based on these two complementary studies, patients with locally advanced tumors that involved only the SMV or PV, as defined by imaging, were operated on with the intent to achieve a complete, margin negative resection. The present study evaluates our institution's experience with SMV/PV reconstruction by comparing patients who required venous reconstruction to those who required no venous reconstruction. Specific end points analyzed in this study included operative time, blood loss, morbidity and mortality rates, hospital and intensive care unit lengths of stay, tumor staging, incidence of positive resection margins, and overall survival rates after surgery.

METHODS

This study is a retrospective analysis of a consecutive series of patients with a diagnosis of pancreatic

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adenocarcinoma who were referred to a single surgeon (T.J.H.) at Indiana University following evaluation with EUS and dual-phase helical CT scanning. All patients in this study were considered to be candidates for complete surgical resection following a complete history and physical examination and analysis of their imaging data. Imaging criteria for operative exploration with curative intent were as follows: (1) absence of liver metastasis or fine needle aspiration–confirmed celiac adenopathy; (2) no evidence of involvement of the superior mesenteric artery (SMA) or celiac axis; and (3) evidence of vascular flow through the SMV/PV. Although partial venous involvement of the SMV/PV was considered potentially resectable, complete encasement leading to vascular thrombosis and cessation of blood flow in the SMV/PV as detected either by EUS or dual phase helical CT was a contraindication to operations with curative intent. Prior to tumor resection, patients underwent carefully operative exploration by either laparoscopy or laparotomy to search for evidence of locally advanced or metastatic disease. Operative criteria for tumor resection were as follows: (1) absence of liver metastases; (2) no peritoneal dissemination or drop metastases in the pelvis; (3) lack of invasion of the transverse mesocolon; (4) absence of metastases to the celiac lymph node station (No. 9); (5) no involvement of the SMA, celiac artery, or common hepatic artery; and (6) the ability to obtain adequate vascular control of the SMV, PV, splenic vein, and inferior mesenteric veins to allow for a safe venous reconstruction.

PD, either standard or pylorus sparing, was the procedure used in this study.[10] All anatomic dissections including the hepatoduodenal ligament, transaction of the pancreatic neck, duodenojejunal flexure, uncinate process, and mobilization of the infrapancreatic SMV were carried out before obtaining vascular control for venous resection.[11] Routine frozen-section examinations of the pancreatic neck, bile duct, and retroperitoneal soft tissue margins were completed. If a positive pancreatic neck or bile duct margin was encountered, further resection was done to achieve a negative histologic margin. Regional lymph nodes around the common hepatic artery (No. 8), celiac trunk (No. 9), hepatoduodenal ligament (No. 12), and anterior (No. 17), and posterior pancreaticoduodenal areas (No. 13) were routinely dissected.[9] If intraoperative assessment showed involvement of these nodes, the SMA (No. 14) and para aortic nodes (No. 16) were also dissected.

Venous reconstruction was done only after en bloc resection of the head of the pancreas, duodenum, distal common bile duct, and retroperitoneal soft tissue margin was completed. Systemic heparinization (100 U/kg) was routinely used before complete vascular isolation, but it was not administered for partial occlusion using a side-biting vascular clamp for lateral venorraphy. The SMA was not routinely clamped. If the venous resection required an interposition graft or patch venorraphy, the saphenous vein was harvested in the groin. Vascular reconstruction was performed using a continuous running suture of 5-0 Prolene with end-to-end anastomoses. Splenic vein patency was routinely maintained either by incorporation into the proximal or distal resection margins or reimplantation of the vein into the interposition graft. Gastrointestinal reconstruction was achieved by a retrocolic end-to-side duct to mucosal pancreaticojejunostomy, and end-to-side hepaticojejunostomy, and an antecolic end-to-side pylorojejunostomy or gastrojejunostomy.

Postoperative care was administered in a standardized fashion following a clinical care pathway specifically developed by our institution for patients following PD. The care pathway outlines the criteria for admission to the intensive care unit, use of prokinetic agents, early mobilization, ambulation, pulmonary toilet, drain care, and is designed to achieve a projected length of stay of 8 days postoperatively. Long-term anticoagulation was not instituted in any of these patients after their venous reconstruction. Postoperative course including hospital length of stay, intensive care unit length of stay, morbidity, mortality, and overall survival were assessed for all patients. Delayed gastric emptying was defined as the inability to achieve full oral intake without the need for intravenous nutritional supplementation by postoperative day 10. A pancreatic fistula was defined as drainage of more than 50 ml of fluid with an amylase concentration greater than three times the upper limit of normal serum level after postoperative day 10. Perioperative mortality included all deaths within 30 days of the operative procedure. Intraoperative factors such as the length of operation, intraoperative blood loss, and transfusions given were obtained from the anesthesiology operative record sheet. Tumor factors such as size, differentiation, incidence of perineural or perivascular invasion, total number of lymph nodes assessed per surgical specimen, incidence of lymph node involvement by tumor, histologic status of the resection margins, and pathologic staging of the tumor were obtained from each patient's pathology report. Patients who received postoperative adjuvant chemoradiotherapy were identified. All patients were followed up until December 2002 either by direct clinic visit or through telephone interviews.

The raw data were transcribed into a Microsoft Excel spreadsheet and later imported into VassarStats (http://faculty.vassar.edu/lowry/VassarStats.html), a

Web-based statistical computation site for evaluation. Chi square tests were employed for nominal variables. For continuous variables, summary statistics were reported in the form of mean ± standard deviation. Comparison between groups was done by means of Student's *t* test (two-tailed). Rates of cumulative survival were calculated by the Kaplan-Meier method and plotted. Statistical significance was taken at a *P* value of less than 0.05.

RESULTS

Patient Populations Studied

After initial CT and EUS staging, 42 patients with adenocarcinoma of the pancreatic head were thought to have potentially resectable disease and referred for surgical treatment. Twenty-six potentially resectable patients (61%) had no evidence of venous involvement on preoperative imaging, whereas 16 patients (39%) had tumors that appeared to involve the PV, SMV, or its confluence. Of the 26 patients with potentially resectable disease with no venous involvement, three (12%) were found at surgery to be unresectable due to a positive celiac lymph node (N = 1), venous encasement of the SMV precluding reconstruction (N = 1), or unrecognized liver metastases (N = 1). All three of these patients underwent palliative procedures. Two patients had gastrojejunostomy and choledochojejunostomy (double bypass), and one had a choledochojejunostomy. The remaining 23 patients underwent PD (8 standard, 15 pylorus sparing) and make up the group that had no venous resection.

Of the initial 42 patients referred, 16 had locally advanced tumors based on preoperative imaging. Three of these patients (19%) were found at surgery to be unresectable because of either SMA involvement (N = 1), venous encasement of the SMV/PV confluence precluding safe reconstruction (N = 1), or liver metastasis (N = 1). One patient had a palliative Roux-en-Y choledochojejunostomy. The remaining 13 patients had SMV/PV involvement that was resectable and underwent PD (6 standard, 7 pylorus sparing) with portal venous reconstruction. Venous reconstruction consisted of segmental resection and end-to-end anastomosis in six patients (46%), lateral venorraphy (one requiring saphenous vein patch) in five patients (38%), and resection with interposition saphenous vein graph in two patients (15%). These patients make up the PV/SMV resection group.

Patient Demographics, Comorbid Conditions, and Perioperative Variables

Patients, on average, were in their late 60s with a slight preponderance of males. Operative comorbidity was generally quite low in this cohort of patients (Table 1). For all 36 patients analyzed who had PD (13 with venous resection, 23 without), the mean operative

Table 1. Patient demographics, comorbity, and intraoperative and postoperative variables for 13 patients with PV/SMV resection and 23 patients who had no venous resection during a Whipple operation for adenocarcinoma of the head of the pancreas

	PV/SMV resection (N = 13)	No venous resection (N = 23)	*P* value
Male/female	7/6	14/9	0.74
Age	68 ± 13 yr	67 ± 8.6 yr	0.83
Diabetes	2 (15%)	3 (13%)	0.39
Coronary artery disease	2 (15%)	5 (22%)	0.74
COPD	2 (15%)	3 (13%)	0.68
Mean operative time	408 ± 114 min	342 ± 42 min	0.01
Intraoperative blood loss (ml)	1567 ± 1081 ml	848 ± 538 ml	0.01
Transfusions	2.3 ± 2.9 units	1.3 ± 1.9 units	0.12
ICU stay	1.9 ± 2.9 days	0.8 ± 1.8 days	0.003
No. of patients with complications	7 (54%)	10 (43%)	0.29
Reoperation	2 (15%)	1 (4%)	0.54
Wound infection	2 (15%)	3 (13%)	0.61
Pancreatic fistula	2 (15%)	3 (13%)	0.54
Delayed gastric emptying	5 (38%)	5 (22%)	0.44
Intra-abdominal abscess	2 (15%)	1 (4%)	0.54
Hospital length of stay	14.0 ± 7 days	10.0 ± 5 days	0.03
Hospital readmission	0	4 (17%)	0.27

COPD = chronic obstructive pulmonary disease; ICU = intensive care unit.

time was 361 ± 78 minutes, estimated blood loss was 1094 ± 828 mL, and volume of red blood cells transfused perioperatively was 1.9 ± 2.5 units. Operations averaged 1 hour longer and incurred roughly twice as much blood loss in patients undergoing PV/SMV resection when compared to patients who had no venous resection ($P = 0.01$). Perioperative transfusion requirements ($P = 0.12$) and the use of intensive care unit resources ($P = 0.003$) were higher in patients with PV/SMV resection than in patients having the standard PD. The overall mean hospital length of stay was 4 days longer in patients with PV/SMV resection ($P = 0.03$). Despite these discrepancies, we found no differences in postoperative complication rates (reoperation, wound infection, pancreatic fistula, delayed gastric emptying, intra-abdominal abscess, or pancreatitis), reoperation rates, or hospital readmission rates between these two groups of patients.

Tumor Factors and Survival

Mean tumor size, histopathologic tumor differentiation, number of lymph nodes harvested in the surgical specimen, incidence of positive lymph node involvement, and incidence of perineural invasion were not significantly different between our two patient groups (Table 2). Histopathologic evidence of vascular invasion was present in all 13 patients who had PV/SMV resection (9 adventitial involvement, 1 intimal involvement, 3 perivascular space invasion). Perivascular invasion was seen histologically in only two patients in the standard PD group ($P < 0.001$). Microscopic surgical margins were found to be histologically positive in 3 (23%) of 13 patients requiring PV/SMV resection and 4 (17%) of 23 patients undergoing standard PD, a difference that was not statistically significant ($P = 0.42$). One patient died perioperatively in each group, both from intra-abdominal sepsis and multiple-organ system failure. Twice as many patients (62% vs. 30%) in the PV/SMV resection group received adjuvant chemoradiotherapy. The 1-year actual survival rate was 83% in the PV/SMV resection group and 60% in the standard PD group. Overall median survival for the PV/SMV resection group was 13 months, which was no different from that in patients undergoing PD without venous resection (Fig. 1.) The overall mean follow-up of both groups of patients is short (mean 13 months), reflecting the limited time we have been doing PV resections at our institution.

DISCUSSION

Fortner's regional pancreatectomy is generally regarded as the initial example of an aggressive operative approach for pancreatic carcinoma, including

Table 2. Tumor size, histopathologic features, and survival in 36 patients who had resection with curative intent for adenocarcinoma of the head of the pancreas

	PV/SMV resection (N = 13)	No venous resection (N = 23)	*P* value
Mean tumor size	3.3 ± 1.5 cm	2.7 ± 1.1 cm	0.20
Histopathologic type			
Well differentiated	2 (15%)	3 (13%)	0
Moderately differentiated	6 (46%)	11 (48%)	0
Poorly differentiated	5 (38%)	9 (39%)	0
Total lymph nodes harvested	7 ± 4.4	6.9 ± 3.8	0.74
Patients with + node	7 (54%)	13 (56%)	0.57
Patients with − node	6 (46%)	10 (44%)	0.57
Perineural invasion	8 (62%)	11 (48%)	0.50
Vascular invasion	13 (100%)	2 (9%)	>0.001
Histopathologic stage*			
I	0	6 (26%)	0.07
II	4 (31%)	5 (22%)	0.69
III	9 (69%)	12 (52%)	0.48
Positive histologic margins	25%	17%	0.42
Perioperative mortality rate	1 (8%)	1 (4%)	1.0
Adjuvant chemoradiotherapy	8 (62%)	7 (30%)	0.09
1 yr actual survival	83%	60%	0.39
Median survival	13 mo	12 mo	
Mean follow-up	13.4 ± 7 mo	13.2 ± 5 mo	

*American Joint Committee on Cancer (AJCC) staging system.

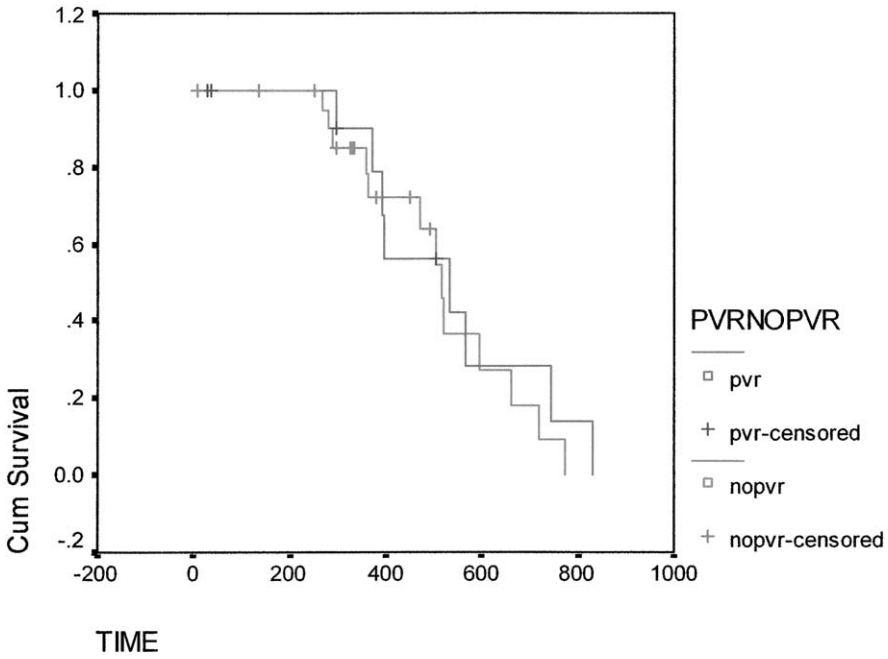

Fig. 1. Overall survival: Portal vein resection (*PVR*) vs. no portal vein resection (*NOPVR*).

resection and reconstruction of the mesenteric vascular system.[12] The significant morbidity (67%) and mortality (23%) of the procedure, coupled with the low survival rates (median survival = 13 months; 3-year survival rate = 3%) never justified the general acceptance of vascular resection and reconstruction for the vast majority of patients with pancreatic carcinoma. In Fortner's defense, he operated on many patients with locoregionally advanced pancreatic carcinoma without the benefits of modern imaging techniques currently in use for preoperative staging and patient selection. Furthermore, his experience was carried out prior to the recent well-documented technologic advances that helped to decrease surgical morbidity and mortality rates for the standard Whipple operation.[13,14] These declining morbidity and mortality rates have fueled a renewed interest in extending the standard Whipple operation to include major vascular resection, resulting in more patients being considered candidates for complete surgical resection (R0 resection). Arterial resection for tumor extirpation continues to be used only in highly selected cases because of the difficulty in obtaining a negative surgical margin and its corresponding increase in perioperative morbidity and mortality rates.[4,15,16] In contrast, venous resection for tumor removal can be done with a morbidity and mortality similar to that for the standard PD and an acceptable rate of positive resection margins.[4,7,11]

This series of patients represents a highly select population who were chosen on the basis of state-of-the-art preoperative imaging studies including dual-phase helical CT scanning and high-quality EUS. This selection bias is underlined by the fact that only 3 (12%) of 25 patients with resectable disease and 3 (19%) of 16 patients with locally advanced disease as assessed by preoperative imaging were found to have unresectable tumors at the time of exploration. Based on the current surgical experience with pancreatic carcinoma (Table 3), the key to improving long-term survival is the ability to perform venous resection only in situations where a high likelihood of achieving negative surgical resection margins exists.[4,15] All patients in this series with positive resection margins had involvement of the retroperitoneal extrapancreatic plexus, a notoriously difficult anatomic area to assess prior to transection of the pancreatic neck and commitment to the operative procedure.[17] Our 23% incidence rate of positive retroperitoneal soft tissue margins compares favorably to rates at other institutions that have reported their experiences in the literature.[4,7,11,17,18] Future improvement in this area will be the product of advances in preoperative imaging techniques including EUS, CT, and MRI that will help to better identify patients with retroperitoneal extrapancreatic plexus invasion.

Our results support the emerging body of literature that shows PV/SMV resection can be safely done in the majority of selected patients.[4,6,7,11,13] The

Table 3. Reported series of SMV/PV resection for pancreatic adenocarcinoma

	No. of patients	Mean tumor size (cm)	Poorly differentiated histology	Positive histologic margins	Positive lymph nodes	Median survival (mo)
Leach et al.[4]	31	3.5*	NA	4 (13%)	13 (42%)	22†
Harrison and Brennan et al.[11]	42	3.5	11 (26%)	10 (24%)	24 (59%)	13
Roder et al.[18]	22	3.9	10 (46%)	15 (68%)	17 (77%)	8
Nagakawa et al.[19]	72	NA	NA	53 (73%)	NA	19
van Geenen et al.[6]	34	2.5	20 (59%)	20 (59%)	NA	14
Bachellier et al.[7]	21	4.8	1 (5%)	8 (38%)	16 (76%)	12
Present study	13	3.3	3 (23%)	3 (23%)	8 (62%)	13

NA = not available.
*Median tumor size.
†(71%) 22 of 31 patients received neoadjuvant chemoradiotherapy.

duration of the operation was longer, estimated blood loss and transfusion requirements were greater, use of intensive care unit resources was higher, and a longer postoperative hospital stay was required in patients undergoing PV/SMV resection than in those patients undergoing a standard PD. Despite these differences, operative morbidity rates, mortality rates, need for reoperation, readmission rates, and the incidence of negative surgical resection margins were similar between the two groups, observations that have been confirmed by others.[7,11] It should be emphasized, in our experience, that venous resection can rarely be done to render inoperable patients operable. More realistically, it is used in select cases to extend the number of patients who are candidates for complete R0 resection and experience the benefits of excellent long-term palliation from PD.[4,6,7,11] Venous resection has never been shown to improve survival rates compared to standard PD without venous resection, an observation that was substantiated in this study.[6,7,11] These data mirror the difficulties in improving long-term survival rates in this disease by more extensive surgery and reflect the sobering long-term survival statistics associated with all operative treatment of patients with pancreatic adenocarcinoma.[19–21] Nevertheless, based on its documented safety and its ability to achieve survival rates that are equivalent to those in patients undergoing standard PD, isolated PV/SMV involvement in patients with adenocarcinoma of the pancreatic head should not be considered a deterrent to complete surgical resection.

CONCLUSION

PV/SMV resection during PD increases the operative time, estimated blood loss, transfusion requirements, length of intensive care unit stay, and overall hospital stay but does not significantly add to the operative morbidity rates, mortality rates, or incidence of positive histologic margins. Kaplan-Meier life table analysis shows similar survival curves for patients who do not undergo venous reconstruction.

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Influence of Mild Obesity on Outcome of Simultaneous Pancreas and Kidney Transplantation

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The influence of body mass index (BMI) on outcome of simultaneous pancreas-kidney transplantation (SPK) has not been well described. We retrospectively reviewed 88 consecutive primary SPKs performed at our institution between March 15, 1995 and August 28, 2001. All patients received antibody induction and maintenance immunosuppression with tacrolimus, mycophenolate mofetil, and steroids. Systemic-enteric implantation was performed in all patients. Primary end points were patient, pancreas, and kidney survival. Secondary end points were rates of anastomotic leakage, pancreas thrombosis, major infection, rejection, repeat laparotomy, and length of hospital stay. Values are shown as mean ± standard deviation, range, or percentage. Fifty-two patients (59.1%) were nonobese with a BMI ⩽24.9 (mean 21.7 ± 2.2, range 15.4 to 24.9). Thirty-six patients were mild to moderately obese with a BMI ⩾25 (mean 27.7 ± 2.2, range 25 to 35.1). Distribution of recipient age, sex, and ethnicity was similar between groups. Kidney and pancreas preservation times were similar between nonobese and obese patients. One-, three-, and five-year actuarial patient (nonobese: 95%, 95%, 95% vs. obese: 95%, 95%, 89%), kidney graft (nonobese: 91%, 91%, 87% vs. obese: 97%, 91%, 85%), and pancreas graft (nonobese: 78%, 78%, 73% vs. obese: 70%, 62%, 62%) survival were comparable between nonobese and obese (P = NS). The mean rates of pancreas thrombosis, major infection, pancreas rejection, kidney rejection, relaparotomy, and length of hospital stay were similar in the two groups. The overall duodenojejunal anastomotic leakage rate was 8%. Obese patients had a 17% incidence of leakage (6 of 36) compared to a 2% incidence of leakage in nonobese patients (P = 0.012). Six of seven leaks occurred in obese patients. Mean BMI in the seven patients with a leak (27 ± 1.9) was significantly higher than in patients who did not develop a leak (24 ± 3.7; P = 0.05). Although obesity had no effect on patient or graft survival, it was associated with a significantly higher leakage rate. There should therefore be a higher degree of suspicion for the presence of duodenojejunal anastomotic leaks in obese SPK recipients. (J Gastrointest Surg 2003;7:1096–1101)

Key words: Obesity, kidney, pancreas, transplantation

Obesity has been described as a significant risk factor for death in the general population and has been specifically associated with an increased risk of cardiovascular mortality.[1] Obesity has also long been considered a risk factor in general surgery procedures[2,3]; however, the extent to which it affects a variety of postoperative complications has been subject to debate.[4] Similarly, there is a great deal of literature regarding the influence of obesity on the outcome of renal transplantation; however, this has been a controversial subject with much conflicting information. Several centers have reported decreased patient survival,[5–7] decreased graft survival,[1,6,8,9–11] increased postoperative complications,[5–8] and increased immunologic graft loss[6] in obese kidney transplant recipients, whereas other series have demonstrated similar outcomes between obese and nonobese patients undergoing renal transplantation.[12–14] Although some have contended that the inferior graft survival in obese patients is related to an increased rate of graft loss due to death with a functioning

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graft,[7,13] others have shown an increased risk of graft loss independent of patient survival.[10,15]

In contrast, the influence of obesity on outcome of simultaneous pancreas-kidney transplantation (SPK) has received little attention. Because the SPK procedure is inherently longer and more technically complex than kidney transplantation alone and requires more aggressive immunosuppression, and because long-standing diabetes is itself a significant risk factor for cardiovascular mortality, we suspected that obesity might have a significant impact on the outcome of SPK. This provided the rationale for the current investigation.

PATIENTS AND METHODS

Patients

The records of 88 consecutive patients undergoing primary SPK at our institution between March 15, 1995 and August 28, 2001 were retrospectively reviewed. Information was obtained from medical records and a pancreas transplant database after institutional review board approval was granted. All patients had confirmed type 1 diabetes as determined by serum C-peptide levels. All patients had undergone preoperative cardiac stress testing or coronary angiography when necessary. Transplantation was not performed in the presence of an abnormal stress test or coronary angiogram. For the purpose of analysis, patients were divided into two groups as follows based on body mass index (BMI) at the time of transplantation: nonobese, BMI $\leqslant$ 24.9; obese, BMI $\geqslant$ 25. Patients were followed until September 5, 2002, or until they died; mean follow-up was 3.5 $\pm$ 2.1 years (range 5 days to 7.6 years, including early postoperative death). No patients were lost to follow-up.

Surgical Technique

Kidneys were implanted retroperitoneally in standard fashion. All pancreata were implanted intraperitoneally using donor iliac artery reconstruction of the superior mesenteric artery and splenic arteries, systemic venous drainage, and enteric drainage of exocrine secretions by side-to-side, two-layer, hand-sewn duodenojejunostomy.

Immunosuppression

All patients received a single intraoperative dose of intravenous methylprednisolone, 500 mg, and antibody induction with either OKT3 or an interleukin-2 receptor antagonist (daclizumab or basiliximab) administered prior to graft reperfusion and continued postoperatively. Maintenance immunosuppression consisted of tacrolimus (TAC), mycophenolate mofetil (MMF), and prednisone taper. Details of the immunosuppresion regimen are shown in Table 1.

Rejection Surveillance

Kidney biopsies were performed when clinically indicated by elevation in baseline serum creatinine levels in the absence of obvious drug nephrotoxicity or technical problems. Ultrasound-guided percutaneous biopsies of the pancreas were performed when clinically indicated in the setting of elevated serum amylase, lipase, or glucose levels. Protocol kidney or pancreas biopsies were not performed.

Rejection Therapy

Biopsy-proved episodes of mild to moderate kidney rejection and mild pancreas rejection were treated with intravenous methylprednisolone, 500 mg daily for 3 to 6 days. Steroid-resistant rejection, vascular kidney rejection, and moderate or severe pancreas rejection were treated with OKT3, 5 mg daily for 14 days.

Patient Data and Statistical Analysis

The BMI of each patient on the date of transplantation was calculated as follows: body weight (kg) divided by height (m^2). Survival curves were computed using the Kaplan-Meier method and compared using the log-rank test. Proportions were compared using the chi-square test, and comparisons of mean values were performed using the *t*-test for independent samples (two-tailed). Values are shown as

Table 1. Standard immunosuppression regimens

Induction
3/15/95–5/7/99: OKT3, 5 mg intravenously, before graft reperfusion; 5 mg intravenously per day on postoperative days 1–7
5/7/99–10/31/99: Daclizumab, 1 mg/kg intravenously, before graft reperfusion; postoperatively, 1 mg/kg every 2 weeks for two to five doses, according to study protocol
11/1/99–9/5/02: Basiliximab, 20 mg intravenously before graft reperfusion and on 4 postoperative day 4
Maintenance
Tacrolimus, 0.15 mg/kg by mouth twice a day. Target trough levels—weeks 1–4, 12–15 ng/ml; weeks 5–12, 10–12 ng/ml; weeks 13–52, 8–10 ng/ml
Mycophenolate mofetil, 1.5 mg by mouth twice a day
Prednisone, 80 mg/day on postoperative days 1–7. Taper to 10 mg every other day until 30 mg reached. 30 mg/day for 7 days. Taper by 5 mg weekly until 10 mg reached. Hold at 10 mg for at least 1 year

mean ± standard deviation, range, or percentage. Significant differences in outcome between groups were further analyzed using stepwise logistic regression. A P value of <0.05 was considered statistically significant. All analyses were performed using SPSS 10.0 software (SPSS, Chicago, IL).

RESULTS

Patient demographics are presented in Table 2. Fifty-two patients were nonobese and 26 were obese. The mean BMI of obese patients (27.7 ± 2.2, range 25 to 35.1) was significantly higher than that in nonobese patients (21.7 ± 2.2, range 15.4 to 24.9; $P < 0.0001$). Fifty-two patients had a BMI <25, 17 had a BMI of 25 to 26.9, 13 had a BMI 27 to 29.9, and six had a BMI ⩾ 30. The percentage of males was greater in the obese group (62%) compared to the nonobese group (40%). Pretransplant dialysis modality, peak panel reactive antibody, degree of human leukocyte antigen mismatch, induction agent used, graft preservation times, and graft anastomotic times were comparable between groups.

Fig. 1 depicts patient survival as determined by Kaplan-Meier analysis. There was no significant difference in patient survival at 5 years after transplant between the obese and nonobese groups. Five of six deaths were attributable to myocardial infarction (3 nonobese, 2 obese). One patient died of hemorrhage resulting from a ruptured iliac artery pseudoaneurysm in the presence of a duodenal anastomotic leak.

Figs. 2 and 3 depict kidney and pancreas graft survival, respectively. Kidney graft survival was comparable in the obese and nonobese groups at 5 years after transplant. Five-year pancreas graft survival was somewhat better in nonobese compared to obese patients (73% vs. 62%, respectively); however, this was not statistically significant. Chronic rejection and death were the most common causes of kidney graft loss in obese patients, whereas acute rejection and death accounted for most of the kidney graft loss in nonobese patients. Thrombosis, acute rejection, and death were the most common causes of pancreas graft failure in both obese and nonobese patients and occurred at similar rates in both groups. Immunologic graft loss (acute rejection + chronic rejection) accounted for 36% of all graft failures in nonobese (7 of 19) and 35% of all graft failures in obese patients (P = NS). The cumulative incidence of at least one kidney or pancreas rejection was 39% in nonobese and 42% in obese patients; this was not significantly different. The distribution of kidney and pancreas rejection grade and mean serum trough TAC levels during the first post-transplant year did not differ significantly between the obese and nonobese groups. Length of hospital stay after transplantation (nonobese 11.5 ± 7.4 days vs. obese 10.2 ± 6.2 days) and readmissions (nonobese 1.35 ± 1.8 vs. obese 1.8 ± 2.1) did not differ between groups. The majority of patients had gained weight at 1 year after transplant, with a similar percentage of nonobese and obese patients demonstrating evidence of weight gain (63.5% vs. 61%, respectively).

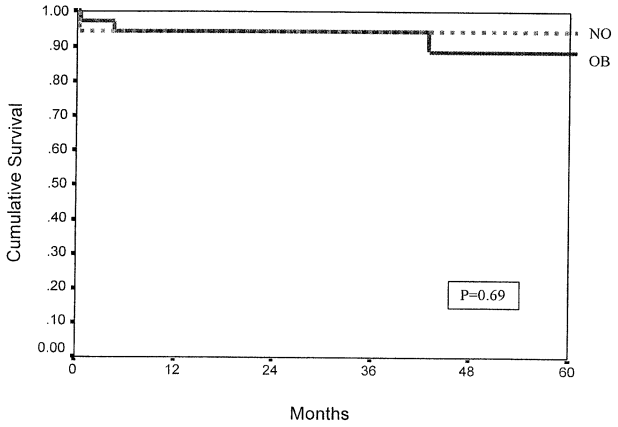

Fig 1. Kaplan-Meier patient survival.

The incidence of postoperative infections is summarized in Table 3. The total incidence of infection was similar between groups. The incidence of wound infection, urinary tract infection, intra-abdominal abscess, pneumonia, and viral infection was comparable

Table 2. Patient demographics

Patient characteristics	BMI ⩽24.9 (n = 52)	BMI ⩾25 (n = 36)	P value
BMI	21.7 ± 2.2 (range 15.4–24.9)	27.7 ± 2.2 (range 25–35.1)	<0.0001
Follow-up (mo)	3.5 ± 2.1	3.5 ± 2.1	NS
Age (yr)	38.8 ± 8.3	38.5 ± 7.3	NS
M/F	21/31	24/12	0.015
Ethnicity (African-American/caucasian)	18/34	11/25	NS

NS = not significant.

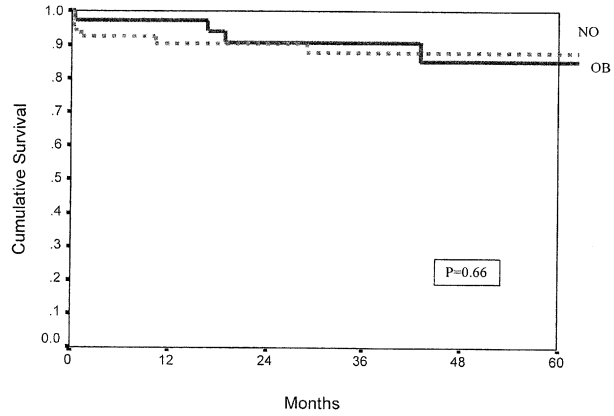

Fig 2. Kaplan-Meier kidney graft survival.

between obese and nonobese patients. The only difference between groups was a higher incidence of duodenojejunal anastomotic leaks in obese patients. Although the overall anastomotic leakage rate was 8%, obese patients had a 17% incidence of leaks compared to a 2% incidence of leaks in nonobese patients ($P = 0.012$). Six of seven leaks occurred in obese patients. The mean BMI in the seven patients with a leak (27 ± 1.9) was significantly higher than that in patients who did not have a leak (24 ± 3.7), $P = 0.05$. Multivariate analysis using stepwise logistic regression demonstrated that BMI ≥ 25 was the only variable that was independently associated with duodenojejunal anastomotic leakage (odds ratio 1.3, $P = 0.03$).

DISCUSSION

Although the impact of obesity on outcome of renal transplantation has been well described, albeit with conflicting reports, there has been only one previous single-center investigation of obesity as a risk factor after SPK. Bumgardner et al.[16] showed comparable actuarial patient survival between patients with a BMI ≤ 27 vs. those with a BMI > 27; however, actuarial kidney and pancreas graft survival were lower in patients with a BMI > 27. The decrease in pancreas and kidney graft survival in Bumgardner's series could not be attributed to earlier death with a functioning graft, an increased incidence of postoperative complications, or differences in the incidence of acute rejection or immunosuppresion levels between obese and nonobese patients. However, late graft loss was noted to correspond to decreased patient survival in the obese group. Our own observations differ from those of Bumgardner et al.[16] for a number of reasons.

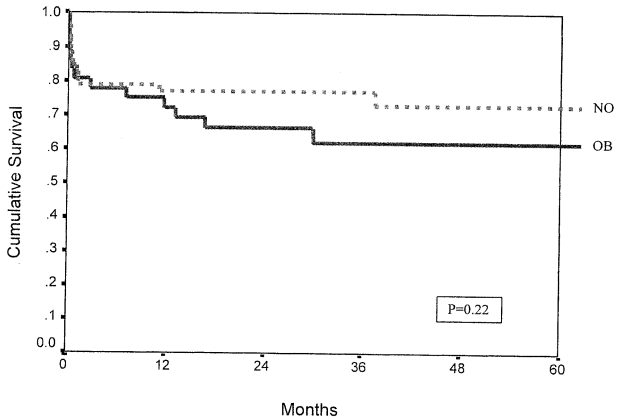

Fig 3. Kaplan-Meier pancreas graft survival.

Table 3. Postoperative infective complications (episodes/patient)

Infection	BMI ≤24.9	BMI ≥25	*P* Value
Wound	0.09 ± 0.3	0.11 ± 0.4	NS
Urinary tract	0.42 ± 0.87	0.31 ± 0.75	NS
Duodenojejunal anastomotic leak	0.02 ± 0.14	0.17 ± 0.38	0.012
Total infections	1.15 ± 1.36	1.06 ± 1.35	NS

NS = not significant.

Although our program did not have a strict weight limit for activation on the SPK list during the period reviewed, we tended to be somewhat more selective in terms of BMI for SPK compared to kidney transplant alone. Additionally, we restricted SPK to only those with documented type 1 diabetes (C-peptide <0.5 ng/ml), whereas some other transplant programs either intentionally or unintentionally perform SPK in patients with type 2 diabetes, who generally tend to have a higher BMI. As a result, the number of patients with a BMI > 27 was too small for analysis in our series; therefore we selected a BMI of 25 as our cutoff for evaluating the impact of BMI on SPK outcome. Although a BMI of 25 technically represents only mild obesity, previous studies have shown that this BMI confers a higher risk of adverse medical events in other patient groups[17,18] and has been shown to be a twofold independent risk factor for decreased graft survival in renal transplantation.[10] Consequently we believed that analysis at this lower BMI cutoff for obesity was still worthwhile, accepting the possibility that our findings might not apply to patients with more severe obesity. The mean BMI of the obese group was significantly higher than that of the nonobese group, suggesting that these groups were indeed sufficiently different for comparison.

As did Bumgardner's group, we demonstrated similar long-term patient survival in obese and nonobese

SPK recipients; however, in contrast, we did not observe any significant differences in either kidney graft or pancreas graft survival between groups. It is likely that selection bias eliminated a significant number of more severely obese SPK candidates, the inclusion of which may have otherwise resulted in a higher incidence of late cardiovascular mortality and a resultant higher incidence of death with a functioning graft in the obese group. Interestingly, although cardiovascular disease accounted for all but one death in our series, only one of five cardiovascular deaths occurred late, whereas four of five occurred within the first month after transplantation. Cardiovascular death occurred irrespective of obese or nonobese status.

Our study further distinguishes itself from others in that it is the only series in the literature that examines the effect of obesity on either kidney transplant or SPK outcome in which all patients received TAC and MMF for maintenance immunosuppression. The recent widespread use of TAC and MMF in SPK has resulted in superior graft survival compared to patients treated with cyclosporine-based immunosuppression.[19] Cyclosporine levels have previously been shown to be comparable between mildly obese and nonobese SPK recipients.[10] However, because the pharmacokinetic profiles of TAC and MMF differ significantly from those of cyclosporine and azathioprine, the potential for attainment of different drug concentrations in nonobese and obese patients and the possible consequences of this on kidney and pancreas graft survival are worthy of consideration. In addition to any effect on graft survival, it is also important to determine whether the use of contemporary immunosuppression results in any other previously undescribed differences in SPK outcome between nonobese and obese patients. Although we did not demonstrate any significant differences in TAC levels between nonobese and obese patients during the first post-transplant year, the potential influence of obesity on MMF absorption and metabolism was not readily apparent because serum MMF levels are not routinely measured in clinical practice.

Despite the use of TAC and MMF immunosuppression in this series, it's noteworthy that immunologic causes still accounted for approximately 35% of all graft losses in both obese and nonobese patients. The potential graft survival advantage afforded by TAC/MMF immunosuppression may be partially offset by the fact that 33% of our SPK recipients were African-American, in comparison to 4% in Bumgardner's series and 10% in the International Pancreas Transplant Registry.[20] African-American ethnicity is a well-known risk factor for increased incidence of rejection and decreased graft survival.[21–23] However, because ethnicity was evenly distributed between obese and nonobese patients (nonobese 35% African-American vs. obese 30% African-American), nonobese and obese kidney and pancreas graft survival remained similar.

The only significant difference noted between obese and nonobese patients in our series was a higher incidence of duodenojejunal anastomotic leaks in the obese group. Multivariate analysis demonstrated that mild obesity was the only variable that was independently associated with this complication. The reasons for this are unclear, particularly since the duodenojejunostomy tends to be the technically easiest part of the SPK procedure. It is possible that differential absorption or metabolism of MMF, which has been shown to interfere with the healing of intestinal anastomoses in an animal model,[24] may account for the observed difference in the leakage rate between obese and nonobese patients. Further study of the absorption, distribution, and enterohepatic recirculation of MMF in obese and nonobese SPK recipients is needed to support or refute this hypothesis. This finding was not seen in Bumgardner's study; however, these investigators did not specify use of either bladder or enteric drainage of exocrine secretions. It is notable that the patients they reviewed underwent transplantation between 1988 and 1994, a period of time when most centers were still using primarily bladder drainage.

In summary, we have demonstrated similar long-term patient, kidney graft, and pancreas graft survival between mildly obese and nonobese SPK recipients with systemic-enteric drainage in the TAC/MMF era. The rates of kidney and pancreas rejection and infective episodes were also comparable between groups. The only significant difference observed between nonobese and obese patients was an unexplained higher rate of duodenojejunal anastomotic leakage in the obese group. A heightened awareness of the increased potential for this complication in SPK recipients with a BMI $\geqslant 25$ receiving TAC/MMF immunosuppression should allow for earlier diagnosis and intervention, thereby minimizing associated morbidity and mortality.

Our study is somewhat limited by the small number of patients, by a relatively low BMI cutoff defining obesity, and by its retrospective design. Further prospective investigation of SPK recipients receiving TAC/MMF immunosuppression and undergoing enteric exocrine drainage is warranted to more clearly define the effect of mild obesity on the long-term outcome of SPK with contemporary immunosuppression and surgical techniques.

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