Utilization of Cholecystectomy—A Prospective Outcome Analysis in 1325 Patients

L. William Traverso, M.D., Rocelyn Lonborg, Ph.D., Karen Pettingell, Ph.D., L. Frederick Fenster, M.D.

The advent of laparoscopic techniques has resulted in an increased incidence of cholecystectomy, creating a need to reevaluate utilization. The new outcomes research movement emphasizes patient-derived data as well as traditional clinical outcomes. One of the purposes of this prospective study was to seek possible correlations between a variety of variables, both patient- and physician-derived, to the patient's degree of overall satisfaction with the outcome. From July 1992 to May 1997, five different data collection forms were prospectively implemented—three physician-derived (preoperative, intraoperative, and postoperative) and two patient-derived sets of data. In the postoperative patient instrument, patients were asked to rate their degree of satisfaction with the outcome of their surgery on a scale of 1 to 5, with 5 being "extremely satisfied" and 1 being "not at all satisfied." We then sought differences between those patients rating their satisfaction as 5 vs. those rating their satisfaction as 1 to 3. Age, sex, and the presence of comorbid conditions did not correlate with eventual satisfaction. The following were correlated with a statistically significant better degree of satisfaction: the preoperative presence of known gallstones or a preoperative physician-derived history of typical biliary pain. No preoperative patient-derived data were associated with satisfaction; however, the postoperative presence of abdominal pain predicted dissatisfaction. Not surprisingly, continued problems with abdominal pain strongly correlated with dissatisfaction, but this finding supports the accuracy of our assessment instrument. Furthermore, the more typical and clear-cut the clinical presentation, the greater the patient satisfaction with the outcome of cholecystectomy. Satisfaction and pain relief are strongly associated. In patients with pain preoperatively, measurement of either pain relief or satisfaction may be adequate to assess correct utilization of this operative procedure. (J GASTROINTEST SURG 2000;4:1-5.)

KEY WORDS: Cholecystectomy, outcome analysis, utilization, history, economics, adverse effects, gallbladder

The outcomes research movement has emphasized that "patient-derived" data should be evaluated as well as "physician-derived" data. Does this trend for obtaining patient-derived data provide useful information to assess the proper utilization of an operative procedure? Cholecystectomy provides surgeons an opportunity to assess one of their most common procedures. One of the most desirable outcomes is patient satisfaction. Can patient satisfaction (or lack thereof) after cholecystectomy be predicted by any preoperative finding? How do patient-derived or physician-derived findings compare in terms of their usefulness in predicting patient satisfaction? What are the factors that make for a satisfied patient? The answers to these questions will help to simplify the potentially vast amount of data that could be recorded. To make outcome assessment successful, the evaluation methodology must not be time consuming or expensive.

METHODS

Between July 1, 1992, and May 9, 1997, a total of 1545 elective cholecystectomies were performed at our institution. Up until July 1994, we had excluded patients who were scheduled to undergo an open

From the Department of Surgery (L.W.T.) and the Office of Value Assessment (R.L., K.P., and L.F.F.), Virginia Mason Medical Center, Seattle, Wash.

Presented at the Thirty-Ninth Annual Meeting of The Society for Surgery of the Alimentary Tract, New Orleans, La., May 17-20, 1998.

Reprint requests: L. William Traverso, M.D., Section of General Surgery, Virginia Mason Medical Center, 1100 Ninth Ave., P.O. Box 900 (C6-GSUR), Seattle, WA 98111.

cholecystectomy but included those converted from a planned laparoscopic to an open procedure. However, after July 1994, all patients undergoing cholecystectomy, either open or laparoscopic, were included. Therefore 1325 elective cholecystectomies were entered into the database. These procedures were performed mainly by five general surgeons (84% of the cholecystectomies) assisted by a surgical resident. During this period, 15% were planned as an open procedure.

Prior to July 1, 1992, we performed a retrospective analysis of 50 consecutive cholecystectomies to design five different data forms—three physician-derived sets of data completed at the preoperative, intraoperative (at completion of the procedure), and follow-up office visit up to 3 weeks postoperatively. In addition, two patient questionnaires were administered preoperatively (without assistance from medical personnel) and 3 months postoperatively (via mail).

We have made periodic revisions of some of the data elements or questions asked in an effort to determine those items that best predict good outcomes. Because of these revisions and the fact that some questions on each form were left unanswered, the number of responses was inconsistent. Surgeons seemed more likely to answer a question if the response was positive. Therefore, throughout this report, emphasis should be focused on percentages. The three physi-

Table I. *Physician-derived data*—Prospectively acquired potential clinical predictor variables*

Potential predictors of satisfaction	No. of patients with predictor	No. of patients with data	Frequency (%)
Preoperative sheet			
Age ≥50 yr	488	965	51
Female	709	953	74
Tests show gallstones	832	996	84
Colicky pain in upper abdomen	836	995	84
Colicky pain not in upper abdomen	94	1000	9
Noncolicky steady abdominal pain	134	999	13
History of gallstone pancreatitis	67	995	7
Previous upper abdominal surgery	157	995	16
Diabetes mellitus	57	996	7
Heart disease	157	996	16
Chronic obstructive pulmonary disease	28	993	3
Corticosteroid use	17	996	2
Chronic liver disease	12	996	1
Chronic renal disease	9	996	1
Alcoholism	18	996	2
Intraoperative sheet			
Stones found in gallbladder	920	1068	86
Bile leak (liver, cystic, or bile duct)	21	1063	2
Common bile duct damage	4	1068	0.4
Bleeding	15	1068	1.4
Converted	87	1068	8
Intraoperative cholangiogram positive in 1067 patients undergoing intraoperative cholangiography	105	1067	10
Postoperative sheet			
Complication since surgery	61	1002	6
Bile leak	8	1002	0.8
Damage to bile duct	4	1002	0.4
Pancreatitis	2	1002	0.2
Stones in gallbladder according to pathology report	738	1002	74
ERCP required	51	883	6
Resumed activity at 1 wk	647	862	75

ERCP = endoscopic retrograde cholangiopancreatography.
*Listed are the frequency (number positive/number patients with data).

cian-derived data collection forms were designed to be part of the permanent record and were approved by the records committee of our medical center. It was hoped that this would avoid duplication of record keeping by the surgeons and hence improve accuracy and completeness.

Frequencies were calculated for potential predictors of satisfaction using all of the physician- and patient-derived data forms. Satisfaction was reported to us directly from the patients through the mailed 3-month postoperative questionnaire. Three months was chosen for practical reasons—we wanted to achieve an acceptable response rate, which tends to decline with greater time lapses, and we wanted to solicit perceptions about the care while the experience was relatively fresh in the patient's mind. We achieved a 52% response rate (n = 691) at the 3-month time period without introducing the bias of follow-up calls or reminders. We felt that the "placebo response" generally was dissipated by 3 months after a one-time surgical intervention.[1,2]

Patients were asked to rank their satisfaction on a scale of 1 to 5, with 5 being "extremely satisfied" and one being "not at all satisfied." After an initial calculation, approximately 7 out of 10 rated their satisfaction as 5, whereas less than 10% rated their satisfaction as 1 to 3. We then sought differences between the extremes of those patients ranking their satisfaction as highest (5) vs. those ranking their satisfaction as lowest (1 to 3). Therefore, in the remainder of this report, patients were considered "satisfaction high" if they rated their experience a 5 and "satisfaction low" if they responded 1 to 3.

The frequency of potential predictors of satisfaction was then compared to satisfaction (yes or no) using a univariate comparison of two proportions to yield a Z statistic, that is, the frequency of those having or not having a predictor that was with high or low satisfaction.

RESULTS

The first two tables show the observed frequencies of potential predictors and the number of patients involved in these calculations for physician-derived data (preoperative, intraoperative, and postoperative) in Table I and for patient-derived data (preoperative and postoperative) in Table II. After statistical analysis, only the predictors that were shown to have a statistically significant relationship to satisfaction are listed in Table III for physician-derived data and in Table IV for patient-derived data.

Table II. *Patient-derived data*—Prospectively acquired potential clinical predictor variables*

Potential predictors of patient-derived satisfaction	No. of patients with predictor	No. of patients with data	Frequency (%)
Preoperative sheet			
Severe upper abdominal pain	801	880	91
Severe pain at other locations	374	831	45
Severe bloating, etc.	79	94	84
Postoperative sheet (patient) (outcome events)			
Satisfaction†			
High (level 5)	482	691	70
Low (level 1-3)	61	691	9
Pain relief			
In those 437 who said they had colicky upper abdominal pain preoperatively and answered postoperative questionnaire	345	437	79
In those 185 who said they had non–upper abdominal colicky pain preoperatively and answered postoperative questionnaire	146	185	79
In those 483 where doctor thought they had colicky upper abdominal pain preoperatively and patient answered postoperative questionnaire	392	483	81

*Listed are the frequency (number positive/number patients with data).
†Satisfaction ratings of 4 were not evaluated, so frequencies in this table do not equal 100%.

Table III. *Physician-derived data*—Only those predictor variables from Tables I and II that were found to be *significantly* ($P < 0.05$) associated with satisfaction are listed

Predictor variable	Predictor frequency	With predictor satisfied	Without predictor satisfied	*P* value	Z statistic
Preoperative sheet					
Chronic obstructive pulmonary disease	12/445 (2.6%)	7/12 (58%)	388/433 (90%)	0.002	3.044
Tests showed gallstones	366/445 (82%)	333/366 (91%)	62/79 (78%)	0.002	3.113
Symptoms were thought to be colicky upper abdominal pain	377/445 (85%)	343/377 (91%)	52/68 (76%)	<0.001	3.389
Symptoms were thought to be steady noncolicky abdominal pain	54/443 (12%)	40/54 (74%)	353/389 (91%)	<0.001	3.499
Intraoperative sheet					
Well-defined stones found in gallbladder at operation	407/478 (85%)	371/407 (91%)	54/71 (76%)	0.001	3.74
Postoperative sheet					
Complication since surgery	24/452 (5.3%)	18/24 (75%)	385/428 (90%)	0.022	2.29

Table IV. *Patient-derived data*—Only those predictor variables from Tables I and II that were found to be *significantly* ($P < 0.05$) associated with satisfaction are listed

Predictor variable	Predictor frequency	With predictor satisfied	Without predictor satisfied	*P* value	Z statistic
Preoperative sheet					
None significant					
Postoperative sheet					
Pain relief (in those who said preoperatively they had colicky upper abdominal pain)	280/337 (83%)	268/280 (96%)	32/57 (56%)	<0.001	8.64
Pain relief (in those who said preoperatively they had colicky non–upper abdominal pain)	116/138 (84%)	109/116 (94%)	10/22 (45%)	<0.001	5.77
Pain relief (in those where physician said colicky upper abdominal pain)	323/377 (86%)	309/323 (96%)	34/54 (63%)	<0.001	7.69

DISCUSSION

This prospective analysis suggests that elective cholecystectomy will be most successful in providing ultimate patient satisfaction if the surgeon determines preoperatively that two items are present—documented gallstones and a history of biliary-type pain. However, a majority of patients without these features were also satisfied (see Table III).

Our data collection instruments showed that satisfaction is strongly associated with the absence of postoperative complications. Although this finding was not surprising, it did verify the data collection methodology, which was not sensitive enough to predict lack of satisfaction in those few patients with the specific complication of bile duct injury. Of interest was the fact that satisfaction was still possible even in the 8% of patients who were converted to an open procedure. When patients perceived that their biliary pain was relieved after cholecystectomy, this finding was strongly associated with satisfaction. A caveat is warranted here when relying on subjective information from a patient asked about satisfaction linked to pain relief. Can a placebo response exist? We tried to balance memory with a placebo response to make our data less susceptible to an inaccurate response. The classic study by Fish et al.[2] found that the placebo response of internal mammary artery ligation to treat angina pectoris was strong but had disappeared by 60 days after surgery. We waited 90 days to ensure the placebo effect had dissipated but that the patient's perioperative memory to evaluate satisfaction had not lapsed.

Also, can the 52% response rate of patients introduce bias into this study? Perhaps the disgruntled pa-

tient would not spontaneously return the questionnaire. We did not provide reminders for two reasons. First, over the years of this study, it was not possible to check on all 1325 patients to ensure a response at the time they received the questionnaire and second, a response actively acquired may have biased the spontaneously obtained data.

Surprisingly, none of the preoperative patient-derived predictors listed in Table II were observed to be associated with postoperative satisfaction. Our methodology may help to explain this finding. The patient's preoperative data collection instrument was closely correlated with the questions on the preoperative physician's instrument. However, patients answered the questions on their forms just prior to seeing a physician. The physician instrument was completed with the surgeon asking the questions. This suggests that the physician-aided interpretation of the clinical history was best to decide whether surgery would predict pain relief and provide satisfaction. Even though the questions were written for patients in the most general manner, the patient's interpretation may have been affected by a person's emotions more than medical knowledge.

This prospective collection of data does strongly associate pain relief with satisfaction, but in our continued effort to provide an adequate assessment of our utilization of cholecystectomy, perhaps we could decrease the complexity of the multiple questionnaires and save time and effort by ensuring that the physican-derived predictors of satisfaction were present prior to cholecystectomy.

Ellwood[2] yearned for a "national database" of outcomes from which we could assess the effectiveness of care rather than using only the very expensive randomized, controlled trials of highly selected populations in academic centers. This was the beginning of the "science of patient information" as the outcomes research movement has subsequently used the increasingly available large computerized administrative databases as well as an increasing volume of sophisticated and validated questionnaires. The latter have emphasized patient-derived data about their outcomes to a much greater degree than in the past, including "health-related quality-of-life" instruments. Although some have questioned the value of this observational type of outcomes research, many believe it has produced useful information and has served to focus efforts at establishing causal relationships and to focus on quality improvement efforts.

The results of our study emphasize the importance of information derived from the patient-doctor relationship. The outcome of patient satisfaction can be adequately predicted by a good clinical history and an understanding of the natural history of gallstone disease. If the patient has biliary pain as defined at the National Institutes of Health Consensus Conference on Gallstones[4] and gallstones are documented preoperatively, then patient satisfaction will be high because the patient's perceived pain relief will also be high. The clinical history derived through a patient-doctor relationship is key and not from a database detached from this relationship. In previous reports from our institution,[5-7] we found that a clinical history of biliary pain in patients with or without gallstones was better than any laboratory test for predicting pain relief, for example, biliary scintigraphy. In addition, we have found that a clinical history of common bile duct stones (jaundice, acholic stools, dark urine) was better than any serum liver function test or ultrasound finding at predicting the presence of stones in the common bile duct at the time of cholecystectomy.[8] The clinical history is an important element in utilizing cholecystectomy because it is derived by a doctor who is cognizant of the natural history of the disease and has discussed the problem with the patient.

Outcomes provide a performance evaluation of our surgical procedures. Side benefits are the benchmarks for comparison (as in Table I) that then allow other surgeons to rank their patients and their performance. Hopefully a simple repository of outcome information can be accumulated based on just the necessary information to predict pain relief (and therefore satisfaction) after cholecystectomy. This simple system would not require elaborate health forms to be completed by the patient or the surgeon. The system could then allow surgeons to participate anonymously and gain insight into their results while comparing them with those from other geographic areas or hospital types.

REFERENCES

1. Beecher HK. Surgery as placebo. A quantitative study of bias. JAMA 1961;176:1102-1107.
2. Fish RG, Crymes TP, Lovell MG. Internal mammary artery ligation for angina pectoris. Its failure to produce relief. N Engl J Med 1958;259:418-420.
3. Ellwood PM. Shattuck lecture—Outcomes management. A technology of patient experience. N Engl J Med 1988;318: 1549-1556.
4. Traverso LW. Clinical manifestation and impact of gallstone disease. Am J Surg 1993;165:405-409.
5. Gilliland TM, Traverso LW. Cholecystectomy: Modern standards for comparison to alternative treatments for symptomatic cholelithiasis with emphasis on long-term symptom relief. Surg Gynecol Obstet 1990;170:39-44.
6. Gilliland TM, Traverso LW. Cholecystectomy provides long-term symptom relief in patients with acalculous gallbladders. Am J Surg 1990;159:489-492.
7. Fenster LF, Thirlby RC, Lonborg R, Traverso LW. What symptoms are cured by cholecystectomy? Am J Surg 1995;169:533-538.
8. Koo KP, Traverso LW. Do preoperative indicators predict the presence of common bile duct stones during laparoscopic cholecystectomy? Am J Surg 1996;171:495-499.

Common Bile Duct Stone Characteristics: Correlation With Treatment Choice During Laparoscopic Cholecystectomy

Robert A. Duensing, M.D., Russell A. Williams, M.D., John Craig Collins, M.D., Samuel E. Wilson, M.D.

Determining the most appropriate management approach for patients with unsuspected choledocholithiasis may be difficult because of the subjective nature of this decision in the absence of clinical data. Treatment of incidental choledocholithiasis during laparoscopic cholecystectomy was reviewed during a 25-month period. Operative cholangiograms were analyzed retrospectively to determine if associations exist between common bile duct stone characteristics and the intraoperative treatment selected by the operating surgeon. Cholangiographic data included quantification of common bile duct stones, stone dimension, position, and presence of radiopaque contrast flow into the duodenum. Two hundred thirty-six laparoscopic cholecystectomy patients underwent operative cholangiography; 25 (11%) demonstrated choledocholithiasis. Seven patients were converted to open common bile duct exploration (group I), 16 patients were referred for postoperative endoscopic retrograde cholangiopancreatography (group II), and two patients were observed (group III). Evaluation of the operative cholangiograms revealed multiple common bile duct stones (>1) in 86% (6 of 7) in group I, 25% (4 of 16) in group II, and none in group III. All patients in group I had at least one stone larger than 5 ml in greatest diameter, whereas only 33% (6 of 18) in groups II and III combined had stones larger than 5 ml. Group I had significantly ($P = 0.027$) more representation of delayed or no contrast flow during operative cholangiography compared to groups II and III. The intraoperative decision to proceed with laparoscopic cholecystectomy and rely on postoperative endoscopic retrograde cholangiopancreatography for stone retrieval rather than open common bile duct exploration was associated with (1) a single common bile duct stone, less than or equal to 5 ml in size on operative cholangiogram and (2) normal contrast flow into the duodenum. Open common bile duct exploration was more frequently associated with the demonstration of multiple or large (>5 ml) stones. A periampullary stone did not discriminate among treatment choices. (J GASTROINTEST SURG 2000;4:6-12.)

KEY WORDS: Bile duct stones, management, characteristics, decisions

The treatment of choledocholithiasis in patients who require laparoscopic cholecystectomy is controversial. The core of the debate centers on the timing of endoscopic stone retrieval with respect to laparoscopic cholecystectomy. Several prospective and retrospective studies support either selective preoperative endoscopic retrograde cholangiopancreatography (ERCP) with or without endoscopic sphincterotomy (ES) or intraoperative cholangiography followed by postoperative endoscopic stone retrieval as needed.[1-7] The management spectrum is further expanded by the advent of various laparoscopic common bile duct (CBD) exploration techniques.

Faced with an array of therapeutic options, it is still the surgeon who must decide in each case which CBD stones are most amenable to postoperative transsphincteric endoscopic retrieval, and which are better managed by immediate open or laparoscopic CBD exploration. Most laparoscopic surgeons recognize that large and/or multiple CBD stones, or those that ap-

From the Department of Surgery, University of California, Irvine Medical Center, Orange, Calif.; and the Surgical Service, Long Beach Veterans Affairs Medical Center, Long Beach, Calif.

Presented at the Annual Scientific Meeting of the Southern California Chapter of the American College of Surgeons, Santa Barbara, Calif., January 19-21, 1996.

Reprint requests: Russell A. Williams, M.D., Professor of Surgery, UCI Medical Center, 101 The City Drive South, Orange, CA 92868.

pear impacted in the distal CBD on intraoperative cholangiogram, may be difficult if not impossible to retrieve at ERCP, even with ES. The decision to perform CBD exploration or arrange for postoperative ERCP is an example of complex surgical decision making. Because of a paucity of objective data from which to construct meaningful guidelines, often the decision is based on subjective impressions. In an effort to facilitate the selection of the most appropriate treatment choice for incidental CBD stones encountered during laparoscopic cholecystectomy, we performed this retrospective analysis of associations among CBD stone characteristics, intraoperative surgical therapeutic decisions, and outcomes.

MATERIAL AND METHODS

The study population comprised 404 consecutive patients scheduled for laparoscopic cholecystectomy from June 1, 1992, through June 30, 1994, within the integrated surgical residency program of the University of California (UC), Irvine. Patients were treated at UC Irvine Medical Center or Long Beach Veterans Affairs Medical Center. All patients considered for laparoscopic cholecystectomy were diagnosed with either acute or chronic cholecystitis or symptomatic biliary hyperamylasemia. Clinical evidence to support the diagnoses was based on a history of biliary colic or acute upper abdominal pain and appropriate physical findings, leukocytosis, elevated serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, amylase, or lipase. Radiographic evidence included cholelithiasis, gallbladder wall thickening, pericholecystic fluid, or biliary sludge on ultrasonography.

Preoperative suspicion of significant choledocholithiasis was based on strong clinical evidence, including history and physical examination, laboratory parameters, and transabdominal ultrasound. Biliary scintiscanning was used rarely. The decision to obtain selective preoperative ERCP or proceed with laparoscopic cholecystectomy was based on the judgment of the individual surgeon. Although variations in management for each patient were not controlled, the indications leading to the decision to perform open CBD exploration versus postoperative ERCP were relatively uniform within our small group of surgeons. Sphincterotomy (ES) was carried out as needed to retrieve stones from the CBD. Preoperative ERCP was reserved for patients with clinical and sonographic evidence of choledocholithiasis supported by elevated bilirubin or alkaline phosphatase levels. Specifically, patients were selected for preoperative ERCP if increased values of at least two of the following parameters were present in conjunction with ultrasonographic presence of cholelithiasis and upper abdominal tenderness: conjugated bilirubin, alkaline phosphatase, dilated CBD or hypoechoic shadow within the CBD on ultrasound examination, or obstructed CBD on biliary scintigraphy. Self-limited biliary hyperamylasemia was not considered an indication for ERCP. Patients who required preoperative ERCP were excluded from this review.

Routine intraoperative cholangiography was performed in the majority of our patients. Intraoperative cholangiograms were obtained by manual injection of half-strength water-soluble radiopaque contrast medium recorded by one or more static supine portable radiographs. All films were interpreted intraoperatively by the operating surgeon. Patients whose cholangiograms were consistent with choledocholithiasis were either referred for postoperative ERCP and stone retrieval after completed laparoscopic cholecystectomy, converted to laparotomy with open CBD exploration, or, rarely, observed in an outpatient setting after laparoscopic cholecystectomy, based on the judgment of the surgeon. Laparoscopic CBD exploration was not performed in any patient in this series.

A retrospective analysis of the intraoperative cholangiograms in those patients with evidence of choledocholithiasis during laparoscopic cholecystectomy was performed. Measurements included maximal stone diameter by caliper in a single-plane, quantification of radiolucencies considered to represent stones, and classification of stone location in the CBD as proximal third, middle third, distal third, or periampullary. In addition, patency of the ampulla of Vater was estimated based on whether the manually injected contrast medium had immediate, delayed, or no flow through the papilla into the duodenum. Because the focus of this study was on CBD stone characteristics, those patients who did not undergo operative cholangiography during laparoscopic cholecystectomy were not included in this review.

Comparisons between groups were made by means of Fisher's exact test, Kruskal-Wallis test, and Wilcoxon rank-sum test for pairwise comparison (normal and centered) where appropriate. Statistical analysis was performed by a UC Irvine professor of mathematics specializing in statistics.

RESULTS

Over 25 months, 404 patients were diagnosed with acute or chronic cholecystitis, or biliary hyperamylasemia, at the two institutions. Of these, 47 patients (9%) were suspected of having choledocholithiasis preoperatively and were referred for ERCP; these patients are not considered in this review. The remaining

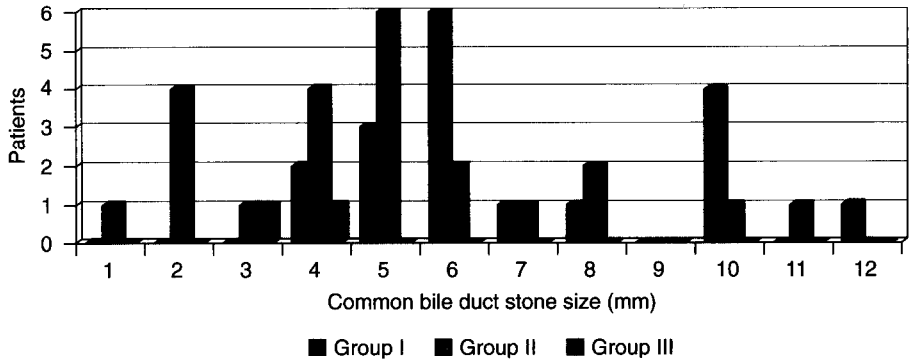


Fig. 1. Relationship between common bile duct (CBD) stone size and number of patients in each treatment group shows the trend toward larger stones in patients undergoing open CBD exploration (P = 0.003). Group I = open CBD exploration (n = 7 patients, 18 CBD stones); group II = postlaparoscopic cholecystectomy endoscopic stone retrieval (n = 16 patients, 23 CBD stones); group III = laparoscopic cholecystectomy and observation (n = 2 patients, 2 CBD stones).

357 patients were not suspected of harboring CBD stones and were scheduled for laparoscopic cholecystectomy. Intraoperative cholangiography was performed in 236 patients (63%), 25 of whom (11%) were shown to have choledocholithiasis. A separate group of 36 patients were converted to laparotomy for standard cholecystectomy because of severe inflammation, fibrosis, gangrene, or other technical reasons that made laparoscopy unsafe in the surgeon's judgment.

Based on cholangiographic evidence of choledocholithiasis, 7 (28%) of 25 patients were converted to open CBD exploration (group I), 16 patients (64%) were referred for postoperative ERCP after laparoscopic cholecystectomy (group II), and two patients (8%) were observed as outpatients after completed laparoscopic cholecystectomy in anticipation of spontaneous stone passage. All open CBD explorations and laparoscopic cholecystectomies were successfully completed. Morbidity occurred in one patient in group I because of intraoperative bleeding after conversion; groups II and III had no morbidity. Operative mortality (30-day) was zero for all groups. Seven (44%) of the 16 patients in group II demonstrated choledocholithiasis on postoperative ERCP; all stones were successfully retrieved endoscopically. CBD stones were not found on postoperative ERCP in nine patients (56%) in group II. This implies either misinterpretation at the time of operative cholangiography or spontaneous stone passage before follow-up ERCP. All nine patients in group II remained asymptomatic without biliary colic for 13 months after ERCP. One patient in group I presented 3 weeks after open cholecystectomy with abdominal pain and nausea, and was subsequently discovered to have choledocholithiasis on ultrasound examination. A single 4 ml stone was retrieved endoscopically, and the patient remained without symptoms at 1 year. CBD stones were not documented on subsequent follow-up for the remaining patients in group I and all of patients in group II patients (mean 16 months and 13 months, respectively). Both patients in group III remain asymptomatic after 6 months' follow-up, and neither has required additional procedures. Further follow-up of the two patients in group III was not possible because of inability to locate the patients at the time of this review.

Retrospective analysis of the intraoperative cholangiograms obtained from patients in groups I, II, and III revealed the following CBD stone characteristics: multiple (>1) CBD stones were present in six (86%) of seven patients in group 1, 4 (25%) of 16 in group II, and in neither patient in group III (group I vs. group II, P = 0.01; group I vs. group III, P = 0.008). All patients in group I had at least one stone more than 5 mm in greatest diameter, with a mean diameter of 7.0 mm. Out of all of the 18 CBD stones found in group I, 13 (72%) were larger than 5 mm. Six (38%) of 16 patients in group II had stones larger than 5 mm with a mean diameter of 4.9 mm. Of the total of 23 CBD stones in group II, seven (30%) measured larger than 5 mm (group I vs. II, P <0.009). The two patients in group III had stone sizes of 4 and 3 ml (Fig. 1). In group I, four (57%) of seven patients had a stone in the distal CBD, adjacent to the ampulla of Vater, whereas 10 (63%) of the 16 patients in group II had periampullary stones (P >0.1). Considering all stones, six (33%) of the total of 18 and 9 (39%) of the 23 CBD stones found in groups I and II, respectively, were periampullary. Locations for the solitary stones in the two patients in group III were the middle and distal CBD (Table I). Contrast flow through the biliary ductal system into the duodenum during intraoperative cholangiography was delayed in four and

Table I. Treatment groups and common bile duct stone characteristics on operative cholangiogram

Cases by treatment group	Largest CBD stone (mm)*	Number of CBD stones†	Periampullary stone (yes/no)‡	Greatest CBD diameter (mm)
Group I	Average = 7.0			Average = 10.6
1	6	2	No	7
2	8	5	Yes	18
3	10	4	Yes	9
4	10	1	Yes	9
5	10	2	Yes	14
6	10	2	No	9
7	12	2	No	8
Group II	Average = 4.9			Average = 10.3
1	4	1	No	8
2	5	1	Yes	11
3	2	3	Yes	12
4	10	1	No	9
5	10	2	No	17
6	6	3	Yes	8
7	8	1	Yes	10
8	4	1	Yes	8
9	4	1	No	7
10	5	1	Yes	14
11	7	1	Yes	12
12	6	1	Yes	13
13	5	1	No	9
14	3	3	No	10
15	4	1	No	10
16	5	1	No	6
Group III	Average = 3.5			Average = 6.5
1	4	1	No	7
2	3	1	No	6

Group I = open common bile duct exploration; group II = LC and postoperative ERCP and endoscopic sphincterotomy; group III = LC and observation.
LC = laparoscopic cholecystectomy; CBD = common bile duct.
*Group I vs. group II, $P < 0.003$; group I vs. group III, $P = 0.02$.
†Group I vs. group II, $P = 0.009$; group I vs. group III, $P = 0.03$.
‡Group I vs. group II, $P = 0.045$, group I vs. group III, $P = 0.008$.

absent in two patients in group I. Contrast flow was delayed in one and absent in five patients in group II. Both patients in group III had normal contrast flow into the duodenum. Last, the mean CBD diameter as measured on intraoperative cholangiography was 10.6 mm (range 7 to 18 mm) in group I, 10.3 mm (range 6 to 17 mm) in group II (group I vs. group II, P = significant), and less than 8 mm in group III (see Table I).

DISCUSSION

Before the development of laparoscopic techniques, management decisions for the treatment of choledocholithiasis were relatively straightforward; patients in whom CBD stones were suspected during cholecystectomy underwent intraoperative cholangiography followed by open CBD exploration if stones were present. The management of choledocholithiasis discovered intraoperatively in patients undergoing laparoscopic cholecystectomy, however, can be complex. The combined endoscopic and laparoscopic approach to choledocholithiasis has provided surgeons and endoscopists with additional tools to more efficiently treat their patients. Currently, treatment options include conversion to laparotomy and open CBD exploration, completion of laparoscopic cholecystectomy with stone retrieval by postoperative ERCP/ES, laparoscopic CBD exploration, or observation anticipating spontaneous passage after laparo-

scopic cholecystectomy in selected cases. Each of these techniques may be effective in clearing CBD stones. Choosing the most appropriate method can be difficult and is largely based on the surgeon's experience, operative skill, and the local availability of necessary technology and endoscopic expertise.

The success of laparoscopic CBD exploration and stone retrieval has been reported to range from 44% to more than 80%.[8-11] This technique, however, has not yet achieved general popularity, most likely because of the requirements for specific equipment and the demand for technical expertise. Morbidity for laparoscopic CBD exploration has been reported to range from 0%[9] to 23%,[10] and it may add 1.5 to 3.5 hours of procedure time.[12,13] In addition, both transcystic and direct laparoscopic CBD intubation carry a small risk of ductal injury. CBD stones may be removed transcystically during laparoscopy using glucagon and/or irrigation techniques. Because of incomplete recording techniques, an accurate assessment of the success rate in this review is not possible. Nevertheless, technology and training in laparoscopic stone retrieval continue to develop, and some biliary surgeons believe it to be an excellent tool for intraoperative CBD stone retrieval that will place less reliance on perioperative ERCP.

Another approach to choledocholithiasis found at laparoscopic cholecystectomy is intraoperative ERCP/ES, but this requires readily available ERCP equipment and expertise, and its efficacy, morbidity, and mortality have not been sufficiently determined.[14,15] Other techniques used to clear CBD stones include catheter saline flushing during laparoscopic cholecystectomy and what has been referred to as the "rendezvous" technique.[12] The rendezvous technique involves placement of a double-lumen transcystic catheter through the ampulla of Vater during intraoperative cholangiography. The catheter is left in place and will allow serial postoperative cholangiograms and facilitate successful endoscopic catheterization if postoperative ERCP is needed. So far, these alternative techniques are infrequently used, and until laparoscopic CBD exploration becomes more universal, most surgeons must decide whether to convert to open CBD exploration, refer the patient for postoperative ERCP, or occasionally observe postoperatively in anticipation of spontaneous stone passage.

Historically, open CBD exploration succeeds in clearing CBD stones in 89% to 97% of patients.[16] This compares to reports ranging from 66.6% to 100% successful ductal clearance by ERCP/ES in patients after laparoscopic cholecystectomy.[1] Although it may seem disappointing to convert a patient's laparoscopic procedure to an open laparotomy from laparoscopic cholecystectomy in order to perform an open CBD exploration, a recent review suggests that open CBD exploration may be safer in patients under the age of 60 years than endoscopic ductal manipulation with sphincterotomy.[17] However, these authors examined the results of preoperative ERCP/ES before open cholecystectomy and not all studies were controlled for age or comorbidity. At best, data regarding the morbidity and mortality of ES compared with open CBD exploration in young, healthy patients remain controversial. Moreover, no prospective studies to date specifically compare the efficacy and complications of open CBD exploration to that of postoperative ERCP/ES with respect to age stratification. Reports of ERCP/ES after laparoscopic cholecystectomy demonstrate an overall 96.8% stone retrieval success rate, minimal morbidity, and no mortality.[1]

There is still room for controversy regarding the risk/benefit ratio between open CBD exploration and ERCP/ES. Indications that will guide the surgeon's intraoperative decision and in turn result in optimal management in terms of efficacy, morbidity, and cost remain vague. Recommendations in the literature for conversion to open CBD exploration during laparoscopic cholecystectomy include cholangiographic evidence of "large" and/or "multiple" CBD stones, a small CBD with a large stone, a long tapered CBD distal to a large stone, an "impacted" or large periampullary stone, and the suspicion of other ductal abnormalities.[14,18,19] Other indications are failed laparoscopic CBD exploration or unavailable equipment, lack of specific endoscopic (ERCP) expertise, and CBD exploration incidental to laparotomy for other technical reasons when choledocholithiasis is suspected.[6] Prospective studies examining the dimensions and numbers of large or multiple stones found during laparoscopic cholecystectomy, and corresponding outcomes of treatment, are limited at the time of this report. With regard to stone characteristics in general, Stain et al.[19] found that when 20 or more stones are removed from the CBD, the presence of retained CBD stones is likely whether removal is accomplished endoscopically or by open technique. Spontaneous passage of biliary stones is a recognized phenomenon in acute pancreatitis,[20,21] and its occurrence with stones in the CBD is supported by the frequent absence of choledocholithiasis on postoperative ERCP after stones are noted on intraoperative cholangiogram.[2,12,22] Still, a recent review found no significant correlation between stone size or number and the probability of spontaneous passage.[2] In the present study, significant associations between surgeons' treatment decisions and CBD stone characteristics were found. Compared with a decision to proceed to open CBD exploration, the decision to com-

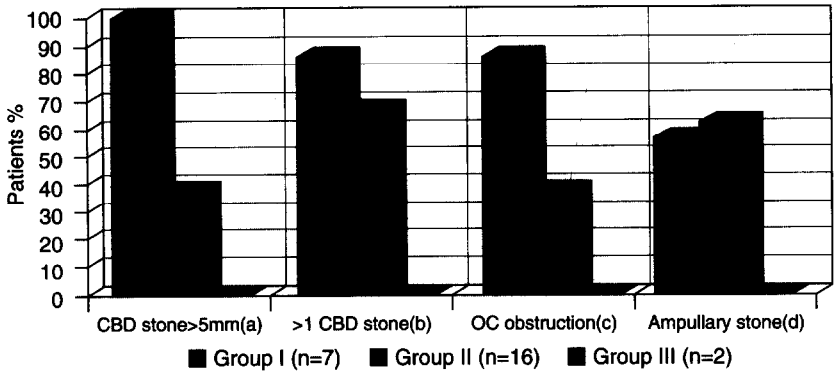


Fig. 2. Percentage of patients in each treatment group with common bile duct *(CBD)* stones larger than 5 mm *(>5 mm) (a)*, more than one *(>1)* CBD stone *(b)*, operative cholangiogram *(OC)* contrast flow obstruction *(c)*, and ampullary stone *(d)*. Group I = open CBD exploration; group II = postlaparoscopic cholecystectomy endoscopic stone retrieval; group III = laparoscopic cholecystectomy and observation. (a) = group I vs. group II, $P = 0.007$; group I vs. group III, $P = 0.028$; (b) = group I vs. group II, $P = 0.010$; group I vs. group III, $P = 0.008$; (c) = group I vs. group II, $P = 0.045$; group I vs. group III, $P = 0.008$; (d) = group I vs. group II, $P > 0.5$.

plete the laparoscopic cholecystectomy and rely on postoperative ERCP for stone retrieval occurred in a greater proportion of patients with a single CBD stone or in whom the largest stone was less than or equal to 5 mm in greatest dimension (Fig. 2). Additionally, patients in the group converted to laparotomy for open CBD exploration had a significantly greater number of stones as well as larger stones when compared to those selected for postlaparoscopic ERCP or observation (see Table I). All but one patient treated by open CBD exploration had both multiple CBD stones and at least one stone larger than 5 mm; the exception was a patient converted to open CBD exploration based on the finding of a single 10 mm stone, with obstruction suggested by delayed flow of contrast material through the ampulla of Vater. Patients who had normal contrast flow into the duodenum on intraoperative cholangiogram more frequently underwent postoperative ERCP for stone retrieval, or observation, than open CBD exploration. AT ERCP, stones were identified in only 44% of patients in whom operative cholangiograms were interpreted as demonstrating choledocholithiasis. Whether the remaining patients (56%) passed stones before ERCP was performed or the operative cholangiograms were misinterpreted cannot be determined with the materials available for this review. However, all of the patients remain without associated symptoms, which may lend support to reliance on postoperative ERCP in the questionable presence of a stone on cholangiogram. Although conventional practice may predict the contrary, the presence of a stone in the periampullary region did not discriminate among treatments, nor did the degree of dilation of the CBD (see Table I). The two patients treated by periodic outpatient visits after laparoscopic cholecystectomy each had a single, small (3 or 4 mm) CBD stone and free flow of contrast into the duodenum on laparoscopic cholecystectomy. Based on the asymptomatic nature of the choledocholithiasis and their cholangiographic characteristics, it was elected to observe these two patients in anticipation of spontaneous passage. With this option, postoperative ERCP remains an option should patients become symptomatic from persistent choledocholithiasis. Because of insufficient experience and small patient populations, further characterization of the natural history of expectant management is needed before it can be advocated generally for reliable patients with single, small CBD stones.

CONCLUSION

Formal treatment guidelines are best defined by a randomized prospective study. Still, the data obtained in our review of the actual management of suspected choledocholithiasis discovered at laparoscopic cholecystectomy demonstrate significant correlations between management decisions and characteristics of the stones as determined by laparoscopic cholecystectomy. In this series, decisions were validated by uniformly satisfactory outcomes. These observations are important because at present laparoscopic CBD exploration is not a treatment option for most surgeons practicing laparoscopic biliary surgery. Thus, in light of this and previous studies, it is appropriate to have

confidence in postoperative endoscopic stone retrieval techniques in the setting of a single, small CBD stone (5 mm or less in diameter) and normal cholangiographic flow of contrast medium, given a successful record of ductal clearance by the ERCP endoscopist. The decision to perform open CBD exploration in this study most frequently occurred in patients with multiple, large (>5 mm) stones and an obstructed common duct. This may be the best option for patients with these features on cholangiogram. However, multiple factors must be weighed in the decision to rely on follow-up ERCP, and the success of the local endoscopist is a significant component. Because of our focus on stone characteristics, other important factors that may influence ERCP success, such as biliary anatomy, were not addressed but should be considered. Until more objective data are obtained and laparoscopic CBD exploration becomes more widely practiced, the decision whether to perform open CBD exploration or postoperative ERCP for stone retrieval largely remains a matter of surgical judgment and should be considered individually.

REFERENCES

1. Graham SM, Flowers JL, Scott TR, Bailey RW, Scovill WA, Zucker KA, Imbembo AL. Laparoscopic cholecystectomy and common bile duct stones. The utility of planned perioperative endoscopic retrograde cholangiography and sphincterotomy: Experience with 63 patients. Ann Surg 1993;218: 61-67.
2. Hainsworth PJ, Rhodes M, Gompertz RHK, Armstrong CP, Lennard TWJ. Imaging of the common bile duct in patients undergoing laparoscopic cholecystectomy. Gut 1994;35:991-995.
3. Frazee RC, Roberts J, Symmonds R, Hendricks JC, Snyder S, Smith R, Custer MD, Stoltenberg P, Avots A. Combined laparoscopic and endoscopic management of cholelithiasis and choledocholithiasis. Am J Surg 1993;166:702-706.
4. Franceschi D, Brandt C, Margolin D, Szopa B, Ponsky J, Priebe P, Stellato T, Eckhauser ML. The management of common bile duct stones in patients undergoing laparoscopic cholecystectomy. Am Surg 1993;59:525-531.
5. Leitman IM, Fisher ML, McKinley MJ, Rothman R, Ward RJ, Reiner DS. The evaluation and management of known or suspected stones of the common bile duct in the era of minimal access surgery. Surg Gynecol Obstet 1993;176;527-533.
6. Miller RE, Kimmelstiel FM, Winkier WP. Management of common bile duct stones in the era of laparoscopic cholecystectomy. Am J Surg 1995;169:273-276.
7. Heinerman PM, Boeckl O, Pimpl W. Selective ERCP and preoperative stone removal in bile duct surgery. Ann Surg 1989;209:267-272.
8. Quattlebaum JK, Flanders HI. Laparoscopic treatment of common bile duct stones. Surg Laparosc Endosc 1991;1:26-32.
9. Petelin JB. Laparoscopic approach to common duct pathology. Surg Laparosc Endosc 1991;1:33-41.
10. Hunter JG. Laparoscopic transcystic common bile duct exploration. Am J Surg 1992;163:53-58.
11. Phillips EH, Carroll BJ, Pearlstein AR, Daykhovsky L, Fallow MJ. Laparoscopic choledochoscopy and extraction of common bile duct stones. World J Surg 1993;17:22-28.
12. Fitzgibbons RI, Ryberg AA, Ulualp KM, Nguyen NX, Litke BS, Camps J, McGinn TR, Jenkins JX, Filipi CJ. An alternative technique for treatment of choledocholithiasis found at laparoscopic cholecystectomy. Arch Surg 1995;130:638-642.
13. Fielding GA, O'Rourke NA. Laparoscopic common bile duct exploration. Aust N Z J Surg 1993;63:113-115.
14. Wilson TG, Jeans PL, Anthony A, Cox MR, Toouli J. Laparoscopic cholecystectomy and management of choledocholithiasis. Aust N Z J Surg 1993;63:443-445.
15. Deslanders E, Gagner M, Pomp A, Rheault M, Leduc R, Clermont R, Gratton J, Bernard EJ. Intraoperative endoscopic sphincterotomy for common bile duct stones after laparoscopic cholecystectomy. Gastrointest Endosc 1993;39:54-58.
16. Miller JS, Ferguson CM. Current management of choledocholithiasis, Am Surg 1990;56:66-70.
17. Phillips EH. Controversies in the management of common duct calculi. Surg Clin North Am 1994;74:931-948.
18. O'Rourke NA, Askew AR, Cowen AE, Roberts R, Fielding GA. The role of ERCP and endoscopic sphincterotomy in the era of laparoscopic cholecystectomy. Aust N Z J Surg 1993;63:3-7.
19. Stain SC, Cohen H, Tsuishoysha M, Donovan AJ. Choledocholithiasis. Endoscopic sphincterotomy or common bile duct exploration. Ann Surg 1991;213:627-633.
20. Acosta JM, Ledesma CL. Gallstone migration as a cause of acute pancreatitis. N Engl J Med 1974;290:484-487.
21. Kelly TR. Gallstone pancreatitis: Pathophysiology. Surgery 1976;80:488-492.
22. Duensing RA, Williams RA, Collins JC, Wilson SE. Managing choledocholithiasis in the laparoscopic era. Am J Surg 1995;170:619-623.

Villous Tumors of the Duodenum: Reappraisal of Local vs. Extended Resection

Michael B. Farnell, M.D., George H. Sakorafas, M.D., Michael G. Sarr, M.D., Charles M. Rowland, M.S., Gregory G. Tsiotos, M.D., David R. Farley, M.D., David M. Nagorney, M.D.

Benign villous tumors of the duodenum are often managed by transduodenal local excision. Risk of local recurrence, coupled with improving safety of radical pancreaticoduodenectomy, has prompted reexamination of the roles of conservative and radical operations. The aim of this study was to determine long-term outcome after local and extended resection in order to identify factors to consider in planning operative strategy. Eighty-six patients (mean age 64 years) with villous tumors of the duodenum managed surgically from 1980 to 1997 were reviewed. Histologic findings, size, presence of polyposis syndromes, and extent of resection were correlated with outcome. Villous tumors were benign adenomas in 64 patients (74%), contained carcinoma in situ in three (4%), and invasive carcinoma in 19 (22%). The presence of cancer was not known preoperatively in 9 (47%) of the 19 with invasive carcinoma. Operative treatment included transduodenal local excision in 53 patients, pancreaticoduodenectomy in 20, pancreas-sparing duodenectomy in five, full-thickness excision in four, and other in six. Among the 50 patients with benign tumors managed by local excision, 17 had a recurrence with actuarial rates of 32% at 5 years and 43% at 10 years; four of the recurrences (24%) were adenocarcinomas. The recurrence rate was influenced by the presence of a polyposis syndrome but not by tumor size. Recurrence of benign villous tumors after local excision is common and may be malignant. Pancreaticoduodenectomy is appropriate for villous tumors containing cancer and may be considered an alternative for select patients with benign villous tumors of the duodenum. If local excision is performed, regular postoperative endoscopic surveillance is mandatory. (J GASTROINTEST SURG 2000;4:13-23.)

KEY WORDS: Villous tumors, villous adenomas, periampullary neoplasms, villoglandular polyps, polyposis syndromes

Villous tumors of the duodenum (VTD) have been recognized with increasing frequency in recent years, perhaps because of the widespread use of upper gastrointestinal endoscopy both for evaluation of patients with gastrointestinal complaints and surveillance of patients with polyposis syndromes. Although pancreaticoduodenectomy for VTD containing invasive carcinoma is generally considered the procedure of choice, management of benign VTD remains controversial. For benign VTD, the debate focuses on the role of transduodenal local excision vs. pancreaticoduodenectomy. Although transduodenal local excision is an organ-preserving operation, the complex anatomy of the ampullary region, the well-recognized coexistence of carcinoma within VTD, and the relatively high rate of recurrence confound surgical management. In contrast, pancreaticoduodenectomy may be associated with perioperative morbidity, mortality, and long-term complications affecting quality of life.

Based on our prior experience in 36 patients with VTD,[1] we advocated transduodenal local excision for periampullary VTD without invasion. Our endorsement of local excision for VTD without invasion was based on the lack of development of malignancy during follow-up in our previous series and suggested that pancreaticoduodenectomy, as recommended by others,[2,5] was not essential for local control. Accumulation of additional experience since our prior report, coupled with improving safety of pancreaticoduodenectomy, prompted us to reassess the respective

From the Department of Surgery (M.B.F., G.H.S., M.G.S., D.R.F., and D.M.N.) and the Section of Biostatistics (C.M.R.), Mayo Clinic and Mayo Foundation, Rochester, Minn.; and the Truman Medical Center (G.G.T.), Kansas City, Mo.
Presented at the Fortieth Annual Meeting of The Society for Surgery of the Alimentary Tract, Orlando, Fla., May 16-19, 1999, and published as an abstract in *Gastroenterology* 116:A1310, 1999.
Reprint requests: Michael B. Farnell, M.D., Professor of Surgery, Department of Surgery, Mayo Clinic, Rochester, MN 55905.

Table I. Relationship between clinical presentation and histologic type of villous tumor of the duodenum

Clinical finding	Histologic type (no. of patients)			
	Benign (n = 64)	Carcinoma in situ (n = 3)	Invasive carcinoma (n = 19)	Overall (n = 86)
Pain	29 (45%)	1 (1/3)	5 (26%)	35 (41%)
Nausea/emesis	9 (14%)	0	5 (26%)	14 (16%)
Anemia	5 (8%)	0	4 (21%)	9 (10.5%)
Weight loss	8 (12%)	0	7 (37%)	15 (17%)
Jaundice	4 (6%)	0	4 (21%)	8 (9%)
Cholangitis	4 (6%)	0	0	4 (5%)
Pancreatitis	9 (14%)	2 (2/3)	1 (5%)	12 (14%)
Melena	2 (3%)	0	2 (10.5%)	4 (5%)
Asymptomatic—incidental finding	21 (33%)	1 (1/3)	5 (26%)	27 (31%)

roles of local excision and pancreaticoduodenectomy for this problematic neoplasm.

PATIENTS AND METHODS

Data on 86 consecutive patients with histologically confirmed VTD treated surgically at our institution from 1980 to 1997 were collected. As in our previous series,[1] histologic criteria for carcinoma in situ and invasive carcinoma were consistent with those reported by Komorowski and Cohen.[6] Patients with predominantly invasive duodenal or ampullary carcinoma with incidentally identified benign adenomatous changes were excluded. Periampullary villous tumors included both those arising from the ampulla as well as the duodenum within 1 cm of the major papillary orifice. Patient demographics, clinical features, presence of associated polyposis syndromes, diagnostic procedures, operative treatment and hospital course were abstracted from patient records. Follow-up information to death or July 1998 was obtained for 95% (n = 82) of patients from medical records, survey questionnaires, or telephone interviews, and length of follow-up ranged from 3 months to 16 years (mean 5.6 ± 3.9 years).

STATISTICAL METHODS

Data are presented as mean ± standard deviation (SD). The cumulative probability of recurrence over time was estimated using the Kaplan-Meier method of analysis. The log-rank test was used to test for group differences in time to recurrence. Fisher's exact test was used to test for association among pairs of categorical variables. Group differences in continuous variables were assessed using the Wilcoxon rank-sum test. A significance level of 0.05 was used for all tests.

RESULTS

Villous tumors of the duodenum occurred in 43 men and 43 women. Age at operation ranged from 31 to 85 years (mean 64 ± 11 years). Sixteen patients (19%) had associated polyposis syndromes: familial adenomatous polyposis (FAP) was present in six (7%), Gardner's syndrome in nine (11%), and Peutz-Jegher's syndrome in one (1%). Patients with an associated polyposis syndrome were younger than those without (52 ± 10 years vs. 67 ± 9 years; $P < 0.001$).

A wide spectrum of presenting symptoms was noted (Table I). Abdominal pain, nausea, weight loss, and pancreatitis were the most common presenting symptoms. Anemia, obstructive jaundice, and weight loss were most prevalent in patients with VTD containing invasive carcinoma. In 27 patients (31%), the VTD was asymptomatic and the diagnosis was made incidentally during endoscopic screening in 11 patients with an associated polyposis syndrome, whereas in the other 16 patients endoscopy was performed for other reasons.

Diagnosis

The diagnosis was confirmed preoperatively in all patients by means of upper gastrointestinal endoscopy and biopsy. Results of upper gastrointestinal barium contrast studies in 23 patients were positive in 14, and abdominal CT scans in 44 patients showed the tumor in 17 (Fig. 1). Percutaneous transphepatic cholangiography, performed in four patients with obstructive jaundice, showed the tumor in only one patient.

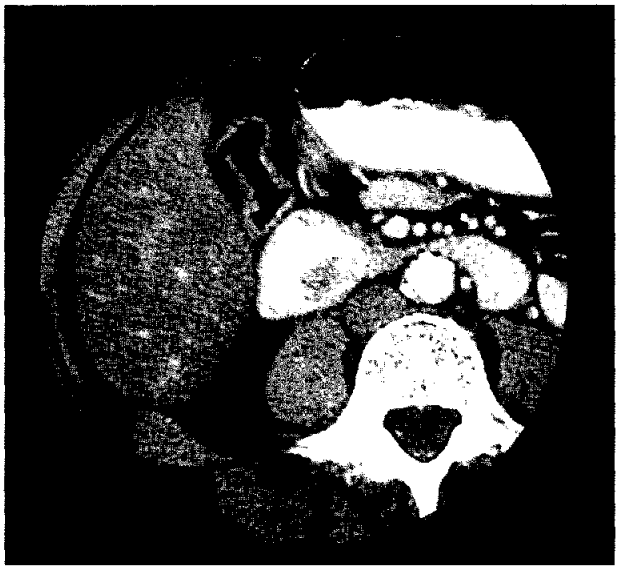


Fig. 1. Abdominal CT scan with oral and intravenous contrast demonstrating filling defect in medial wall of second portion of duodenum.

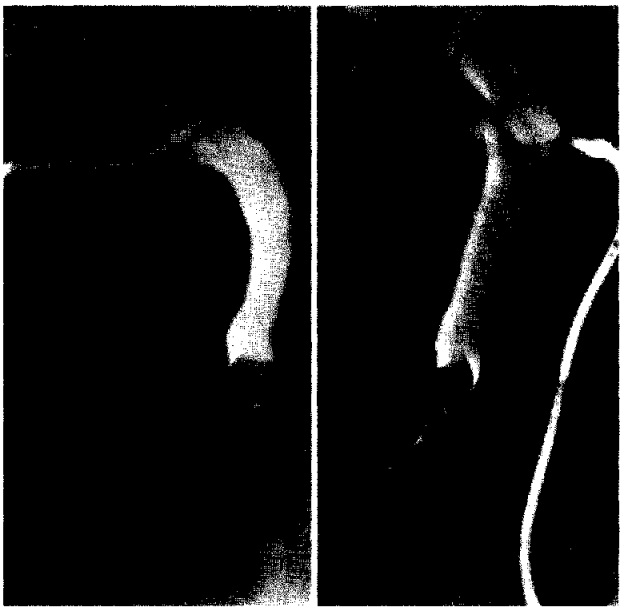


Fig. 2. Postoperative T-tube cholangiograms showing luminal filling defect with characteristics of neoplasm. Subsequent upper esophagogastroduodenoscopy confirmed the presence of an ampullary villous tumor. These images demonstrate the propensity of ampullary villous tumors to extend proximally into the bile duct.

In three patients, the VTD was found incidentally by T-tube cholangiography during or after cholecystectomy and common bile duct exploration for choledocholithiasis (Fig. 2). Subsequent upper gastrointestinal endoscopy confirmed the diagnosis of VTD.

The presence of cancer in VTD was not known preoperatively in 9 of the 19 patients with VTD containing invasive carcinoma (false negative biopsy, 47%) and in all three patients with VTD containing carcinoma in situ.

Distribution of Villous Tumors of the Duodenum

VTD were solitary in 76 patients (88%) and multiple in 10 (12%). Seven (70%) of the 10 patients with multiple VTD had an associated polyposis syndrome (Gardner's syndrome in four, FAP in two, and Peutz-Jeghers syndrome in one). One patient with Gardner's syndrome had 30 VTDs involving all portions of the duodenum. Among those patients with *solitary tumors*, 53 (70%) were periampullary (within 1 cm of the ampulla), 10 (13%) occurred in the third portion of the duodenum, nine (11%) in the nonperiampullary second portion, three (4%) in the first portion, and one in the fourth portion.

Patients with associated polyposis syndromes more often had tumors at multiple sites in the duodenum than those with the sporadic form (44% vs. 6%; $P < 0.001$). Although the periampullary area was the preferred location of *solitary-sporadic VTD* in 72%, 56% of those patients with associated polyposis syndromes had only periampullary tumors ($P = 0.44$).

Size and Histology of Villous Tumors of the Duodenum

VTD ranged from 5 to 90 mm in greatest diameter (mean 31 mm). Histologically, VTD were benign adenomas in 64 patients (75%; 48 of whom had atypia), adenomas with carcinoma in situ in three (4%), and adenomas with foci of invasive carcinoma in 19 (22%). Mean size for each histologic type was 31, 15, and 33 mm, respectively, and did not correlate with the presence of cancer ($P = 0.53$). Although resection margins were available for malignant lesions, margin verification histologically for benign VTD was rarely reported.

Operative Procedures

Benign VTD were treated by transduodenal local excision in 50 patients (combined with sphincteroplasty with or without pancreatic duct septotomy in 28), by pancreaticoduodenectomy in six, by pancreas-sparing duodenectomy in five, and by full-thickness

Table II. Postoperative hospital course per procedure

	Local excision (n = 53)	Pancreatico-duodenectomy (n = 20)	Pancreas-sparing duodenectomy (n = 5)	Full-thickness excision (n = 4)	Other (n = 4)
Complications					
Pancreatitis	3*	0	0	0	0
Leak	3†	7‡	3§	1	0
Delayed gastric emptying	2	1	3	1	0
Ileus	2	0	0	0	0
Fluid overload	1	0	0	0	0
Delirium tremens	0	0	1	0	0
Abscess	0	2‖	1¶	0	0
Total hospital stay**					
Mean (SD) (days)	12.3 (2.4)	15.5 (6.2)	26.8 (1.2)	16.2 (9.3)	9.7 (4.8)
Median (days)	8	15	17	15	7.5
Range (days)	5-155	9-30	7-60	7-28	7-17
ICU stay**					
No. of patients (%)	5 (9%)	8 (40%)	1 (20%)	2 (50%)	2 (50%)
Mean no. of days (SD)	6.6 (7.4)	2.4 (1.6)	11	1.5 (0.7)	2.5 (0.7)
Median (days)	2	2	11	1.5	1.5
Range (days)	2-19	1-5	11	1-2	2-3

*One patient with pseudocyst formation, drained percutaneously.
†Leak from duodenotomy.
‡Bile leak (3 patients), leak from pancreatojejunostomy (3 patients), and leak from both choledochojejunostomy and pancreatojejunostomy (1 patient).
§Anastomotic leak with drainage of bile and pancreatic fluid (see text).
‖Requiring open drainage in one patient and percutaneous drainage in one.
¶Drained percutaneously.
**Survivors only.

excision in three patients with VTD on the antimesenteric wall of the duodenum.

Two of the three cases of VTD with carcinoma in situ were treated by local excision. The presence of carcinoma in situ within the VTD was recognized only on permanent histologic sections. These two patients underwent no further surgery and received no other type of treatment. The third patient underwent pancreaticoduodenectomy because dilatation of the pancreatic duct was suspicious for underlying malignancy.

Of the 19 patients with VTD containing invasive carcinoma, 13 were managed with pancreaticoduodenectomy. Eight of the 10 patients with VTD containing invasive carcinoma known preoperatively were treated by pancreaticoduodenectomy; the other two were unresectable (liver metastasis, local extension) and underwent operative palliation. Among the nine other patients in whom cancer within the VTD was not known preoperatively, an initial attempt at local excision was converted to pancreaticoduodenectomy in five patients because of suspicious findings or frozen-section diagnosis of VTD containing invasive carcinoma, to segmental resection in two patients, and to full-thickness excision in two patients, one or whom had portal hypertension/cirrhosis and the other who had extensive nodal metastases.

Operative Morbidity and Mortality

One patient died after pancreaticoduodenectomy from bleeding and sepsis related to a leak at the hepaticojejunostomy. In total, 30 complications occurred in 23 patients, giving an overall morbidity rate of 27%. The complications, total length of hospital stay, and days in the intensive care unit are shown in Table II. Morbidity was 17% after local excision, 40% after pancreaticoduodenectomy, 80% after pancreas-sparing duodenectomy (4 of 5 patients), and 50% after full-thickness excision (2 of 4 patients).

Long-Term Follow-Up

Results of long-term follow-up (mean duration 5.6 years [range 0.3 to 16 years]) were available for 82 patients (95%). Twenty-one patients (26%) had died during this time period—10 from metastatic disease and 11 from unrelated causes. No recurrences were observed in any of the patients with benign VTD managed by pancreaticoduodenectomy, pancreas-sparing duodenectomy, or full-thickness excision. Benign villous tumors recurred in 17 of 52 patients who underwent transduodenal local excision for benign VTD or VTD containing carcinoma in situ, all in the group with benign VTD. Most recurrences were observed within 5 years, but late recurrences—even after

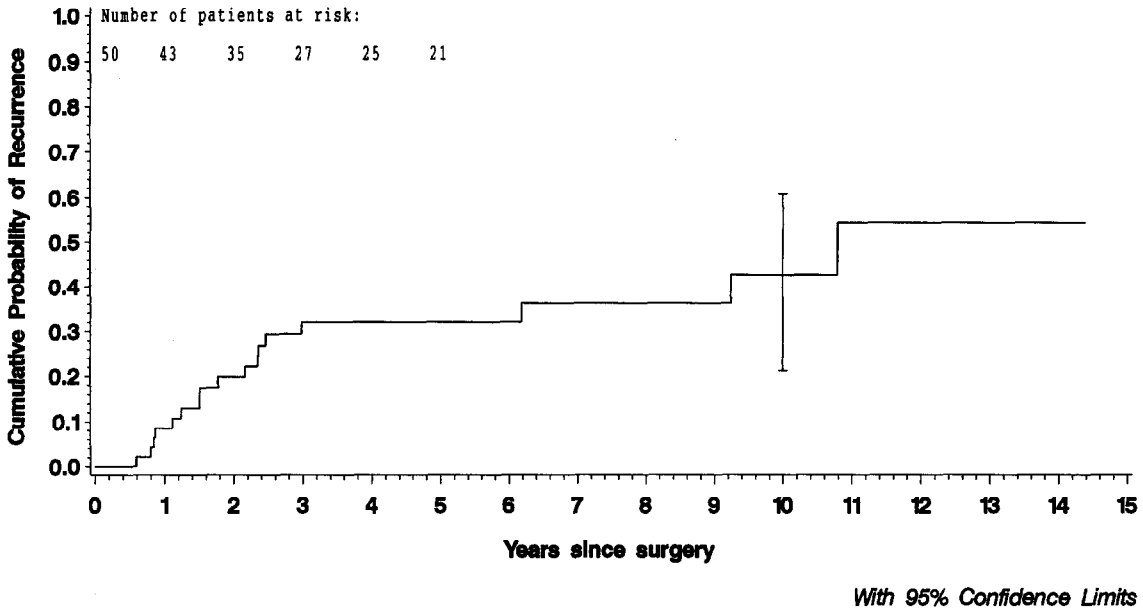


Fig. 3. Cumulative probability (Kaplan-Meier) of any recurrence in 50 patients with benign villous tumors of the duodenum (both sporadic and polyposis-associated) treated by local excision. Recurrence rates were 32% at 5 years and 43% at 10 years.

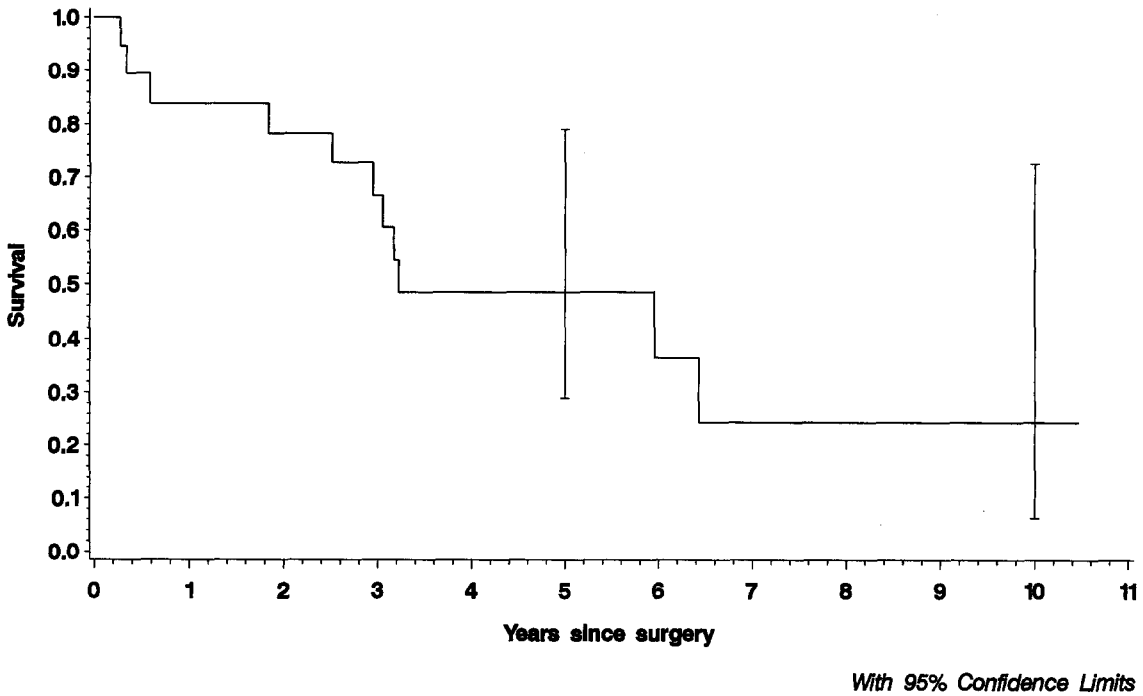


Fig. 4. Survival among 19 patients with villous tumors of the duodenum containing invasive cancer. Includes patients undergoing potentially curative and palliative procedures.

10 years—were also noted. The recurrence rate was 32% at 5 years and 43% at 10 years (Fig. 3). Eleven of 19 patients with VTD containing invasive carcinoma have died; the 5-year survival for this group of patients was 49% (Fig. 4).

In four patients with benign VTD treated by local excision, the tumor recurred as an adenocarcinoma 1.5, 2.5, 3, and 6 years later representing 23% (4 of 17) of recurrences or approximately 8% of all patients undergoing local excision for benign VTD. Three of

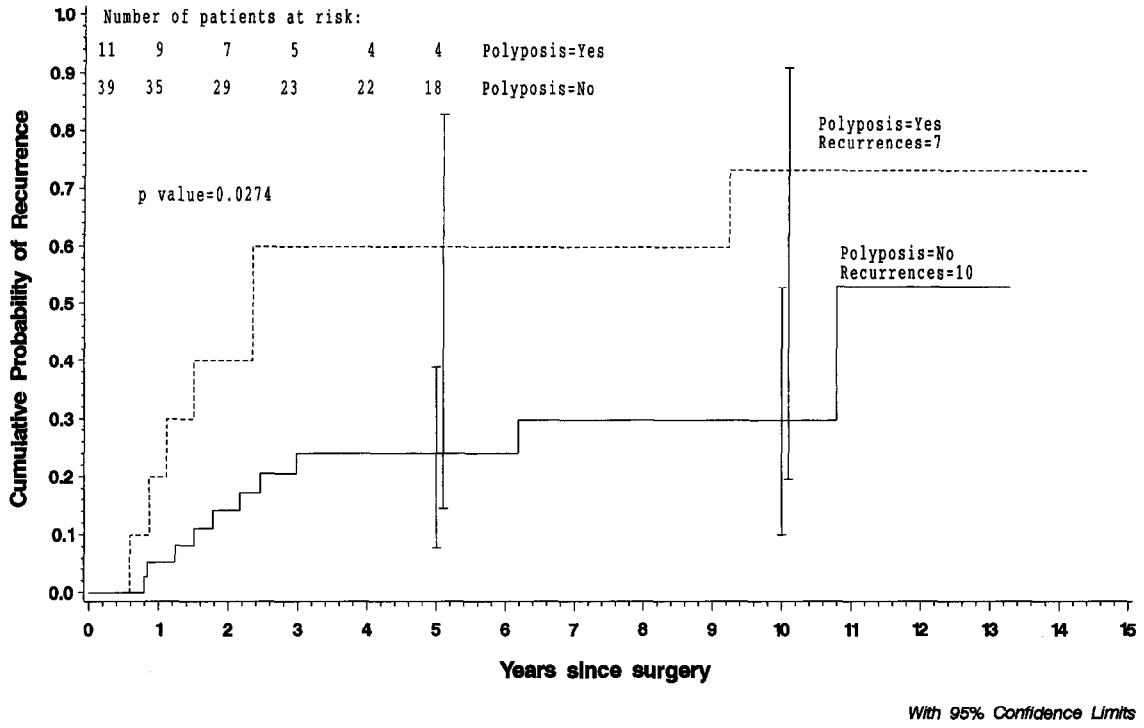


Fig. 5. Cumulative probability of recurrence (Kaplan-Meier) of benign villous tumors of the duodenum after local excision in 11 patients with associated polyposis syndrome vs. 39 patients with sporadic tumors. Recurrence rates at 5 years were 60% and 24%, respectively ($P = 0.0274$).

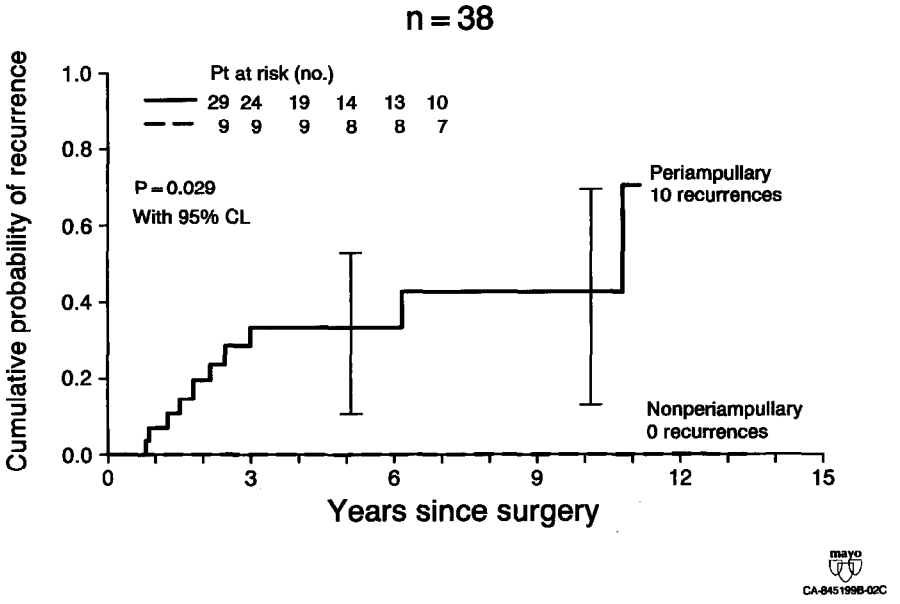


Fig. 6. Cumulative probability of recurrence (Kaplan-Meier) of solitary, sporadic benign villous tumors of the duodenum after local excision in 29 patients with periampullary location vs. nine patients with tumors remote from the papilla. Recurrence rates at 5 years were 34% and 0%, respectively ($P = 0.029$). Patients with associated polyposis syndrome or multiple tumors are excluded.

the four patients with malignant recurrences had invasion, one of which was unresectable. Although the numbers are small, there are no obvious differences in size of tumor between those with and without recurrence as adenocarcinoma.

The remaining 13 VTD recurrences were treated by endoscopic resection (n = 10), local reexcision (n = 2), and pancreaticoduodenectomy (n = 1). Four patients had a recurrence after endoscopic excision; three of them were treated by transduodenal reexcision and one by pancreas-sparing duodenectomy.

Seven of the 11 patients with an associated polyposis syndrome who had benign VTD treated by local excision have had a recurrence; recurrence rates were 60% at 5 years and 73% at 10 years and are significantly different from the rates in patients without an associated polyposis syndrome (24% and 30% at 5 and 10 years, respectively; $P = 0.0274$) (Fig. 5). Patients with solitary sporadic, benign villous tumors managed by transduodenal local excision were significantly more likely to develop a recurrence when the tumor was located periampullary rather than remote from the papilla (34% and 0%, respectively; $P = 0.029$) (Fig. 6).

DISCUSSION

The present study confirms many of the observations made in our earlier report[1] and the experience of others.[2-5,7] Most VTD are solitary periampullary tumors, 25% of which are malignant at the time of surgical excision. Approximately 20% of patients have an associated polyposis syndrome. As previously noted, we found that tumor size was not predictive of the presence of malignancy in a VTD. Most important, recurrence of VTD after transduodenal periampullary resection is quite common (43% at 10 years) and VTD may recur as invasive cancer. These observations raise many questions about appropriate treatment of VTD, especially in younger patients.

Diagnosis

Although endoscopic examination is important for the diagnosis of VTD, a very real risk for sampling error is present in periampullary adenoma, geographic variability in the distribution of dysplasia within the periampullary adenoma is common, and endoscopic biopsies miss the diagnosis of malignancy within a VTD in 40% to 60% of patients.[2,5,8-13] Indeed, in our present study, a false negative diagnosis of malignancy was made preoperatively based on endoscopic biopsy in 12 (55%) of 22 patients found to have a malignancy after complete tumor excision.

Treatment

Sporadic Villous Tumors of the Duodenum. Although there is uniform agreement that VTD should be resected, opinions differ regarding the optimal method. The presence of invasive carcinoma within a VTD is an indication for a more radical resection (pancreaticoduodenectomy); however, the treatment of benign VTD with or without atypia or VTD containing carcinoma in situ remains controversial. The difficulty in identifying underlying malignancy preoperatively, the potential morbidity and mortality of pancreaticoduodenectomy, imprecision in confirmation of negative margins, and the high rate of recurrence with local excision combine to make proper management problematic.

In our previous report on periampullary VTD,[1] we advocated transduodenal resection with sphincteroplasty and pancreatic duct septotomy for benign VTD because of a low incidence (17%) of local recurrence. A meticulous technique previously described[1,14] was thought to be crucial with regard to achieving good results. In our current study comprised of a larger group of patients, the 30% rate of recurrence at 5 years for benign VTD after local excision, coupled with the observation that in 4 of 17 the recurrences (23%) were adenocarcinomas, calls into question the role of local excision even for benign VTD.

Based on our initial study, our bias was to perform transduodenal excision for benign VTD when feasible. The data presented herein suggest that when contemplating local excision for benign VTD, recognition of factors affecting the propensity for recurrence needs to be considered. Hard areas on palpation, an ulcerated tumor, dilation of the common bile duct and/or the pancreatic duct on preoperative imaging, and severe dysplasia on preoperative biopsies should all be considered suspicious for underlying malignancy, and a pancreaticoduodenectomy should be entertained, especially in medically fit patients. When these factors are absent, local excision seems most reasonable in compliant patients willing to undergo lifelong endoscopic surveillance at regular intervals or in patients unfit for a more radical operation. Similarly, a regular lifelong endoscopic surveillance program, as suggested by others,[8,9] may also be a viable option in appropriate patients in an attempt to avoid "prophylactic" pancreaticoduodenectomy. Whether preoperative endoscopic ultrasound will allow better selection of candidates for local excision, as suggested by Rattner et al.,[15] is yet to be determined. For benign VTD located on the antimesenteric surface or for those in the first, distal third, or fourth portion of the duodenum, full-thickness excision or segmental resection, when feasible, is a reasonable option.

In the past, pancreaticoduodenectomy was considered a procedure associated with prohibitively high morbidity (70%) and mortality (15% to 30%) and thus inappropriate for benign disease.[16-18] Recently, with increased experience, many institutions have reported improved morbidity (20% to 40%) and a much lower mortality (2% to 4%) of pancreaticoduodenectomy.[19-25] An important study by McCleod et al.[26] showed that quality of life and gastrointestinal function in patients after pancreaticoduodenectomy without recurrent tumor were excellent and similar to that of patients following cholecystectomy. Accordingly, pancreaticoduodenectomy should be considered as an option for benign VTD in selected medically fit patients.

For VTD harboring invasive cancer that arises in the first, second, or proximal third portion, pancreaticoduodenectomy as a formal cancer operation is warranted. We prefer the pylorus-preserving technique for sporadic VTD, both provided the tumor does not arise in the first portion of the duodenum and the patient does not have a polyposis syndrome. For the 19 patients with VTD containing invasive cancer, the 5-year survival rate was 49%, which is similar to previous reports.[1,27] When the VTD arises in the distal third or fourth portion of the duodenum, an extended segmental resection may be as effective as a pancreaticoduodenectomy, since the classic pancreaticoduodenectomy does not remove the nodal basin of these distal duodenal cancers.

VTD containing only carcinoma in situ remain controversial. In our series of 86 patients, only two such patients were treated by transduodenal local excision, and both remain recurrence free 7 and 8 years postoperatively. Such a small experience prevents any firm recommendations based on data.

Recurrence following local excision can be treated by either local reexcision (either open or endoscopic) or, as we prefer, by pancreaticoduodenectomy. Unfortunately, recurrence may be in the form of invasive carcinoma as occurred in 4 (23%) of our 17 patients. In such circumstances pancreaticoduodenectomy, when feasible, is the only reasonable option.

Villous Tumors of the Duodenum Associated With Polyposis Syndrome. The duodenum and periampullary region are second only to the colon and rectum as a site of malignancy in patients with FAP[28-33] With the advent of prophylactic total colectomy, periampullary carcinoma is now the leading cause of death in FAP affecting up to 12% of patients.[30-33] In patients with FAP, the relative risk of periampullary or duodenal cancer is 250 times that of the general population,[34-35] and mortality rates for periampullary cancer are more than 300 times that of the general population.[28,36] For these reasons, patients with FAP should be enrolled in a formal surveillance esophagogastroduodenoscopy program once the diagnosis is established.

Our current recommendation for patients with FAP but without a known VTD is yearly enteroscopy with ampullary biopsies. Patients with positive ampullary biopsies should undergo endoscopic retrograde cholangiopancreatography (ERCP), snare ampullectomy, prophylactic sphincterotomy, and thermal ablation of the periampullary adenoma with reexamination every 3 months until histologic resolution. Henceforth, patients should be reexamined endoscopically and biopsies performed on an annual basis. Recognition of high-grade dysplasia should prompt consideration of surgical intervention. Because the entire duodenal mucosa is at risk, consideration should be given to pancreaticoduodenectomy[7,37] or to pancreas-sparing duodenectomy.[38,39] Indeed, of the 11 patients with associated polyposis syndromes and benign VTD treated by local excision alone, seven developed a recurrence.

Our experience with pancreas-sparing duodenectomy for the treatment of benign VTD is limited to five patients. This procedure was associated with a high morbidity and two patients required therapeutic interventions (percutaneous abscess drainage in one and laparotomy for stent placement in one). Additional experience with pancreas-sparing duodenectomy will need be obtained before its role in the management of patients with FAP and VTD is established.

CONCLUSION

The surgical management of VTD is selective and based on clinical presentation, information from preoperative diagnostic evaluation, the presence of a polyposis syndrome, and intraoperative findings. Although local excision has a lower rate of complications, it has a high rate of recurrence (32% at 5 years, 43% at 10 years). If the VTD is treated by local excision, regular endoscopic surveillance is mandatory.

Pancreaticoduodenectomy prevents recurrence and is appropriate for selected patients at high risk with both benign VTD as well as VTD containing carcinoma. For patients with VTD associated with polyposis syndromes, the entire duodenal mucosa is at risk for malignancy. Dysplasia warrants complete removal of the duodenal mucosa with pancreaticoduodenectomy. More experience with pancreas-sparing duodenectomy is necessary before this procedure can be generally recommended.

REFERENCES

1. Bjork KJ, Davis CJ, Nagorney DM, Mucha P. Duodenal villous tumors. Arch Surg 1990;125:961-965.
2. Chappuis CW, Divincenti FC, Cohn I. Villous tumors of the duodenum. Ann Surg 1989;209:593-599.
3. Asbun HJ, Rossi RL, Munson JL. Local resection for ampullary tumors: Is there a place for it? Arch Surg 1993;128:515-520.
4. Zinzindohoue F, Gallot D, Majery N, Malafosse M. Pour un traitement radical des nappes villeuses du duodenum. Ann Chir 1996;50:330-332.
5. Ryan DP, Shapiro RH, Warshaw AL. Villous tumors of the duodenum. Ann Surg 1986;203:301-306.
6. Komorowski RA, Cohen EB: Villous tumors of the duodenum: A clinicopathological study. Cancer 1981;47:1377-1386.
7. Penna C, Bataille N, Balladur P, Tiret E, Parc R. Surgical treatment of severe duodenal polyposis in familial adenomatous polyposis. Br J Surg 1998;85:665-668.
8. Nugent KP, Spigelman AD, Williams CB. Surveillance of duodenal polyps in familial adenomatous polyposis: Progress report. J R Soc Med 1994;87:704-706.
9. Bleau BL, Gostout CJ. Endoscopic treatment of ampullary adenomas in familial adenomatous polyposis. J Clin Gastroenterol 1996;22:237-241.
10. Galandiuk S, Hermann RE, Jagelman DG, Fazio VW, Sivak MV. Villous tumors of the duodenum. Ann Surg 1988;207: 234-239.
11. Goldberg M, Zamir A, Nissan S. Wide local excision as an alternative treatment for periampullary carcinoma. Am J Gastroenterol 1987;82:1169-1171.
12. Gray G, Browder W. Villous tumors of the ampulla of Vater: Local resection versus pancreaticoduodenectomy. South Med J 1989;82:917-920.
13. Shemesh E, Nass S, Czerniak A. Endoscopic sphincterotomy and endoscopic fulguration in the management of adenoma of the papilla of Vater. Surg Gynecol Obstet 1989;169:445-448.
14. Sakorafas GH, Sarr MG. Local excision of villous tumours of the duodenum. Eur J Surg Oncol 1999;25:90-93.
15. Rattner DW, Fernandez de-Castillo C, Brugge WR, Warshaw AL. Defining the criteria for local resection of ampullary neoplasm. Arch Surg 1996;131:366-371.
16. Shapiro TM. Adenocarcinoma of the pancreas: A statistical analysis of bypass vs Whipple resection in good risk patients. Ann Surg 1975;182:715-721.
17. Crile G. The advantages of bypass operation over radical pancreatoduodenectomy in the treatment of pancreatic carcinoma. Surg Gynecol Obstet 1970;130:1049-1053.
18. Hertzberg J. Pancreatico-duodenal resection and by-pass operation in patients with carcinoma of the head of the pancreas, ampulla, and distal end of the common bile duct. Acta Chir Scand 1974;140:523-527.
19. Strasberg SM, Drebin JA, Soper NJ. Evolution and current status of the Whipple procedure: An update for gastroenterologists. Gastroenterology 1997;113:983-994.
20. Cameron JL, Pitt HA, Yeo CJ, Lillemoe KD, Kaufman HS, Coleman J. One hundred and forty-five consecutive pancreatoduodenectomies without mortality. Ann Surg 1993;217:430-438.
21. Trede M, Schwall G, Saeger HD. Survival after pancreatoduodenectomy. 118 consecutive resections without an operative mortality. Ann Surg 1990;210:447-458.
22. Fernandez del Castillo C, Rattner DW, Warshaw AL. Standards for pancreatic resection in the 1990s. Arch Surg 1995;130: 295-300.
23. Gordon TA, Burleyson GP, Tielsch JM, Cameron JL. The effects of regionalization on cost and outcome for one general high-risk surgical procedure. Ann Surg 1995;221:43-49.
24. Crist DW, Stizmann JV, Cameron JL. Improved hospital morbidity, mortality and survival after the Whipple procedure. Ann Surg 1987;206:358-365.
25. Pellegrini CA, Heck CF, Raper S, Way LW. An analysis of the reduced morbidity and mortality rates after pancreaticoduodenectomy. Arch Surg 1989;124:778-781.
26. McLeod RS, Taylor BR, O'Connor BI, Greenberg GR, Jeejeebhoy KN, Royall D, Langer B. Quality of life, nutritional status, and gastrointestinal hormone profile following the Whipple procedure. Am J Surg 1995;169:179-185.
27. Monson JRT, Donohue JH, McEntee GP, McIlrath DC, van Heerden JA, Shorter RG, Nagorney DM, Ilstrup DM. Radical resection for carcinoma of the ampulla of Vater. Arch Surg 1991;126:353-357.
28. Offerhaus GJA, Giardiello FM, Krush AJ, Booker SV, Tersmette AC, Kelley NC, Hamilton SR. The risk of upper gastrointestinal cancer in familial adenomatous polyposis. Gastroenterology 1992;102:1980-1982.
29. Jagelman DG, DeCosse JJ, Bussey HJR. Upper gastrointestinal cancer in familial adenomatous polyposis. Lancet 1988;1: 1149-1151.
30. Arvanitis ML, Jagelman DG, Fazio VW, Lavery IC, McGannon E. Mortality in patients with familial adenomatous polyposis. Dis Colon Rectum 1990;33:639-642.
31. Goedde TA, Rodriguez-Bigas MA, Herrera L, Petrelli NJ. Gastroduodenal polyps in familial adenomatous polyposis. Surg Oncol 1992;1:357-361.
32. Belchetz LA, Berk T, Bapat BV, Cohen Z, Gallinger S. Changing causes of mortality in patients with familial adenomatous polyposis. Dis Colon Rectum 1996;39:384-387.
33. Presciuttini S, Sala P, Roddetti C, Petrouisti M. The Italian registry of familial polyposis. Causes of death and post-surgical survival in familial adenomatous polyposis: Results from the Italian registry. Semin Surg Oncol 1994;10:225-234.
34. Iwama T, Mishima Y, Utsunomiya J. The impact of familial adenomatous polyposis on the tumorigenesis and mortality at the several organs. Ann Surg 1993;217:101-108.
35. Iwama T, Tomita H, Kawachi Y, et al. Indications for local excision of ampullary lesions associated with familial adenomatous polyposis. J Am Coll Surg 1994;179:462-464.
36. Sanabria JR, Croxford R, Berk TC, Cohen Z, Bapat BV, Gallinger S. Familial segregation in the occurrence and severity of periampullary neoplasms in familial adenomatous polyposis. Am J Surg 1996;171:136-141.
37. Wallace MH, Phillips RKS. Upper gastrointestinal disease in patients with familial adenomatous polyposis. Br J Surg 1998; 85:742-750.
38. Chung RS, Church JM, vanStokl R. Pancreas-sparing duodenectomy: Indications, surgical technique, and results. Surgery 1995;117:254-259.
39. Maher MM, Yeo CJ, Lillemoe KD, Roberts JR, Cameron JL. Pancreas-sparing duodenectomy for infra-ampullary duodenal pathology. Am J Surg 1996;171:62-67.

Discussion

Dr. K. Warren (Boston, Mass.). We have always favored pancreatic resection, although we do recognize that there are some benign polypoid lesions of the duodenum and the ampulla of Vater that can be excised locally. Dr. Rodney Smith, many years ago, pointed out that local excision has many problems. First, to be sure that all of the tumor has been removed, multiple biopsies are necessary. Then, after the tumor has been excised, the bile duct and pancreatic duct have to be reinserted, and it is more difficult to accomplish this through the duodenum than to perform a Whipple procedure. I do not want to imply that a Whipple procedure should be used in all cases but I think you made the point that it should be the treatment of choice.

Dr. M. Farnell. One of the problems we had in reviewing these data is that information on margins was available in very few cases of benign villous tumors that were locally resected. This speaks to your point about the difficulty in assessing margins. These friable tumors often end up being sent to the pathology laboratory in pieces, and it is difficult for the pathologist to give precise information with regard to margins.

Dr. C. Fernandez-del Castillo (Boston, Mass.). This experience is probably the largest series of villous tumors of the duodenum. I think your series confirms that preoperative biopsies, unfortunately, are plagued with a very high incidence of false negative results. Therefore the surgeon needs to have access to a very good frozen section laboratory in order to obtain information intraoperatively, and the surgeon must be prepared to perform a Whipple resection even if he or she has chosen initially to perform a local excision as opposed to a pancreatoduodenectomy.

Endoscopic ultrasound has been proposed by other groups as a useful tool in the evaluation of these tumors. By providing information on the depth and invasion of the tumor, it can anticipate that there will be an invasion even when no cancer has been found on the preoperative biopsies. I wonder if the Mayo Clinic has experience using this modality. My second question focuses on the benign tumors that were treated with local excision and recurred. You showed that the risk of recurrence in patients with polyposis syndromes is very high, and that is no surprise. But even in sporadic tumors, the risk of recurrence is still 25% to 30%. Can you tell us something more about those patients who had a recurrence? What was their margin status, and what was the size of the tumors? This information could help us predict which patients are more likely to have a good outcome with a local excision versus a Whipple procedure.

Finally, most of us would agree with your point that a pancreaticoduodenectomy is a better operation for most patients and that the surgeon really should have a definite reason for performing a local excision, perhaps because the patient is elderly and frail. But there is a dilemma in patients with polyposis syndromes. Often they have a single lesion with dysplasia and many of these patients are young. Do you think there is a place for periodic surveillance as opposed to performing a Whipple procedure at an early age in this group of patients?

Dr. Farnell. Our experience with endoscopic ultrasound is only anecdotal. We are, of course, beginning to integrate this modality into our armamentarium. We find it useful particularly in those periampullary tumors in which ERCP is not technically feasible to assess both the proximal extent and the depth of invasion. I did not show you examples of cholangiography in some of these patients that showed extension of the tumor proximally into the bile duct. Those of you who deal with this problem know that this can be a difficult situation.

You asked about local excision in patients who have sporadic tumors. We did find that dysplasia and size did not correlate with the likelihood of recurrence and unfortunately we do not have information on margins for the reasons I have already addressed. Our patients with familial polyposis who have a small tumor are often seen first by our endoscopist who enrolls them in a surveillance program and performs a biopsy. If a microadenoma is found, sphincterotomy and ablation are performed and biopsies are done at 2- to 6-month intervals until histologic findings revert to normal. It is only those patients who have macrovillous tumors with dysplasia or who cannot be managed endoscopically that we surgeons are asked to consider for operation.

Dr. S. Helton (Chicago, Ill.). As a subgroup, can you comment on the recurrence rate for those tumors that were not periampullary, that is, those on the antimesenteric wall or in the third portion of the duodenum. With this long a follow-up, did you observe the development of any additional tumors in the pancreas or biliary tree in these patients?

Dr. Farnell. My last Kaplan-Meier graph (see Fig. 6) compared the benign locally excised tumors that were periampullary with those that were nonperiampullary. In the group that was nonperiampullary, there was a zero recurrence rate. Accordingly, when the tumor is not periampullary, either a full-thickness excision for tumors on the antimesenteric border, a segmental excision for those that are distal, or a local excision are all reasonable options. It is the periampullary tumors that are the most likely to recur following local excision. In regard to your second question, I do not have information with regard to additional tumors in the pancreas or in other areas.

Dr. A. Warshaw (Boston, Mass.). I want to ask about the pathogenesis of these lesions. There are two possibilities at least, I would think. One would be a genetic field defect as demonstrated in the polyposis syndromes. The other, based on the observed concentration of the lesions at the periampullary region and the fact that they do not occur at the minor ampulla, would be that something in the combination of bile and pancreatic juice causes them. Are all of the recurrences at the reconstructed periampullary region at the neoanastomosis, whether or not the original tumor was associated with the polyposis syndrome? If so, does that lend credence to this chemical toxicity mechanism?

Dr. Farnell. The recurrences were indeed in the periampullary area or slightly downstream. There does seem to be an abnormality in the mucosa as well as a carcinogenic

effect of bile. Patients with polyposis in particular will often have what our endoscopists refer to as a "goatee" type of tumor, issuing from the papilla and extending distally, implying that the high local concentration of bile contributes to the genesis of the tumor.

Dr. S. Jones (Charlottesville, Va.). You mentioned the difficulty with margins and that is certainly a problem, but there is another dimension to that issue. Even if the margins are negative, there is still tissue being left behind that is at risk for developing a neoplasm. In other words, this is a DNA problem; if the remainder of the duodenal mucosa is left behind in a patient with a genetic tendency for this disease, the margin issue is moot because eventually these patients are at risk for developing another lesion. My own concern in this condition is not whether to perform a Whipple operation. That is the proper treatment. My concern has been whether to perform a pylorus-sparing operation, because 2 to 3 cm of duodenal mucosa is left. I have not seen a recurrence in that tissue, and I noticed you performed Whipple operations in 40 patients with no recurrences. So I was reassured by your data that performing the pylorus-sparing operation in this disease is safe.

Dr. L. Way (San Francisco, Calif.). Let us just narrow the focus to those patients who had benign ampullary tumors. We are disappointed in the recurrences because of problems with margins and other factors. However, is it not appropriate to emphasize that the treatment is local excision *plus* surveillance? If I were to put myself in the shoes of a patient with a benign tumor, I would choose local excision as long as I could count on the surveillance program to detect any recurrences. It is probably better to have a local recurrence that is detected on surveillance than to perform a Whipple procedure in all patients to avoid the 25% failure rate. So the question is, what is the follow-up regimen you recommend and why did it fail in some instances?

Dr. Farnell. We recommend yearly examinations after local excision of a sporadic tumor, but that is not always possible. I remind you that four of the patients who had benign tumors excised locally had recurrences as carcinoma, so compliance can be a problem. In addition, McLeod et al.[26] showed that the quality of life after a Whipple operation compares favorably with that following cholecystectomy. They concluded that the only issue to consider with a Whipple operation is the initial morbidity and mortality of the procedure rather than quality-of-life issues after surgery; patients who undergo pancreaticoduodenectomy tend to do very well.

Sodium Salicylate Inhibits Proliferation and Induces G1 Cell Cycle Arrest in Human Pancreatic Cancer Cell Lines

Richard A. Perugini, M.D., Theodore P. McDade, M.D., Frank J. Vittimberga, Jr., M.D., Andrew J. Duffy, M.D., Mark P. Callery, M.D., F.A.C.S.

The mutations most common in pancreatic cancer decrease the ability to control G1 to S cell cycle progression and cellular proliferation. In colorectal cancer cells, nonsteroidal anti-inflammatory drugs inhibit proliferation and induce cell cycle arrest. We examined whether sodium salicylate, an aspirin metabolite, could inhibit proliferation in human pancreatic cancer cell lines (BxPC3 and Panc-1). Quiescent cells were treated with medium containing 10% fetal calf serum, with or without salicylate. Cellular proliferation was measured by MTT assay and bromodeoxyuridine incorporation. The fractions of cells in G0/G1, S, and G2/M phases of the cell cycle were quantitated by fluorescence-activated cell sorting. Results were compared between groups by two-tailed *t* test. Cyclin D1 expression was determined by Western blot analysis and prostaglandin E_2 expression by enzyme-linked immunosorbent assay. Serum-starved cells failed to proliferate, with most arrested in the G1 phase. Salicylate significantly inhibited serum-induced progression from G1 to S phase, cellular proliferation, and the expression of cyclin D1. The concentrations at which 50% of serum-induced proliferation was inhibited were 1.2 mmol/L (Panc-1) and 1.7 mmol/L (BxPC3). The antiproliferative effect of sodium salicylate was not explained by inhibition of prostaglandin E_2 production. This study provides further evidence in a noncolorectal cancer model for the antineoplastic effects of nonsteroidal anti-inflammatory drugs. (J GASTROINTEST SURG 2000;4:24-33.)

KEY WORDS: Pancreatic adenocarcinoma, NSAID, sodium salicylate, cell cycle, cyclin D1

Pancreatic cancer is the fifth most common cause of cancer death in the Western world and affected approximately 26,700 Americans in 1997.[1] Less than 5% of patients with adenocarcinoma of the pancreas will be alive 5 years after their diagnosis. Yeo et al.[2] demonstrated that postoperative chemoradiation improves survival in patients who undergo pancreaticoduodenectomy for adenocarcinoma of the pancreas. However, the beneficial effect is limited, as median survival improved from 13.5 months to 19.5 months. Novel therapies are needed as adjuncts to surgery in our approach to adenocarcinoma of the pancreas.

In adults, few cells are actively undergoing cell division, from a minimum of 0.01% of vascular endothelial cells to 14% of gastrointestinal epithelial cells.[3] Instead the majority of cells are in a stage called G0, which allows for terminal differentiation. Cancer can be thought of as a loss of the normal regulatory mechanisms that inhibit inappropriate cell cycle progression.

Many genetic abnormalities found in adenocarcinoma of the pancreas affect the mechanisms controlling G1 to S cell cycle progression and cellular proliferation[4] (Fig. 1). The most frequent genetic abnormality associated with carcinoma of the exocrine pancreas is the K-ras mutation, leading to inappropriate activation of the ras protein.[5,6-8] As the $p21^{ras}$ protein is situated in mitogen-induced signal transduction cascades, oncogenic activation of $p21^{ras}$ is thought to lead to abnormal upregulation of the signal for cell proliferation.[9] The p53 tumor suppressor gene is altered in up to 70% of primary pancreatic adenocarcinomas.[10] Wild-type p53 is a nuclear transcription factor which, through the synthesis of WAF1, inhibits cyclin D/cyclin-dependent kinase activity and arrests

From the Department of Surgery, University of Massachusetts Medical School, Worcester, Mass.
Presented at the Fortieth Annual Meeting of The Society for Surgery of the Alimentary Tract, Orlando, Fla., May 16-19, 1999.
Reprint requests: Mark P. Callery, M.D., F.A.C.S., Associate Professor of Surgery and Cell Biology, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655-0333. e-mail: Mark.Callery@banyan.ummed.edu

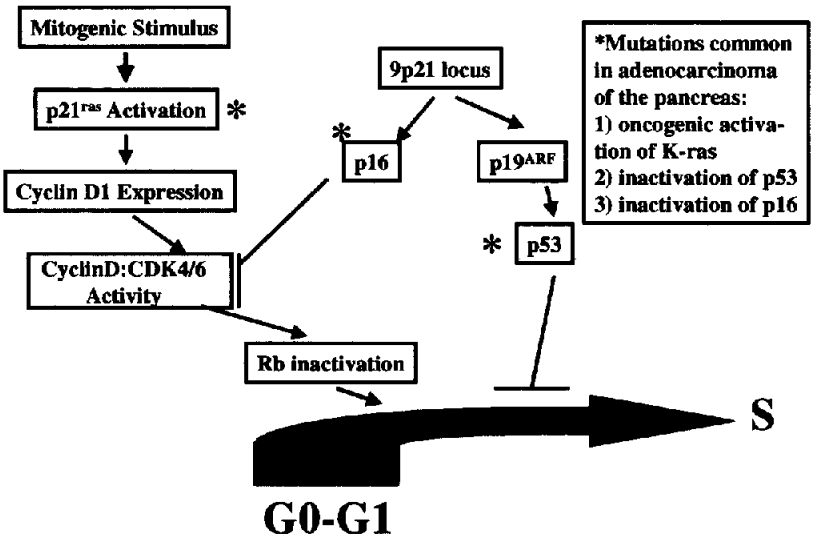


Fig. 1. Events involved in G1 to S phase cell cycle progression. The most frequent mutations in human pancreatic cancer affect the control of G1 to S phase cell cycle progression. The K-ras mutation leads to inappropriate activation of mitogen-induced signal transduction cascades. Loss of p16/Ink4a allows cyclin D:CDK4/6 activity to proceed unchecked, leading to phosphorylation and inactivation of the retinoblastoma gene product. Finally, mutation of p53 inhibits the ability of the cell to arrest in G1 and G2 when DNA damage occurs, and to undergo apoptosis. The 9p21 locus, which codes for p16/Ink4a, also codes for $p19^{ARF}$, a protein necessary for normal p53 function.

the cell cycle at G1.[11-13] The locus that codes for the p16/Ink4a tumor suppressor is a frequent target for homozygous deletion or for other mutation in human pancreatic cancer.[14-16] The p16/Ink4a protein inhibits cyclin D/cyclin-dependent kinase activity and, in doing so, inhibits progression through the G1 phase of the cell cycle.[17] This same locus also codes for $p19^{ARF}$, a protein product required for normal p53 activity.[18] Therefore deletion of a single locus in pancreatic cancer may disable two distinct tumor suppressor pathways, further decreasing control of G1 to S phase cell cycle progression.

In human epidemiologic studies, nonsteroidal anti-inflammatory drugs (NSAIDs) appear to have a protective effect against colorectal cancer.[19,20] Patients who take NSAIDs have up to a 50% reduction in the incidence of colorectal cancer. Several case reports have documented regression of polyps in patients with familial adenomatous polyposis treated with NSAIDs. NSAIDs inhibit cellular proliferation in colorectal cancer cells, with arrest of the cell cycle at the G1 phase and with a reduction in the activity of cyclin-dependent kinases.[21-23] NSAIDs are also noted to increase the rate of apoptosis in colorectal cancer cells.[22-26] The antiproliferative effects of NSAIDs may be independent of their ability to inhibit prostaglandin production.[24,25]

We studied the effect of the NSAID sodium salicylate on the cellular proliferation and control of G1 to S progression in the cultured human pancreatic cancer cells, BxPC3 and Panc-1. Sodium salicylate is the metabolite of aspirin formed after it is deacetylated on its first pass through the portal circulation.[27] Both the BxPC3 and Panc-1 cell lines have deletions of the p16 tumor suppressor gene[16] and overexpress a mutated form of p53.[28] BxPC3, a moderately differentiated human pancreatic adenocarcinoma cell line, expresses wild-type K-ras, whereas Panc-1, a poorly differentiated cell line, has the oncogenic ras mutation.[29,30] To focus on the events involved in G1 to S progression, we incubated the cell line without serum for more than 60 hours in order to induce quiescence (G0 phase).[31] Next we exposed the cells to medium containing 10% serum to stimulate progression to the S phase.

MATERIAL AND METHODS

Cell Culture and Treatments

The human pancreatic adenocarcinoma cell lines BxPC3 and Panc-1 were obtained from American Type Culture Collection (Rockville, Md.). Cells were grown in RPMI (BxPC3) or Dulbecco's modified Eagle medium (DMEM) (Panc-1), supplemented with

10% fetal calf serum (FCS) (Sigma, St. Louis, Mo.), penicillin, and streptomycin. They were maintained in an incubator at 37° C and 5% CO_2. For all experiments, initial cell density was constant at 3×10^4 cells/cm^2. After serum starvation for 60 hours in an attempt to induce quiescence, cells were exposed to medium supplemented with 10% FCS in addition to the appropriate concentration of sodium salicylate.

Proliferation Assay

All proliferation assays were carried out in quadruplicate in four parallel 96-well microtiter plates. Cells were plated at a concentration of 1×10^4 cells per well. Following serum starvation for 60 hours, cells were exposed to medium supplemented with 10% FCS, along with the appropriate amount of sodium salicylate. Cells were incubated at 37° C in 5% CO_2. On a daily basis, one plate was used for a viability assay. Supernate was removed and stored at −70° C to be later assayed for prostaglandin E_2 concentration.

Viability was determined by two methods: monotetrazolium (MTT) assay and bromodeoxyuridine (BrdU) assay. For the MTT assay, cells were placed in growth medium (RPMI or DMEM with 10% FCS) with 1 mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (Sigma). Cells were incubated at 37° C in 5% CO_2 for 3 hours. The medium was aspirated and the formazan crystals, formed from MTT by NADH-generating dehydrogenases in metabolically active cells, were dissolved in 200 μl dimethylsulfoxide. Absorbance at 570 nm, with a reference of 750 nm, was determined by an MRX Microplate Reader (Dynatech, Chantilly, Va.). Viability was defined in relation to that of the quiescent cells prior to treatments by the following equation: [viability (% control) = 100 × (absorbance treated sample)/(absorbance of cells after 60 hours' serum starvation)]. Data were analyzed by a two-tailed t test, with statistical significance defined as $P < 0.05$.

Viability was also measured by the cell proliferation enzyme-linked immunosorbent assay (ELISA), BrdU (Boehringer Mannheim-Roche, Indianapolis, Ind.), which measures DNA synthesis by assessing the incorporation of the nucleotide BrdU. Cells were placed in growth medium (RPMI or DMEM with 10% FCS) with BrdU labeling reagent. Cells were incubated at 37° C in 5% CO_2 for 3 hours. The medium was aspirated and, following fixation of the cells to the plate with FixDenat, anti-BrdU antibody linked to peroxidase was added to each well. After 2 hours of incubation, the antibody solution was removed and the wells were washed three times with wash buffer. Substrate solution was added to each well and the reaction was stopped by the addition of 1 mol/L H_2SO_4 after 10 minutes. Absorbance at 490 nm, with a reference of 750 nm, was determined by an MRX Microplate Reader.

For dose-response curves, varying concentrations of sodium salicylate (0.5 to 5 mmol/L) were used in addition to 10% FCS. Proliferation was determined at 85 hours by both MTT and BrdU assay. Serum-starved control samples (0% proliferation) and control samples treated with FCS without sodium salicylate (100% proliferation) were included. The IC_{50} was defined as the concentration at which 50% of the proliferation induced by FCS was inhibited by sodium salicylate.

Fluorescence-Activated Cell Sorter Analysis of Cell Cycle

Cells were plated in parallel in 25 cm^2 culture flasks at a concentration of 8×10^5 cells per flask. After serum starvation for 60 hours, cells were placed in medium containing 10% FCS along with 5 mmol/L sodium salicylate and incubated at 37° C in 5% CO_2. Samples were harvested daily by trypsinization, with care being taken to include any floating cells. They were washed in phosphate-buffered saline, fixed in 70% ethanol, and stored at −20° C. At the completion of the time course, ethanol was removed from the samples and they were treated with a nuclear staining solution (NP-40, propidium iodide, sodium citrate) at room temperature in the dark for 30 minutes. DNA histograms were obtained by fluorescence-activating cell sorting (FACS), and cell cycle analysis was performed using the Modfit Program (Verity, Topsham Maine).

Western Blot Analysis

After appropriate treatments, cells were harvested by adding ice-cold radioimmunoprecipitation assay buffer (1% NP-40, 50 mmol/L Tris, 150 mmol/L NaCl, 0.25% deoxycholate, 1 mmol/L ethyleneglycol-tetraacetic acid, 1 mmol/L NaF, and added protease inhibitor cocktail [Sigma]). Samples were incubated on ice for 20 minutes and centrifuged at 20,000g for 11 minutes. A Lowry protein quantification assay (Bio-Rad, Hercules, Calif.) was performed on the resulting supernate. Equal amounts of protein were loaded onto a 10% SDS-PAGE gel and electro-phoresed at 100 volts. The gel was transferred to Imobilon-P membrane (Millipore, Bedford, Mass.) at 100 volts for 65 minutes. Membranes were dried by placing them in methanol for 1 minute, then at room temperature for 15 minutes. Membranes were developed with anti-cyclin D1 and anticyclin E antibodies (Santa Cruz, Calif.). Briefly, membranes were blocked by incubating them in 5% milk in Tris-buffered saline with 0.05% Tween-20 (TBST) for 1 to 3 hours. Next, membranes were incubated with primary antibody at room tem-

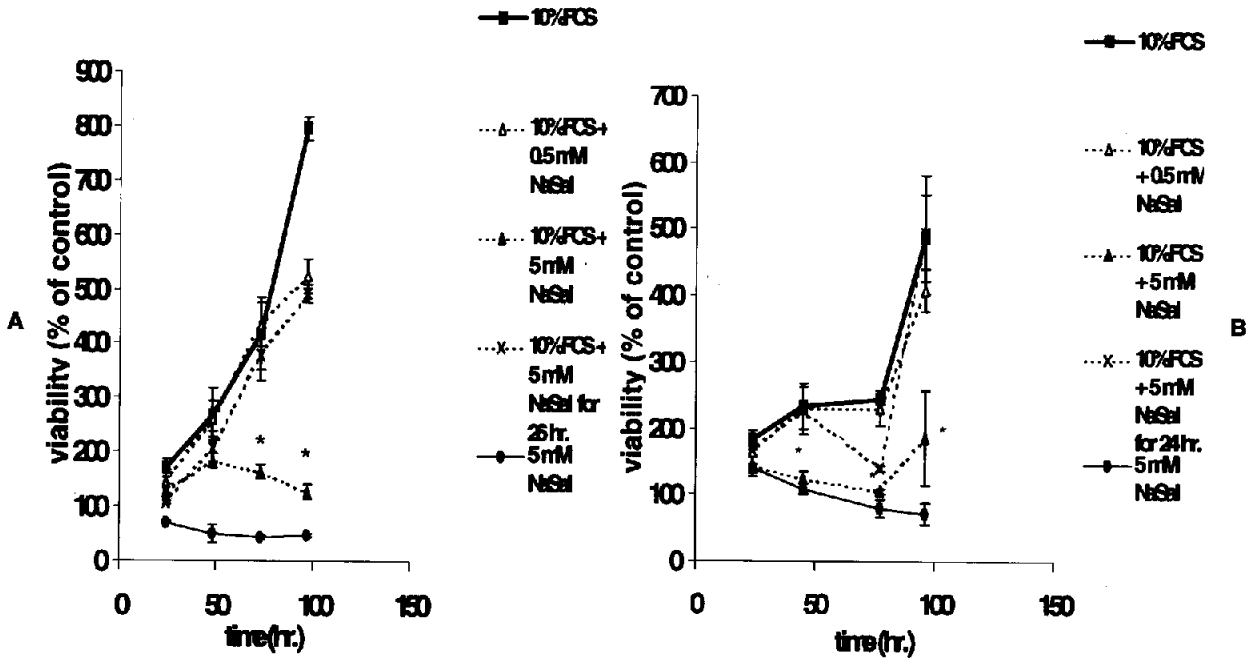


Fig. 2. Proliferation of pancreatic cancer cells in response to serum with and without sodium salicylate *(NaSal)*. NaSal at 5 mmol/L inhibits FCS-induced proliferation of BxPC3 cells **(A)** and Panc-1 cells **(B)**. Cells were serum starved for more than 60 hours. They were then exposed to 10% FCS ± 0.5 or 5 mmol/L NaSal. Viability is expressed as a percentage of the viability of control cells incubated in serum-free medium. Finally, the viability of cells exposed to 5 mmol/L salicylate without serum was assayed to determine the effect of this concentration of salicylate in the absence of serum. When serum-starved cells are exposed to 10% FCS, proliferation occurs, and these wells have approximately 5- to 8-fold more cells than wells maintained in serum-free medium by 96 hours. Incubating the cells with NaSal at 5 mmol/L in addition to 10% FCS inhibits this proliferation. This inhibition is statistically significant (* = $P < 0.05$) at 72 and 96 hours in BxPC3 cells and at 48, 72, and 96 hours in Panc-1 cells. NaSal alone has an effect on cell viability, decreasing relative viability to 30% ± 1% by 96 hours in BxPC3 cells and 72% ± 16% in Panc-1 cells. Growth inhibition by NaSal is reversible, as samples in which NaSal was removed after 26 hours immediately began to proliferate at the same rate as cells not treated with NaSal.

perature for 1 hour. After three washes in 0.05% TBST, membranes were incubated in horseradish peroxidase–linked secondary antibody for 1 hour at room temperature. Membranes were again washed three times with 0.05% TBST and developed using enriched chemiluminescence. Membranes were then exposed to film (Hyperfilm ECL, Amersham, Buckinghamshire, England).

Supernatant Prostaglandin E_2 Concentration

Supernatant concentration of prostaglandin E_2 was determined by Prostaglandin E_2 Enzyme Immunoassay Kit—Monoclonal (Alexis, San Diego, Calif.). Supernates harvested before MTT assay were thawed and diluted 1:20 in enzyme immunoassay buffer. To each well of an ELISA plate coated with goat anti-mouse antibody, we added 50 µl of diluted supernate, 50 µl of acetylcholinesterase tracer, and 50 µl of prostaglandin E_2 monoclonal antibody. Appropriate control and standard wells were prepared. The assay was performed in triplicate. Following 18 hours of incubation at 4° C, supernates were washed off and Ellman's reagent was added to all wells. Absorbance at 405 nm was determined in an MRX Microplate Reader. Results were analyzed by two-tailed *t* test, with statistical significance defined as $P < 0.05$.

RESULTS

Sodium Salicylate Inhibits Serum-Induced Proliferation in BxPC3 Human Pancreatic Cancer Cells

Cells that were serum starved for more than 60 hours (to arrest them in G0/G1) were exposed to 10% FCS, with or without sodium salicylate, at 0.5 or 5 mmol/L. Cells proliferated so that by 96 hours there were approximately 4.5-fold more Panc-1 cells and eightfold more BxPC3 cells in wells cultured in 10% FCS, as compared to paired control cells maintained in serum-free medium (Fig. 2). The viability of cells maintained in serum-free medium did not change.

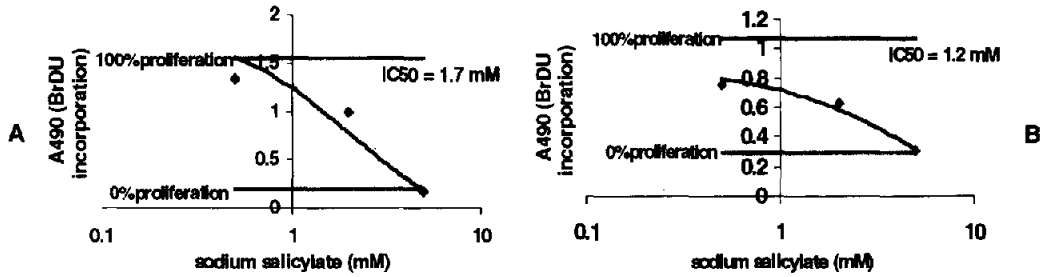


Fig. 3. Dose-response curves for inhibition of BxPC3 **(A)** and Panc-1 **(B)** cell proliferation by sodium salicylate. Quiescent cells were treated with medium with 10% FCS in addition to varying concentrations of sodium salicylate. Control specimens consisted of samples maintained in serum starvation (defined as 0% proliferation) and treated with 10% FCS (defined as 100% proliferation). BrdU proliferation assays were performed 85 hours after treatments. IC_{50} was defined as the concentration of sodium salicylate needed to inhibit 50% of the proliferation induced by FCS.

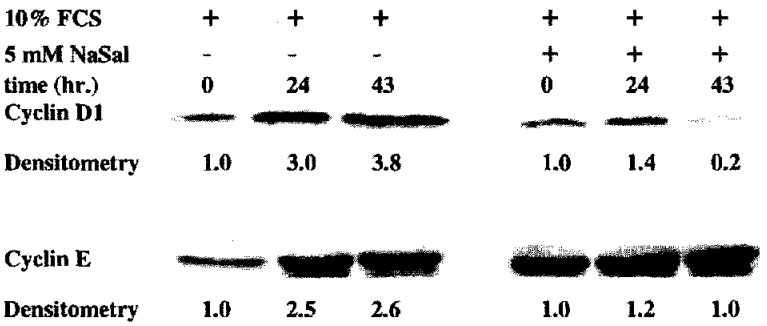


Fig. 4. Expression of cyclin D1 and cyclin E in response to serum with and without sodium salicylate *(NaSal)* in pancreatic cancer cells. Following serum starvation for 66 hours, BxPC3 cells were exposed to 10% FCS with or without 5 mmol/L NaSal. Cells were lysed in radioimmunoprecipitation assay buffer at various time points following exposure to FCS. Exposure to FCS increased the expression of cyclin D1 by 24 hours. This effect persisted for the course of the experiment. When cells were treated with 5 mmol/L NaSal in addition to 10% FCS, upregulation in expression of cyclin D1 was inhibited. By 43 hours, cyclin D1 expression was markedly decreased. The expression of cyclin E, a cyclin downstream of cyclin D1 in the cascade leading to G1 to S phase cell cycle progression, is similarly upregulated on exposure to FCS alone. The addition of 5 mmol/L NaSal inhibits this upregulation. However, cyclin E levels do not decrease with exposure to 5 mmol/L NaSal.

Therefore the increase in viability was due to proliferation of the serum-treated cells rather than death of the serum-starved cells.

Sodium salicylate inhibited proliferation in a dose-dependent manner. Although sodium salicylate at 0.5 mmol/L had no appreciable effect, sodium salicylate at 5 mmol/L inhibited FCS-induced proliferation in both BxPC3 and Panc-1 cells. At 5 mmol/L, sodium salicylate alone (without FCS) had only a small effect on cell viability. The effect of 5 mmol/L sodium salicylate was reversible. BxPC3 immediately began to proliferate after withdrawal of sodium salicylate at the same rate as cells not treated with sodium salicylate. Panc-1 cells demonstrated an initial decrease in viability on withdrawal of sodium salicylate but recovered so that by 100 hours there was no statistically significant difference between cells treated with FCS alone and cells treated with FCS and sodium salicylate for 24 hours. Dose-response assays indicated that the concentration of sodium salicylate at which 50% of FCS-induced proliferation was inhibited (IC_{50}) was 1.7 mmol/L for BxPC3 cells and 1.2 mmol/L for Panc-1 cells (Fig. 3).

Sodium Salicylate Arrests Cells at G0/G1

FACS analysis of DNA content was used to analyze cell cycle changes with the various treatments

Table I. Analysis of cell cycle changes in BxPC3 and Panc-1*

	Treatment	G0/G1	S	G2-M fraction
FACS analysis of cell cycle in BxPC3 cells				
	Serum starved for 60 hr	65 ± 5	25 ± 4	11 ± 1
24 hr				
	10% FCS	45 ± 4	41 ± 3	14 ± 4
	10% FCS + 5 mmol/L NaSal	76 ± 2†	11 ± 2†	13 ± 1
48 hr				
	10% FCS	51 ± 2	35 ± 1	14 ± 1
	10% FCS + 5 mmol/L NaSal	69 ± 3†	20 ± 3†	11.3 ± 0.6†
	10% FCS + 5 mmol/L NaSal (24 hr)	44 ± 11 ‡	24 ± 2	32 ± 13
FACS analysis of cell cycle Panc-1 cells				
	Serum starved for 60 hr	70 ± 3	13 ± 2	17 ± 1
24 hr				
	10% FCS	36 ± 6	59 ± 5	5 ± 5
	10% FCS + 5 mmol/L Sal	56 ± 5†	31 ± 10	12 ± 6
48 hr				
	10% FCS	41.8 ± 0.5	47 ± 8	11 ± 8
	10% FCS + 5 mmol/L Sal	65 ± 8	18.7 ± 0.3	16 ± 8
	10% FCS + 5 mmol/L Sal (24 hr)	53 ± 7	37 ± 3	11 ± 10

*Serum starvation induces G0/G1 arrest in both BxPC3 and Panc-1 human pancreatic cancer cell lines. Exposure to 10% FCS stimulates progression from G1 to S phase of the cell cycle, as evidenced by the decreasing G0/G1 fraction and the increase in the S fraction. When 5 mmol/L sodium salicylate (NaSal) is present, this serum-induced progression to S phase is inhibited in both cell lines.
†$P < 0.05$, comparing 10% FCS to 10% FCS + 5 mmol/L NaSal. The effect of NaSal is reversible. When NaSal is removed after 24 hours (10% FCS + 5 mmol/L Sal [24 hr]), both cell lines progress from G1 to S by 48 hours.
‡$P < 0.05$, comparing 10% FCS + 5 mmol/L NaSal to 10% FCS + mmol/L NaSal for 24 hours.

(Table I). After serum starvation, a majority of cells in both cell lines were in the G0/G1 phase. Exposure to 10% FCS resulted in a progression from G1 to S phase. The G0/G1 fractions decreased (65% to 45% in BxPC3 and 70% to 36% in Panc-1) by 24 hours, with concomitant increases in the S fractions (25% to 41% in BxPC3 and 13% to 59% in Panc-1). When 5 mmol/L sodium salicylate was added in addition to 10% FCS, this G1 to S progression was significantly inhibited in both cell lines. Sodium salicylate caused a significant decrease in the M fraction of G2 fraction at 48 hours in BxPC3 cells exposed to serum. This is further evidence that a population of BxPC3 cells that would have progressed through the cell cycle on the addition of 10% FCS remained arrested at G0/G1.

The cell cycle arrest induced by salicylate was reversible. When sodium salicylate was withdrawn at 24 hours, progression from G1 to S phase was evident by 48 hours in both cell lines. No increase in apoptosis was seen after treatment with salicylate (data not shown).

Western Blot Analysis for Cyclin D1 Expression and Cyclin E Expression

Expression of the cyclin proteins D1 and E was determined by Western blot analysis (Fig. 4). Cyclin D1 expression was upregulated after 24 hours of exposure to 10% FCS. This persisted throughout the course of the experiment. Treatment with sodium salicylate at 5 mmol/L inhibited cyclin D1 expression. There was no increase in expression at 24 hours, and by 43 hours after treatment with 10% FCS and 5 mmol/L sodium salicylate, cyclin D1 was markedly reduced.

Cyclin E is a cyclin necessary to drive a cell from G1 to S phase and whose production is induced by cyclin D/cyclin-dependent kinase 4/6 activity. The expression of cyclin E is increased in response to 10% FCS. The addition of 5 mmol/L sodium salicylate inhibits this increase in cyclin E expression. Therefore sodium salicylate decreases mitogen-induced cyclin D1 expression and its downstream effects.

Sodium Salicylate Inhibition of Prostaglandin E_2 Production Is Insufficient to Inhibit Serum-Induced Proliferation

Supernatant prostaglandin E_2 levels determined by ELISA increased with exposure of BxPC3 cells to serum (Fig. 5). Sodium salicylate inhibited serum-induced prostaglandin E_2 production in a dose-dependent manner, as 0.5 mmol/L sodium salicylate and 5 mmol/L sodium salicylate reduced serum-induced prostaglandin E_2 production by 69% and 83%, respectively. The inhibition of serum-induced prosta-

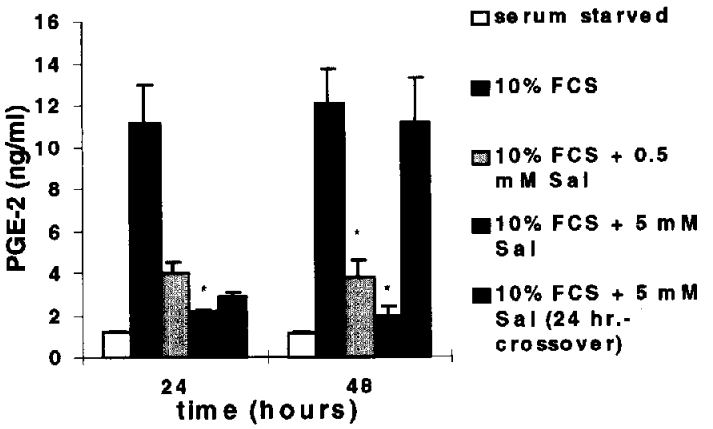


Fig. 5. Supernatant concentrations of prostaglandin E_2 in pancreatic cancer cells treated with serum with and without sodium salicylate *(Sal)*. Concentration of prostaglandin E_2 *(PGE-2)* in the supernatant of cultured cells was determined by ELISA. Cells were serum starved for 66 hours. They were then exposed to serum with or without NaSal (0.5 or 5 mmol/L). To determine reversibility, one sample was exposed to 10% FCS with 5 mmol/L NaSal for 24 hours, at which point the medium was changed to 10% FCS alone. Supernates were harvested at 24 and 48 hours and stored at −80° C until they were assayed for PGE-2. Exposure of quiescent cells to 10% FCS results in an increase in PGE-2 production. Sodium salicylate inhibited PGE-2 production in a dose-dependent manner. At 48 hours, NaSal at 0.5 mmol/L led to a 69% reduction in PGE-2 production by the BxPC3 cell line. The effect of NaSal on PGE-2 production is reversible, as removal of NaSal at 24 hours results in PGE-2 levels at 48 hours equivalent to cells that had not been treated with NaSal (* = $P < 0.05$, n = 3).

glandin E_2 production was reversible. When 5 mmol/L salicylate was removed after 24 hours, the supernatant prostaglandin E_2 levels increased to the levels of serum-treated control cells by 48 hours.

DISCUSSION

The most common genetic abnormalities associated with adenocarcinoma of the pancreas (K-ras, p53, and p16/Ink4a) suggest that altered control of G1 to S phase progression may in part be responsible for neoplastic growth. Indeed virtually all tumors involve abnormalities in one or more cell cycle regulators.[32] Neoplastic growth involves inappropriate proliferation in conditions where cells with normal cell cycle control mechanisms would become arrested. The inhibition of cellular proliferation with induction of G1 cell cycle arrest offers an interesting strategy for therapy in cancer. In essence, agents able to do this would directly compensate for a regulatory mechanism that has been lost in tumor cells. Furthermore, arrest of the cell cycle at G1 may allow cells to enter G0, mature, and terminally differentiate.

Our model used to study events governing G1 to S phase transition appears to be valid for the human pancreatic cancer cell lines used. Serum starvation led to a decrease in the S fraction of cells with an increase in the G0/G1 fraction of cells, implying arrest of cells in the G0/G1 phase. Further, the viability of cells maintained in serum-free medium did not decrease over the course of the experiment. From these two findings we infer that the cells entered quiescence, or the G0 phase. We used serum as a mitogenic stimulus. Exposure to serum resulted in proliferation and G1 to S cell cycle progression of the quiescent cell lines. We have shown that sodium salicylate, a metabolite of aspirin, can decrease cellular proliferation and cause G1 cell cycle arrest in human pancreatic cancer cell lines. Our results are consistent with those of previous studies in colorectal cancer cell lines.[21-24] This effect is immediately reversible on removal of the salicylate.

We questioned whether the concentrations of sodium salicylate used in these assays were physiologically relevant. In humans, serum levels of salicylate less than 1.44 mmol/L are therapeutic, whereas levels greater than 2.17 mmol/L are toxic.[33] We determined that the IC_{50}s for the effect of sodium salicylate on proliferation were 1.7 mmol/L (BxPC3) and 1.2 mmol/L (Panc-1). Thus the levels used would appear to be attainable.

Some investigators studying the effect of NSAIDs on colorectal cancer cells have determined that apoptosis, rather than cell cycle arrest, is the more important mechanism for decreased viability of treated cells with time.[25,26] We have previously reported that higher concentrations of sodium salicylate (>15 mmol/L) do induce apoptosis in the BxPC3 cell line.[34]

In the present study, treatment with sodium salicylate at 5 mmol/L did not lead to an increase in the rate of apoptosis by FACS or by ELISA for interhistone nuclear fragmentation (data not shown). Therefore the decrease in proliferation over time was likely due to an arrest of cell cycle progression rather than programmed cell death.

Sodium salicylate, noted to be far less potent than aspirin as an inhibitor of COX-1 and half as potent an inhibitor of COX-2, significantly reduced the amount of prostaglandin E_2 produced by the BxPC3 pancreatic cancer cells at a dose (0.5 mmol/L), which did not have any effect on cell viability. The concentration of sodium salicylate required for inhibition of prostaglandin E_2 production in our study is consistent with that of prior studies. Mitchell et al.[35] determined the IC_{50}s for inhibition of COX-1 and COX-2 in intact cells were 35 ± 11 μg/ml (0.22 ± 0.07 mmol/L and 100 ± 16 μg/ml (0.6 ± 0.1 mmol/L), respectively. A concentration of 0.5 mmol/L caused an approximately 67% reduction in prostaglandin E_2 production in the BxPC3 cell line. It appears that inhibition of prostaglandin E_2 production is either insufficient or unnecessary for the inhibition of pancreatic cancer cell proliferation by sodium salicylate.

CONCLUSION

We have presented studies demonstrating that sodium salicylate inhibits proliferation in pancreatic cancer cell lines, inducing G1 arrest. These effects are associated with decreased expression of cyclin D1, a protein necessary for mid-G1 phase cell cycle progression. This effect is not due to the ability of sodium salicylate to inhibit prostaglandin E_2 production. This suggests that the chemopreventive effect of NSAIDs is not limited to colorectal cancer but may be generalized to other neoplasms including pancreatic cancer.

REFERENCES

1. Parker SL, Tong T, Bolden S, Wingo PA. Cancer Statistics, 1997. CA Cancer J Clin 1997;47:5-27.
2. Yeo CJ, Abrams RA, Grochow LB, Sohn TA, Ord SE, Hruban RH, Zahurak ML, Dooley WC, Coleman J, Sauter PK, et al. Pancreaticoduodenectomy for pancreatic adenocarcinoma: Postoperative adjuvant chemoradiation improves survival. Ann Surg 1997;225:621-36.
3. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996;86: 353-364.
4. Perugini RA, McDade TP, Vittimberga FJ, Callery MP. Molecular and cellular biology of pancreatic cancer. Crit Rev Eukaryot Gene Expr 1998;8:377-393.
5. Bos JL. p21ras: An oncoprotein functioning in growth factor-induced signal transduction. Eur J Cancer 1995;31A:1051-1054.
6. Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell 1988;53:549-554.
7. Lemoine NR, Hughes CM, Barton CM, Poulsom R, Jeffery RE, Kloppel G, Hall PA, Gullick WJ. The epidermal growth factor receptor in human pancreatic cancer. J Pathol 1992; 166:7-12.
8. Grunewald K, Lyons J, Frohlich A, Feichtinger H, Weger RA, Schwab G, Janssen JWG, Bartram CR. High frequency of Ki-ras codon 12 mutations in pancreatic adenocarcinomas. Int J Cancer 1989;43:1037-1041.
9. Pronk GJ, Bos JL. The role of p21ras in receptor tyrosine kinase signalling. Biochim Biophys Acta 1994;1198:131-147.
10. Redston MS, Caldas C, Seymour AB, Hruban RH, da Costa L, Yeo CJ, Kern SE. p53 mutations in pancreatic carcinoma and evidence of common involvement of homocopolymer tracts in DNA microdeletions. Cancer Res 1994;54:3025-3033.
11. El-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817-825.
12. Xiong Y, Hannon GJ, Zhang H, Casso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature 1993; 366:701-704.
13. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ. The p21 cdk-interacting protein Cip21 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 1993;75:805-816.
14. Chen Z, Zhang H, Savarese T. Gene deletion chemoselectivity: Codeletion of the genes for p16 (INK4), methylthioadenosine phosphorylase, and the alpha- and beta-interferons in human pancreatic cell carcinoma lines and its implications for chemotherapy. Cancer Res 1996;56:1083-1090.
15. Huang L, Goodrow T, Zhang S, Klein-Szanto A, Chang H, Ruggeri B. Deletion and mutation analyses of the p16/MTS-1 tumor suppressor gene in human ductal pancreatic cancer reveals a higher frequency of abnormalities in tumor-derived cell lines than in primary ductal adenocarcinomas. Cancer Res 1996;56:1137-1141.
16. Naumann M, Savitskaia N, Eilert C, Schramm A, Kalthoff H, Schmiegel W. Frequent codeletion of p16/MTS1 and p15/MTS2 and genetic alterations in p16/MTS1 in pancreatic tumors. Gastroenterology 1996;110:1215-1224.
17. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. Nature 1993;366:704-707.
18. Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L, Potes J, Chen K, Orlow I, Lee H-W, et al. The Ink4a tumor suppressor gene product, p19ARF, interacts with MDM2 and neutralizes inhibition of p53. Cell 1998; 92:713-723.
19. Trujillo MA, Garewal HS, Sampliner RE. Nonsteroidal anti-inflammatory agents in chemoprevention of colorectal cancer. At what cost? Dig Dis Sci 1994;39:2260-2266.
20. Giardiello FM, Offerhaus GJA, DuBois RN. The role of non-steroidal antiinflammatory drugs in colorectal cancer prevention. Eur J Cancer 1995;31A:1071-1075.
21. Qiao L, Shiff SJ, Rigas B. Sulindac sulfide inhibits the proliferation of colon cancer cells: Diminished expression of the proliferation markers PCNA and Ki-67. Cancer Lett 1997;115: 229-234.
22. Shiff SJ, Qiao L, Tsai L-L, Rigas B. Sulindac sulfide, an aspirin-like compound, inhibits proliferation, causes cell cycle quiescence, and induces apoptosis in HT-29 colon adenocarcinoma cells. J Clin Invest 1995;96:491-503.
23. Shiff SJ, Koutsos MI, Qiao L, Rigas B. Nonsteroidal antiinflammatory drugs inhibit the proliferation of colon adenocar-

cinoma cells: Effects on cell cycle and apoptosis. Exp Cell Res 1996;222:179-188.
24. Hanif R, Pittas A, Feng Y, Koutsos MI, Qiao L, Staiano-Coico L, Shiff SI, Rigas B. Effects of nonsteroidal antiinflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-dependent pathway. Biochem Pharmacol 1996;52:237-245.
25. Piazza GA, Rahm AK, Finn TS, Fryer BH, Li H, Stoumen AL, Pamukcu R, Ahnen DJ. Apoptosis primarily accounts for the growth-inhibitory properties of sulindac metabolites and involves a mechanism that is independent of cyclooxygenase inhibition, cell cycle arrest, and p53 induction. Cancer Res 1997;57:2452-2459.
26. Elder DE, Hague A, Hicks DJ, Paraskeva C. Differential growth inhibition by the aspirin metabolite salicylate in human colorectal tumor cell lines: Enhanced apoptosis in carcinoma and in vitro-transformed adenoma relative to adenoma cell lines. Cancer Res 1996;56:2273-2276.
27. Higgs GA, Salmon JA, Henderson B, Vane JR. Pharmacokinetics of aspirin and salicylate in relation to inhibition of arachidonate cyclooxygenase and antiinflammatory activity. Proc Natl Acad Sci U.S.A. 1987;84:1417-1420.
28. Barton CM, Staddon SL, Hughes CM, Hall PA, O'Sullivan C, Kloppel G, Theis B, Russel RCG, Neoptolemos J, Williamson RCN, et al. Abnormalities of the p53 tumour suppressor gene in human pancreatic cancer. Br J Cancer 1991; 64:1076-1082.
29. Berrozpe G, Schaeffer J, Peinado MA, Real FX, Perucho M. Comparative analysis of mutations in the p53 and K-ras genes in pancreatic cancer. Int J Cancer 1994;58:185-191.
30. Berger DH, Jardines LA, Chang H, Ruggeri B. Activation of Raf-1 in human pancreatic adenocarcinoma. J Surg Res 1997; 69:199-204.
31. Lavoie JN, L'Allemain G, Brunet A, Muller R, Pouyssegur J. Cyclin D1 expression is regulated positively by the p42/44^{MAPK} and negatively by the p38/HOG^{MAPK} pathway. J Biol Chem 1996;271:20608-20616.
32. Bartek J, Bartkova J, Lukas J. The retinoblastoma protein pathway in cell cycle control and cancer. Exp Cell Res 1997; 237:1-6.
33. Tietz NW. Tietz NW, ed. Fundamentals of Clinical Chemistry. Philadelphia: WB Saunders, 1976, p 1148.
34. McDade TP, Perugini RA, Vittimberga FJ, Carrigan RC, Callery MP. Salicylates inhibit NF-kB activation and enhance TNF-a-induced apoptosis in human pancreatic cancer cells. J Surg Res 1999;83:56-61.
35. Mitchell JA, Akarasereenont P, Thiermermann C, Flower RJ, Vane JR. Selectivity of nonsteroidal anti-inflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proc Natl Acad Sci 1993;90:11693-11697.

Discussion

Dr. J. Matthews (Boston, Mass.). This study builds on findings that COX-2 is overexpressed in colorectal neoplasia. This has led to the use of NSAIDs to inhibit colon cancer growth. NSAIDs appear to work in part by increasing apoptosis. Interestingly, they do so in concentrations that are micromolar and at concentrations that are independent of their effects on COX-2. The evidence for that includes the fact that salicylates and NSAIDs are still antineoplastic in cell lines that are devoid of COX-2, and the fact that NSAIDs that have no effect against COX-2 can also induce apoptosis in cancer cells. The effects that are reported for colorectal neoplasia occur at very low micromolar concentrations, so I would actually turn the conclusions of your study around. Your pancreatic cancer cell lines are resistant to the effects of salicylates compared to other cells. So my questions are (1) why are your cells so resistant to the effects of salicylates, (2) what is the COX-2 status of these cells, and (3) why do you think you are seeing no apoptosis in your cell lines?

Dr. R. Perugini. Sodium salicylate is an interesting NSAID in that it is actually much less potent than other NSAIDs in inhibiting COX-1 and COX-2; it is 100-fold less potent than aspirin at inhibiting COX-1 and twofold less potent at inhibiting COX-2. We are using millimolar concentrations because this is a much less potent NSAID. At the conclusion of the study, I did not believe that the COX activity had much to do with proliferation. The reason we are using high doses is because of the potency of sodium salicylate as opposed to the resistance of the cell lines. To address your questions, we should use NSAIDs that are used in colorectal cancer, specifically sulindac. In regard to apoptosis, sodium salicylate at 15 or 20 mmol/L, which are not really very physiologically relevant doses, induces a significant amount of apoptosis, but at the particular doses we used in this study, it does not.

Dr. J. Drebin (St. Louis, Mo.). I think you are onto some interesting potential mechanisms for identifying agents in pancreatic cancer. My question has to do with your identification of cyclin D as a potential target. Are you hypothesizing a direct effect on cyclin D expression or an effect on some other agents, which themselves then alter cyclin D expression. Is this just a result of cell cycle arrest, as the cyclins themselves are expressed in a cell cycle–dependent fashion? Do you have any information on what would happen if you replaced cyclin D in the cells; would it make them immune to the salicylate effect?

Dr. Perugini. We have not tried to replace cyclin D1 in these cells. If you look at cell cycle progression, by 24 hours the cells have already progressed through G1 and into the S phase. The cylin D1 expression really becomes decreased at 24 hours and thereafter. So I think it is probably not the primary mechanism by which cell cycle progression is inhibited, but just another end effect down the road. The proximal mechanisms remain to be elucidated.

Dr. D. Beauchamp (Nashville, Tenn.). There are COX-2–dependent effects that are required for NSAIDs to have an inhibitory effect on the production of colorectal neoplasia. And I suspect this is true in pancreatic cancer and other malignancies. You have to be very careful about interpreting data where you use pharmacologic inhibitors of COX-2 and

COX-1, and know that you are using these antagonists at dosages that can be achieved physiologically. The work with sulindac sulfone and other agents that are supposedly not inhibitory to prostaglandin production has really shown that it is possible to administer doses in animals or humans that are nontoxic yet inhibit neoplasia. I agree that there are probably nonprostaglandin-mediated effects of the NSAIDs that may play an important role in inhibiting tumorigenesis, but there are also very clear prostaglandin-dependent inhibitory effects that are well demonstrated in both genetic and nongenetic models of tumorigenesis. I would be careful about drawing firm conclusions about the role or lack of a role for COX-2 at this point.

Dr. Perugini. We are merely trying to infer a mechanism. The best way to directly prove whether COX-2 is really related to proliferation in the cell lines is to actually measure COX-2 activity, to prove that COX-2 activity is either affected or unaffected, and to see what it has to do with proliferation in the cell lines, and we have not done that.

A Prospective Analysis of Staging Laparoscopy in Patients With Primary and Secondary Hepatobiliary Malignancies

William R. Jarnagin, M.D., Jessica Bodniewicz, Ellen Dougherty, R.N., M.A., Kevin Conlon, M.D., M.B.A., F.A.C.S., Leslie H. Blumgart, M.D., F.R.C.S., F.A.C.S., Yuman Fong, M.D., F.A.C.S.

Laparoscopy and laparoscopic ultrasound are used widely in cancer staging and are perceived to prevent unnecessary open exploration in many patients. The aim of this study was to analyze the impact of staging laparoscopy in improving resectability in patients with primary and secondary hepatobiliary malignancies. Over a 10-month period (November 1, 1997 to August 31, 1998), 186 patients with primary and secondary hepatobiliary cancers were submitted to operation for potentially curative resection. One hundred four patients staged laparoscopically (LAP) before laparotomy were compared prospectively to 82 patients undergoing exploration without laparoscopy (NO LAP). Assignment to each group was not random but was based on surgeon practice. Demographic data, diagnoses, the extent of preoperative evaluation, and the percentage of patients resected were similar in the two groups. Laparoscopy identified 26 (67%) of 39 patients with unresectable disease. In the NO LAP group, 28 patients (34%) had unresectable disease discovered at laparotomy. In patients with unresectable disease and submitted to biopsy only, the operating times were similar in the two groups (LAP 83 ± 22 minutes vs. NO LAP 91 ± 33 minutes; $P = 0.4$). However, laparoscopic staging significantly reduced the length of hospital stay (LAP 2.2 ± 2 days vs. NO LAP 8.5 ± 8.6 days; $P = 0.006$). Likewise, total hospital charges, normalized to 100 in the NO LAP patients, were significantly lower in the LAP group (LAP 54 ± 42 vs. NO LAP 100 ± 84; $P = 0.02$). Staging laparoscopy identified the majority of patients with unresectable hepatobiliary malignancies, significantly improved resectability, and reduced the number of days in the hospital and the total charges. The yield of laparoscopy was greatest for detecting peritoneal metastases (9 of 10), additional hepatic tumors (10 of 12), and unsuspected advanced cirrhosis (5 of 5) but often failed to identify nonresectability because of lymph node metastases, vascular involvement, or extensive biliary involvement. Eighty-three percent of patients subjected to laparotomy after laparoscopy underwent a potentially curative resection compared to 66% of those who were not staged laparoscopically. (J GASTROINTEST SURG 2000;4:34-43.)

KEY WORDS: Staging laparoscopy, hepatobiliary malignancies

Hepatic resection is the only effective therapy for primary and secondary hepatobiliary malignancies, offering a potential for cure that is not available through other treatment modalities.[1-6] In general, however, resection is considered only when all hepatic disease can be removed completely and there is no extrahepatic disease. Past reports of liver resection in patients with metastatic colorectal cancer revealed a high incidence of unresectable disease that was not suspected on preoperative imaging studies and was discovered only at laparotomy.[7,8] With improvements in imaging techniques, the incidence of unnecessary exploratory operations has declined but remains a significant problem (Table I).[9,10]

From the Department of Surgery, Memorial Sloan-Kettering Cancer Center, New York, N.Y.
Supported in part by grants R01CA76416 (Dr. Fong) and R01CA/DK80982 (Dr. Fong) from the National Institutes of Health.
Presented at the Fortieth Annual Meeting of The Society for Surgery of the Alimentary Tract, Orlando, Fla., May 16-19, 1999.
Reprint requests: William R. Jarnagin, M.D., Department of Surgery, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021. e-mail: jarnagiw@mskcc.org

Table IA. Resectability rates of patients with hepatic colorectal metastases taken to surgery for potentially curative liver resection

Reference	Year	No.	Resected	
			No.	Percent
Fortner et al.[7]	1984	265	75	30
Steele et al.[8]	1991	150	69	46
Gibbs et al.[9]	1998	159	97	61
Jarnagin et al.[10]	1999	534	416	78

Table IB. Selected reports of staging laparoscopy and reduction in unnecessary laparotomy in patients with a variety of hepatobiliary malignancies

Reference	Year	No.	Avoided unnecessary laparotomy (%)
Babineau et al.[12]	1994	29	14 (48)
John et al.[14]	1994	50	32 (64)
Callery et al.[13]	1997	50	22 (44)
Lo et al.[11]	1998	91	15 (16)

The potential benefits of identifying unresectable patients without resorting to a laparotomy are obvious and include reduced length of hospital stay and hospital cost, decreased procedure-related morbidity, and earlier initiation of nonoperative therapy.[11] Staging laparoscopy has been used increasingly to assess extent of disease in patients with intra-abdominal malignancies. Indeed, several early reports have documented the utility of laparoscopy in identifying radiographically occult unresectable disease in patients considered for resection of hepatobiliary cancers (see Table I).[11-14]

The current study is a prospective evaluation of staging laparoscopy at a tertiary care hepatobiliary center and demonstrates that, even in a setting of extensive preoperative imaging and an aggressive approach to tumor extirpation, laparoscopy is a useful tool for assessing resectability.

METHODS

Consecutive patients with potentially resectable hepatobiliary malignancies were staged by laparoscopy and laparoscopic ultrasound. A parallel group of consecutive patients underwent exploratory surgery without laparoscopy. Assignment to each group was not random but was based on surgeon practice. Specifically, two surgeons used laparoscopy and a third did not. Only patients with potentially resectable primary (hepatocellular carcinoma, gallbladder cancer, or hilar or intrahepatic cholangiocarcinoma) or secondary (colorectal or other metastatic) hepatobiliary malignancies, taken to operation for potentially curative resection, were included. Patients with benign liver tumors or a history of previous hepatic resection and those taken to operation for palliative procedures were excluded. On the other hand, prior liver biopsy or upper abdominal surgery (i.e., cholecystectomy) was not grounds for exclusion and these patients were included. Patients usually presented with part of the preoperative investigation having been done by the referring physician. These studies were reviewed by the surgeon and one or more radiologists at a weekly hepatobiliary case management conference; additional studies were obtained as necessary.

In all cases, staging laparoscopy was performed under general anesthesia just prior to planned open exploration and resection. Access to the peritoneal cavity was through a 1 cm incision in the right or left upper quadrant, along the line of the anticipated laparotomy incision, usually in the midclavicular line. A 10 mm Hasson-type trochar was inserted under direct vision (Endopath, Ethicon Endo-Surgery, Cincinnati, Ohio). Pneumoperitoneum was established at a pressure of 15 mm Hg using a high-flow carbon dioxide insufflator (Karl Storz Endoscopy-America, Inc., Culver City, Calif.). A 30-degree angled telescope was then inserted and a second 10 mm trochar was inserted under direct vision in the right anterior axillary line, again along the line of the anticipated laparotomy incision. Additional ports were placed as necessary. The anterior and posterior surfaces of the right and left hepatic lobes, the gastrohepatic omentum, porta hepatis, pelvis, and peritoneal cavity were inspected. The liver was examined sonographically using an Aloka Ultrasound Imaging System (Tokyo, Japan) with a 7.5 MHz flexible laparoscopic probe. Full ultrasonographic examination of all segments of the liver, the portal pedicles, and hepatic veins was generally possible through the right upper quadrant ports. The port in the right anterior axillary line afforded adequate examination of the right posterior sector (segments VI and VII). In some cases where adhesions limited access to the right or left lobe, a small incision in the falciform ligament allowed insertion of the laparoscopic ultrasound probe and examination of the contralateral side. A small number of patients did not undergo laparoscopic ultrasound imaging, either because laparoscopy was not possible or widespread extrahepatic disease was identified.

Laparoscopic examination was considered limited if all areas of interest were not amenable to at least partial inspection and was considered a failure if no relevant areas could be inspected. During examination, suspicious extrahepatic or hepatic lesions, which

might alter management, were sent for biopsy (Jarit Instruments, Hawthorne, N.Y.) and frozen-section histologic analysis. In those patients found to have unresectable disease at laparoscopy, the procedure was either stopped at that point or, in some cases, alternative palliative procedures were performed. In those patients who were subjected to exploratory laparotomy, typically done through an extended right subcostal or chevron incision, the liver was again fully evaluated by palpation and open ultrasound examination, and the peritoneal cavity, pelvis, and retroperitoneum were carefully inspected for evidence of metastatic disease. Again, when unresectable disease was discovered at laparotomy, the procedure was usually terminated but some patients were subjected to palliative procedures.

With few exceptions (see below), the finding of extrahepatic disease in the abdomen (peritoneum, lymph nodes) constituted nonresectability. Likewise, patients with unsuspected advanced cirrhosis were not submitted to resection if the residual liver was considered inadequate for recovery. Patients with hilar cholangiocarcinoma or gallbladder cancer were considered to have unresectable disease if there was extrahepatic or discontiguous intrahepatic metastases, extensive involvement of the biliary tract such that a clear margin of bile duct could not be obtained, or technically insurmountable vascular involvement. Occasionally a tumor adherent to the main portal vein can be resected with portal venous reconstruction, and a small number of patients in both groups underwent exploratory operations with this possibility in mind.[2] In patients with primary tumors of the extrahepatic biliary tract, metastatic disease in the proximal porta hepatis lymph nodes does not necessarily represent unresectable disease, since these nodes are routinely removed, but spread beyond this to the periduodenal, retropancreatic, or celiac nodes precluded resection. Patients with secondary tumors were not submitted to resection if all hepatic disease could not be removed completely. The presence of additional unsuspected tumors in the liver was not necessarily a reason to stop the procedure, and neither the number of tumors nor the presence of bilobar metastases was used as an absolute criterion of resectability, provided that all disease could be resected.[3] A small number of patients with metastatic colorectal cancer and an in situ primary tumor were considered for combined colectomy and hepatic resection.

Data were gathered prospectively and entered into a database. Demographic data, operative findings and procedures performed, length of hospital stay, operating room time, hospital charges, and resectability (proportion of patients submitted to potentially curative resection at open exploration) were analyzed. Hospital charges substituted for cost, which was not available for analysis. The total hospital charges represent the sum of 16 charge categories from the hospital financial database and were calculated from the time of surgery to discharge, as was the length of hospital stay.[15] Given the short period of the study, actual dollar amounts were used without discounting. For presentation purposes, the actual dollar amounts for the NO LAP patients were normalized to 100, and the charges for patients in the LAP group were adjusted accordingly. However, the actual dollar amounts were used for statistical comparisons. Statistical analyses were performed using SPSS for Windows, version 8.0 (Statistical Package for the Social Sciences, SPSS, Inc., Chicago, Ill.). Continuous variables were compared using Student's t test and categorical variables were compared using a chi-square test. Numeric data are expressed as the mean ± standard deviation unless otherwise indicated; P values less than or equal to 0.05 were considered statistically significant.

RESULTS

Over a 10-month period (November 1, 1997 to August 31, 1998), 186 patients were entered into the study. One hundred four patients underwent staging laparoscopy before laparotomy (93 with laparoscopic ultrasound) and 82 underwent exploration without laparoscopy. Laparoscopic examination was complete in 88 (85%) of 104 but was limited in 14 (13%) of 104 and was not possible in 3 (3%) of 104 because of intra-abdominal adhesions. Laparoscopic ultrasound imaging was not performed in 11 patients, either because laparoscopic access to the peritoneal cavity was not possible or because widespread extrahepatic disease was encountered early in the examination.

Patient Demographics

The mean age and distribution of men and women were similar in the two groups (Table II). The proportion of patients with primary and secondary tumors was not significantly different, with just over half of the patients in each group having a primary hepatobiliary malignancy ($P = 0.7$). However, patients with hepatocellular carcinoma were overrepresented in the LAP group compared to the NO LAP group (19% vs. 5%; $P = 0.004$), while the converse was true for hilar cholangiocarcinoma (9% vs. 19%; $P = 0.03$). Significant differences were not observed for any of the other diagnoses. All diagnoses were confirmed histologically. The groups were also similar with respect to extent of preoperative investigation, with 85% and 88%, respectively, undergoing two or more imaging studies (computed tomography [CT], CT portography, magnetic resonance imaging, duplex ultrasound, or positron emission tomography [PET]).

Table II. Distribution of age, sex, diagnoses, extent of preoperative workup, number resected, and procedures performed in the laparoscopy (LAP) and no laparoscopy (NO LAP) groups

	LAP	NO LAP
Age (yr)	64 ± 12	63 ± 13
Male/Female	60/44	49/33
Diagnoses		
Primary tumors	46 (44%)	34 (42%)
Hepatocellular carcinoma	20	4
Hilar cholangiocarcinoma	9	16
Gallbladder carcinoma	9	10
Intrahepatic cholangiocarcinoma	7	3
Sarcoma (primary hepatic)	1	1
Secondary tumors	58 (56%)	48 (58%)
Colorectal	40	35
Breast	5	1
Ovarian	3	0
Neuroendocrine	2	1
Squamous cell carcinoma	2*	1
Sarcoma	2	3
Adrenocortical carcinoma	1	1
Carcinoid	1	1
Melanoma	1	3
Thyroid	1	0
Gastrinoma	0	1
Renal cell carcinoma	0	1
TOTAL	104	82
Two or more preoperative studies	88 (85%)	73 (88%)
Resected	65/104 (63%)	54/82 (66%)
Procedures in resected patients		
Resection of two or more hepatic segments	58 (89%)	43 (80%)
En bloc caudate lobe resection	10 (15%)	6 (11%)
Concomitant biliary reconstruction	9 (14%)	11 (20%)

Resected refers to number and proportion of patients in each group who underwent a potentially curative resection. The percentages listed under *Procedures* reflect percentages with respect to total resected.
*One patient thought to have metastatic squamous cell cancer was found to have a benign lesion (fibronodular hyperplasia) at laparoscopy and was therefore not resected.

The proportion of patients who ultimately underwent a potentially curative hepatic resection was 63% in the LAP group and 66% in the NO LAP group ($P = 0.6$), and the extent and complexity of the resections performed were likewise similar (see Table II).

Operative Findings

The outcome of the laparoscopically staged patients is detailed in Fig. 1. Laparoscopy was not helpful in 53 patients (51%)—42 had no additional findings, seven had additional findings not appreciated at laparoscopy and the planned resection was altered at laparotomy, and four patients had additional findings that did not affect the planned resection. Twenty-six patients (25%) had unresectable disease identified at laparoscopy. Seventeen of these patients were spared an unnecessary laparotomy, whereas nine underwent alternative procedures (cryoablation of liver tumor in 3, hepatic artery pump placement in 1, colectomy in 1, diverting colostomy in 1, biliary bypass in 1, thoracoscopy in 1, and laparotomy for biopsy of extrahepatic disease visualized but not amenable to a laparoscopic biopsy in 1). Ten patients (10%) had additional findings at laparoscopy and underwent potentially curative resection, although it was different from the planned procedure. Twelve patients had unresectable disease not appreciated at laparoscopy, nine of whom underwent laparotomy and biopsy only and three of whom underwent palliative biliary-enteric bypass. Laparoscopy was unsuccessful in three patients, two of whom underwent resection.

Overall, laparoscopic staging identified 26 (67%) of 39 patients with unresectable disease. Additional findings were identified in 40 patients (38%); however, 14 of these patients underwent resection despite the additional findings, whereas 26 were considered to have unresectable disease.

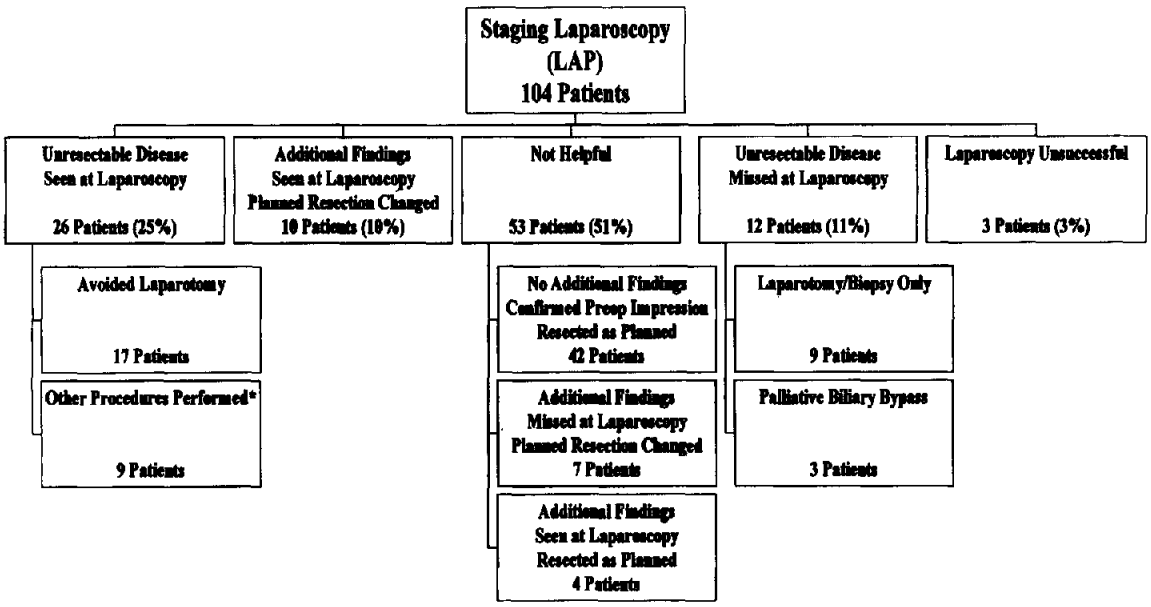


Fig. 1. Flow diagram outlining findings in laparoscopically staged patients *(LAP)*. * = Cryoablation of liver tumor in three, hepatic artery pump placement in one, colectomy in one, diverting colostomy in one, biliary bypass in one, and thoracoscopy in one; one patient required a laparotomy for biopsy of extrahepatic disease visualized but not amenable to a laparoscopic biopsy.

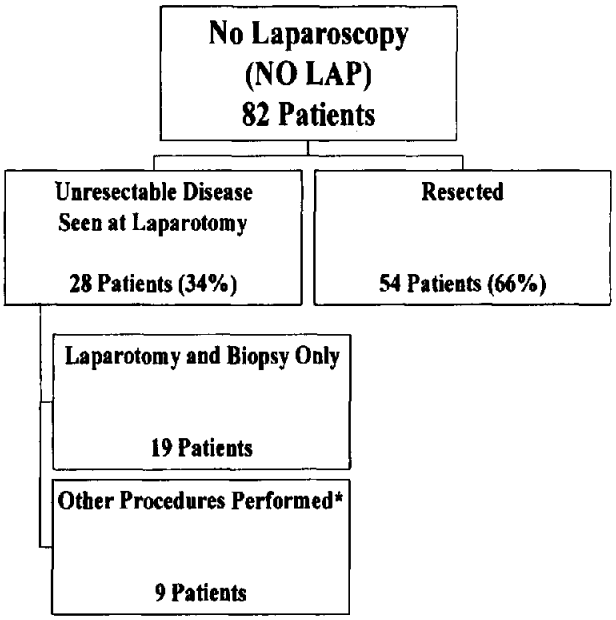


Fig. 2. Flow diagram outlining the findings in patients undergoing exploration without laparoscopy *(NO LAP)*. * = Biliary bypass in six, palliative resection in one, colectomy in one, and abscess drainage in one.

Table III. Summary of operating time, length of hospital stay, and hospital charges* in the laparoscopy (LAP) and no laparoscopy (NO LAP) groups

A. Patients in the LAP group with *unresectable disease* identified laparoscopically and subjected to biopsy only compared to corresponding patients with unresectable disease in the NO LAP group

	Unresectable—Biopsy only		
	LAP (n = 17)	NO LAP (n = 19)	*P* value
Operating time (min)	83 ± 22	91 ± 33	0.4
Length of hospital stay (days)	2.2 ± 2	8.5 ± 8.6	0.006
Total hospital charges	54 ± 42	100 ± 84	0.05
Room and board	34 ± 41	100 ± 103	0.02
Supplies	142 ± 120	100 ± 122	0.3
Recovery room	80 ± 28	100 ± 48	0.1
Operating room	119 ± 46	100 ± 13	0.1

B. Similar analysis extended to include *all* patients in the LAP and NO LAP groups

	All patients		
	LAP (n = 104)	NO LAP (n = 82)	*P* value
Operating time (min)	246 ± 125	184 ± 87	0.0001
Length of hospital stay (days)	8.6 ± 6.6	11.9 ± 8.7	0.004
Resectability†	(63/85) 83%	(54/82) 66%	0.01
Total hospital charges	80 ± 42	100 ± 67	0.004
Room and board	74 ± 68	100 ± 72	0.014
Supplies	100 ± 102	100 ± 84	0.99
Recovery room	89 ± 38	100 ± 35	0.06
Operating room	112 ± 39	100 ± 31	0.02

*Hospital charges are normalized to 100 in the NO LAP patients.
†Refers to proportion of patients submitted to potentially curative resection at open exploration.

Of the 82 patients not staged laparoscopically, 28 had unresectable disease—19 underwent laparotomy and biopsy only and nine underwent other procedures (biliary bypass in 6, palliative resection in 1, colectomy in 1, and abscess drainage in 1) (Fig. 2).

Comparative Results (Staging Laparoscopy vs. No Laparoscopy)

The two groups were then compared with respect to operating time, length of hospital stay, and hospital charges (Table III). The initial analysis compared patients with unresectable disease only, excluding those who underwent alternative palliative procedures (Table III, *A*). Patients in the LAP group identified as nonresectable at laparoscopy and submitted to biopsy only (n =17) were compared to patients with unresectable disease in the NO LAP group, who were also submitted to biopsy only (n = 19). The operating time was not significantly different. However, patients in the LAP group had significant reductions in both length of hospital stay and total hospital charges (Table III, *A*). Hospital room and board charges in the LAP group were reduced by more than 60%, and this was the only individual charge category that was significantly different.

We extended this analysis to include all patients in both groups (Table III, *B*). Operating time in the LAP group was increased by an average of 62 minutes per case. However, hospital length of stay was reduced by more than 3 days (8.6 ± 6.6 vs. 11.9 ± 8.7; $P = 0.004$) and resectability, or the proportion of patients submitted to potentially curative resection at open exploration, was significantly greater (83% vs. 66%; $P = 0.01$), reflecting a reduction in the incidence of unnecessary laparotomies. Moreover, total hospital charges were lower in the LAP group (80 ± 42 vs. 100 ± 67; $P = 0.004$). As in the preceding analysis, the reduction in total charges appears to be largely the result of lower room and board charges. In patients submitted to resection, total charges were lower in the LAP group, but the difference was not statistically significant (83 ± 33 vs. 100 ± 60; $P = 0.06$). The operating room charges were slightly but significantly increased in the LAP group. On the other hand, patients submitted to resection in the LAP and NO LAP groups were not significantly different with respect to length of hospital stay.

Table IV. Analysis of factors that might potentially influence the ability of laparoscopy to identify unresectable disease

A. Laparoscopic identification of unresectable disease stratified by the operative finding precluding resection

Operative findings precluding resection	Proportion of patients with unresectable disease identified at laparoscopy
Extrahepatic disease	10/17 (59%)*
Peritoneal disease	9/10
Lymph node metastases	1/7
Liver related	16/23 (70%)*
Additional tumors	10/12
Cirrhosis	5/5
Vascular invasion	0/2
Extensive biliary involvement	0/3
Tumor benign†	1/1
	$P = 0.4$*

B. Laparoscopic identification of unresectable disease stratified by diagnosis, previous laparotomy, number of tumors, and size of largest tumor

Factor	Proportion of patients with unresectable disease identified at laparoscopy	*P* value
Diagnosis		0.5
Primary tumors	13/21 (62%)	
Secondary tumors	13/18 (72%)	
Previous laparotomy		0.7
Yes	12/19 (63%)	
No	14/20 (70%)	
Number of tumors		0.07
1	10/19 (53%)	
>1	16/20 (80%)	
Size of largest tumor		0.5
≤5 cm	13/18 (72%)	
>5 cm	10/16 (63%)	

*Includes one patient with both extrahepatic disease and additional hepatic disease.
†One patient thought to have metastatic squamous carcinoma was found at laparoscopy to have fibronodular hyperplasia and was not resected.

Factors Affecting Laparoscopic Identification of Unresectable Disease

Several factors that might potentially influence the ability of laparoscopy to identify unresectable disease were analyzed (Table IV). Laparoscopy revealed peritoneal metastases in 9 of 10 patients with this finding, the lone failure occurring in a patient in whom laparoscopy was not possible. However, only one of seven patients with perihepatic lymph node metastases was identified laparoscopically. Similarly, laparoscopy identified the vast majority of patients with unresectable disease due to unsuspected additional hepatic tumors or advanced cirrhosis, but failed in the small number of patients with tumor adherence (not encasement) to major vascular structures or extensive biliary involvement. Laparoscopic identification of unresectable disease ranged from 50% in patients with hilar cholangiocarcinoma to 83% in patients with noncolorectal hepatic metastases. Fifty-nine patients in the LAP group had previously undergone abdominal exploration, but this did not affect the ability of laparoscopy to identify unresectable disease. Similarly, the number of tumors and the size of the largest tumor, both determined at operation, did not significantly affect the ability of laparoscopy to identify unresectable disease. In addition, there was no "learning curve" effect with respect to identifying unresectable disease, since the proportion of patients with unresectable tumors identified laparoscopically was similar throughout the period of study. However, the operating time in the LAP group was significantly lower after the first 2 months of the study (235 ± 162 minutes vs. 299 ± 162 minutes; $P = 0.05$).

Complications

There were two laparoscopy-related complications. One patient incurred a serosal tear of the small bowel, which was repaired laparoscopically, and a second patient developed hypotension related to the

pneumoperitoneum. Of the 17 patients found to have unresectable disease at laparoscopy and submitted to biopsy only, the lone postoperative complication was urinary retention in one patient. Among the corresponding patients in the NO LAP group (n = 19), four had postoperative complications—ileus in two, wound infection in one, and cholangitis in one.

DISCUSSION

The therapeutic benefit of resection for selected patients with primary and secondary hepatobiliary malignancies is well established.[1-6] With few exceptions, resection is appropriate in otherwise fit patients if there is no extrahepatic disease and if a complete tumor resection can be achieved while leaving behind an adequate liver remnant for recovery. Preoperative imaging plays a critical role in assessing the extent of disease and feasibility of resection and in identifying patients with unresectable disease. Despite improvements in imaging technology, a significant number of patients with apparently resectable tumors have unsuspected occult disease and undergo exploratory operations unnecessarily. The consequences are increased time spent in the hospital, delay in nonoperative therapy, and potential procedure-related morbidity.

Several authors have reported marked reductions in the incidence of unnecessary laparotomy using laparoscopic staging. In a series of 50 patients with a variety of hepatobiliary tumors, John et al.[14] reported that laparoscopy and laparoscopic ultrasound spared 32 patients (64%) an unnecessary laparotomy. Likewise, Babineau et al.[12] found unresectable disease in nearly half of a group of 29 patients with primary and metastatic liver tumors, and Callery et al.[13] used laparoscopic staging to identify unresectable disease in 44% of patients. These results differ from our own experience, particularly in patients with metastatic colorectal cancer and hepatocellular carcinoma where resection rates without laparoscopy are 78% and 67%, respectively.[4,10] In addition, a recent report on laparoscopic staging in patients with hepatocellular carcinoma suggested a more modest benefit, with 15 of 91 identified as having unresectable disease at laparoscopy.[11]

These somewhat conflicting results prompted the current prospective evaluation of the benefits of staging laparoscopy and laparoscopic ultrasound in patients with hepatobiliary malignancies. Data from a tertiary care center were examined to determine the positive yield of laparoscopy in the setting of extensive preoperative imaging and an aggressive approach to tumor resection. In addition, the benefits derived from positive laparoscopic findings and avoiding unnecessary laparotomy, as measured by a reduction in the length of hospital stay and hospital charges, were evaluated in a direct comparison to open exploration without laparoscopy.

The results of the present study show a clear benefit of laparoscopic staging in patients with hepatobiliary malignancies. Although this was not a randomized study, the laparoscopically staged patients were similar in nearly all respects to those subjected to open exploration without laparoscopy. All patients were treated by three surgeons who share the same fundamental philosophy regarding the application of resection in this patient population and use a similar operative approach. In addition, all patients were presented and discussed at a weekly multidisciplinary conference on hepatobiliary disease management. Twenty-five percent of the patients in the LAP group had unresectable disease identified laparoscopically. Patients subjected to laparoscopic biopsy alone had a dramatically shorter hospital stay and significantly reduced hospital charges compared to those whose biopsies were performed at laparotomy. Indeed, the hospital stay and charges for the entire group of laparoscopically staged patients were significantly lower compared to those who were not subjected to laparoscopy. These data demonstrate that the increased time and expense of performing laparoscopy in the majority of patients who then undergo resection is justified by the positive yield in the minority of patients.

A major end point of the study was hospital charges. The use of hospital charges to measure the impact of an intervention certainly has precedence in the literature[15,16] but has been criticized because charges are influenced by factors unrelated to actual cost and cannot be used to make comparisons between geographic areas or even neighboring institutions[17]. However, although charges do not equate to cost, they do provide a valid indication of resource consumption.[15]

Additional findings, unsuspected on preoperative imaging, were found in 40 of the LAP patients. Laparoscopy was particularly effective for detecting nonresectability secondary to peritoneal metastases, additional hepatic disease, or advanced cirrhosis, identifying 24 of 27 patients with these findings. By contrast, determining nonresectability because of lymph node metastases, vascular invasion, or extensive biliary involvement proved to be more difficult. The reason for these failures is not clear. Certainly patients with bulky nodal disease or vascular encasement or occlusion, which may be easier to identify at laparoscopy, are generally not considered for exploratory surgery. However, cancer-bearing lymph nodes are not always obviously enlarged, and, in fact, may be relatively small and difficult to detect without palpation. Whether biologic scanning techniques such as ^{18}F-FDG PET scanning will increase the detection rate of occult cancer in such sites remains to be de-

termined. In addition, tumor adherence to major vascular structures and extensive biliary involvement by cancer is often difficult to determine radiographically. This is especially true in primary biliary tract cancers with endoscopic or percutaneous stents in place.[18] Full assessment of these tumors and of the perihepatic lymph nodes often requires an extensive dissection before resectability can be determined unequivocally. In a series of patients with hepatocellular cancer staged with laparoscopy and laparoscopic ultrasound, Lo et al.[11] reported similar difficulties in assessing vascular involvement and invasion of adjacent organs by tumor.[11]

In summary, staging laparoscopy improved resectability, reduced the incidence of unnecessary laparotomy, and reduced the length of hospital stay and hospital charges in patients with hepatobiliary malignancies. Overall, laparoscopy identified two thirds of patients with unresectable disease. No specific factors were identified that significantly affected our ability to identify these patients at laparoscopy. The laparoscopic failure rate because of adhesions was 3%, similar to that reported by John et al.[14] Previous abdominal operation had no impact on the efficacy of laparoscopic staging. Avoiding unnecessary open explorations, even in a small number of patients, greatly outweighed the added time and expense of performing laparoscopy in a large number of patients who did not benefit from the procedure. Laparoscopy is a useful tool in evaluating the resectability of hepatobiliary cancers and should be used routinely.

REFERENCES

1. Bartlett DL, Fong Y, Fortner JG, et al. Long-term results after resection for gallbladder cancer. Implications for staging and management [Review]. Ann Surg 1996;224:639-646.
2. Burke EC, Jarnagin WR, Hochwald SN, et al. Hilar cholangiocarcinoma: Patterns of spread, the importance of hepatic resection for curative operation, and a presurgical clinical staging system. Ann Surg 1998;228:385-394.
3. D'Angelica M, Brennan MF, Fortner J, et al. Ninety-six five-year survivors after liver resection for metastatic colorectal cancer. J Am Coll Surg 1997;185:554-559.
4. Fong Y, Sun RL, Jarnagin WR, Blumgart LH. Hepatocellular carcinoma: An analysis of 412 cases at a Western center. Ann Surg 1999;229:790-799.
5. Harrison LE, Brennan MF, Newman E, et al. Hepatic resection for noncolorectal, nonneuroendocrine metastases: A fifteen-year experience with ninety-six patients. Surgery 1997; 121:625-632.
6. Harrison LE, Fong Y, Klimstra DS, et al. Surgical treatment of 32 patients with peripheral intrahepatic cholangiocarcinoma. Br J Surg 1998;85:1068-1070.
7. Fortner J, Silva JS, Golbey RB, et al. Multivariate analysis of a personal series of 247 consecutive patients with liver metastases from colorectal cancer: I. Treatment by hepatic resection. Ann Surg 1984;199:306-316.
8. Steele G, Bleday R, Mayer RJ, et al. A prospective evaluation of hepatic resection for colorectal carcinoma metastases to the liver: Gastrointestinal tumor study group protocol 6584. J Clin Oncol 1991;9:1105-1112.
9. Gibbs JF, Weber TK, Rodriguez-Bigas MA, et al. Intraoperative determinants of unresectability for patients with colorectal hepatic metastases. Cancer 1998;82:1244-1249.
10. Jarnagin WR, Fong Y, Ky A, et al. Liver resection for metastatic colorectal cancer: Assessing the risk of occult irresectable disease. J Am Coll Surg 1999;188:33-42.
11. Lo CM, Lai EC, Liu CL, et al. Laparoscopy and laparoscopic ultrasonography avoid exploratory laparotomy in patients with hepatocellular carcinoma. Ann Surg 1998;227:527-532.
12. Babineau T, Lewis D, Jenkins R, et al. Role of staging laparoscopy in the treatment of hepatic malignancy. Am J Surg 1994;167:151-155.
13. Callery MP, Strasberg SM, Doherty GM, et al. Staging laparoscopy with laparoscopic ultrasonography: Optimizing resectability in hepatobiliary and pancreatic malignancy. J Am Coll Surg 1997;185:33-39.
14. John TG, Greig JD, Crosbie JL, et al. Superior staging of liver tumors with laparoscopy and laparoscopic ultrasound. Ann Surg 1994;220:711-719.
15. Conlon KC, Ting A, Dougherty E, Brennan MF. The economic effect of the introduction of laparoscopic staging for patients with adenocarcinoma of the pancreas: A prospective analysis of hospital charges. Ann Surg Oncol (in press).
16. Savader SJ, Lillemoe KD, Prescott CA, et al. Laparoscopic cholecystectomy-related bile duct injuries: A health and financial disaster. Ann Surg 1997;225:268-273.
17. Traverso LW. The laparoscopic surgical value package and how surgeons can influence costs. Surg Clin North Am 1996; 76:631-639.
18. Blumgart LH, Benjamin IS. Cancer of the bile ducts. In Blumgart LH, ed. Surgery of the Liver and Biliary Tract. Edinburgh: Churchill Livingstone, 1994, pp 967-995.

Discussion

Dr. H. Pitt (Milwaukee, Wis.). This is a very nice analysis, but I really think we need more information to put it into proper perspective. Much of it has to do with what your philosophy is in managing each of these tumors. First, let me focus on liver metastases. For example, if your philosophy is not to use cryotherapy and not to treat bilateral disease, and to select only patients with a few lesions, then this makes sense. But if your philosophy is to treat more tumors with cryotherapy than three or four, then I am not sure that this makes sense. I also think we need a breakdown by hepatocellular, gallbladder, biliary, and secondary tumors. I can see where this makes a lot of sense, for exam-

ple, in gallbladder cancer where the resectability rate is very low and peritoneal metastasis is very common. I am not so sure that it makes sense in hilar cholangiocarcinoma.

Dr. W. Jarnagin. I agree that larger studies and those focusing on individual diagnostic groups are necessary. Some of the data you request appear in the manuscript, but were a bit too much to add to the oral presentation. Our philosophy of resection is an aggressive one and that plays a role. If you are going to back away from resection because of an unsuspected additional solitary tumor that in and of itself may be resectable, you are correct this will certainly make much more sense.

It is a bit arbitrary to divide patients into those with primary and those with secondary tumors. Patients with primary tumors, from our initial analysis of the data, seemed to benefit more from laparoscopy. But within that group, the patients with hilar cholangiocarcinoma are going to be unresectable for different reasons than those with hepatocellular carcinoma, so additional analysis is necessary.

Dr. S. Helton (Chicago, Ill.). If you look only at your patients with hepatocellular carcinoma, to what degree did the laparoscopy change your management? Second, I do not understand why you have a 2-day length of stay for those patients who were judged unresectable by laparoscopy. Most of the time those patients can go home the same day. The psychological issues can be dealt with if the patients are told beforehand what will happen if they are found to have unresectable disease.

Dr. Jarnagin. There were approximately 30 to 35 patients with hepatocellular cancer in the entire study. Laparoscopy identified five of five patients who were unresectable in that arm. In the no laparoscopy group, there was a smaller number of patients with hepatocellular cancer; I believe there were only five in that group, and three of them underwent resection and two did not. So I do not think the numbers are quite large enough to make an individual comparison just yet. The average length of stay in unresected patients was 2 days because some of the patients in that group underwent biliary drainage procedures performed by radiologists, which increased the length of hospital stay.

Dr. J. Ponsky (Cleveland, Ohio). You clearly demonstrated that laparoscopy could predict unresectability in these patients, who then went on to various types of palliation. Did you look at the survival of or quality of life in those patients who underwent methods of palliation other than surgical palliation, such as transhepatic or endoscopic biliary drainage?

Dr. Jarnagin. In this study we did not look at quality of life issues at all. That is being addressed in other studies, however.

Dr. S. Strasberg (St. Louis, Mo.). This question also pertains somewhat to the discussion we were having at the end of the preceding presentation. If one looks in the literature, one finds two types of articles that have examined this issue. First are studies in which the authors have actually performed laparoscopy, and there have been a number from your institution, as well as from Dr. Warshaw's, ours, and Edinburgh. There is a highly consistent figure that comes from studies in which laparoscopy has actually been performed. It is about 20% to 25% positive. There is another group of studies in which the authors have not actually performed laparoscopy. They have looked at laparotomy findings and asked: "If we had performed laparoscopies in these patients, what would the value of laparoscopy have been?" That figure consistently comes out to be approximately 10%. Why this discrepancy? It might be that we see things at laparoscopy that we do not see at laparotomy. Many times I have been glad that I performed a laparoscopy because I saw a very small lesion and I wondered if I would have seen it with laparotomy. So my question for you is this: "Does laparoscopy not only save futile laparotomies, but does it also save futile resections?" Have you analyzed the lesions you detected that led you not to proceed? How small were these lesions and do you think you would have detected them at laparotomy?

Dr. Jarnagin. I do not know whether laparoscopy prevents futile resections. In our series there were a number of patients with perihepatic nodal involvement that we did not see at laparoscopy. These nodes were not enlarged and possibly the way they were discovered at laparotomy was by feeling them. In terms of the size of the tumors that led us not to pursue resection, it was quite variable. The specific findings that led us away from resection were also variable. Generally, though, the criteria we used for pursuing resection were those stated in the beginning—absence of extrahepatic disease and the ability to clear all hepatic disease with a resection.

Dr. L. Way (San Francisco, Calif.). You were performing laparoscopy and ultrasound imaging. In analyzing your results, did you separate the diagnostic contribution of each of these procedures? If so, what was the contribution of ultrasound and how did you validate your ultrasonographic judgments?

Dr. Jarnagin. In 10 patients, laparoscopic ultrasound identified additional findings beyond those seen by laparoscopic inspection of the liver and porta hepatis. But in that group of 10 patients, seven went on to resection. So, really, laparoscopic ultrasound spared only three patients an unnecessary laparotomy.

Dr. J. Hoffman (Philadelphia, Pa.). First, what was the selection bias in your study: Why did some patients undergo laparoscopy and some not? Second, could many of the peritoneal metastases be found by a minilaparotomy, which might have allowed the patient to go home the next day?

Dr. Jarnagin. Regarding selection bias, it was based on surgeon practice. Specifically, two surgeons used laparoscopy and one did not. Some of the peritoneal disease would have been easily discovered by minilaparotomy.

Preconditioning Protects Against Ischemia/Reperfusion Injury of the Liver

Bengt Nilsson, M.D., Ph.D., Styrbjörn Friman, M.D., Ph.D., Bengt I. Gustafsson, M.D., Ph.D., Dick S. Delbro, M.D., Ph.D.

Ischemic preconditioning (IPC) of an organ may induce protection against the injury caused by longer duration of ischemia and subsequent reperfusion. In a standardized model of such injury in the rat liver, we used the following protocol to investigate whether adenosine played a role in IPC by preventing its enzymatic degradation by dipyridamole pretreatment according to the following protocol: group 1, non-ischemic control rats; group 2, ischemic control rats subjected to 60 minutes of ischemia by clamping of the common hepatic artery followed by 60 minutes of reperfusion; group 3, IPC with 10 minutes of ischemia followed by 15 minutes of reperfusion, prior to the ischemia/reperfusion period as in group 2; group 4, pharmacologic preconditioning with administration of dipyridamole prior to the ischemia/reperfusion period as in group 2. Peripheral liver blood flow was significantly reduced during clamping (groups 2 to 4). After unclamping, blood flow was still reduced in the ischemic rats (group 2) but had returned to preclamp values in the animals that had been subjected to ischemic (group 3) or pharmacologic (group 4) preconditioning. Liver cell injury was significantly increased in the ischemia group (group 2) only. In our experimental model of ischemia/reperfusion injury in the rat liver, we found an equally beneficial effect with ischemic and pharmacologic preconditioning. Adenosine appears to be a crucial factor in IPC. (J GASTROINTEST SURG 2000;4:44-49.)

KEY WORDS: Preconditioning, ischemia, adenosine

Tissue exposed to a brief period of vascular occlusion prior to a subsequent longer episode of ischemia will suffer fewer harmful effects than corresponding tissues that have not been subjected to such ischemic preconditioning (IPC). This phenomenon was first described in canine myocardium.[1] In other experimental models, in the rat, mouse, and dog, IPC has been shown to protect skeletal muscle, brain, small intestine, and liver from ischemic damage.[2-4]

IPC may reduce infarction size in skeletal muscle, improve postischemic capillary perfusion, reduce leukocyte infiltration, and diminish the production of reactive oxygen metabolites in tissues subsequently exposed to ischemia/reperfusion.[3] The exact mechanism underlying the protective effect of IPC remains uncertain. With respect to the heart, it has been suggested that IPC causes release of vasoactive substances from the tissue such as nitric oxide (NO), adenosine, prostacyclin, and bradykinin, which could confer protection against the ischemic insult.[5]

With particular emphasis on the purine nucleoside, adenosine, this agent was found to be released during IPC in the rabbit heart, which seemingly rendered the heart resistant to subsequent infarction via an activation of A_1 receptors on the myocytes. Such protection was also evident by the exogenous administration of adenosine. Adenosine has also been reported to serve as a mediator of IPC in other tissues such as mouse cremaster muscle and rat mesentery.[3] Furthermore, Peralta et al.[6] suggested that IPC of the liver may be mediated by the inhibitory action of NO on the vasoconstrictor property of endothelin. Subsequently

From the Department of Surgery, Sahlgrenska University Hospital, Göteborg, Sweden.
Presented at the Thirty-Ninth Annual Meeting of The Society for Surgery of the Alimentary Tract, New Orleans, La., May 17-20, 1998 (poster presentation).
Supported by the Swedish Medical Research Council (11611 and 17x-04984), the Swedish Medical Society, the Lars-Erik Gelin Fund, and the Göteborg Medical Society.
Reprint requests: Bengt Nilsson, M.D., Ph.D., Institute for Surgical Sciences, Sahlgrenska University Hospital, S-413 45 Göteborg, Sweden. e-mail: bengt.nilsson@mbox353.swipnet.se

these authors suggested that NO is the final mediator of IPC in the rat liver, and that adenosine, in fact, causes the induction of NO synthesis.[4,7] In this organ, IPC is seemingly mediated by activation of A_2 adenosine receptors.[8]

In ischemic tissues, adenosine triphosphate is sequentially degraded via adenosine diphosphate to adenosine monophosphate, which is dephosphorylated to adenosine by the enzymatic action of 5'-nucleotidase.[9] Dipyridamole is a nucleoside transport blocker that causes an increase in endogenous interstitial adenosine levels by suppressing the cellular reuptake of this substance[10] and has for many years had a role as a coronary vasodilating agent.[11]

The aim of the present study was to further investigate adenosine as a mediator of IPC of the liver. Specifically we wanted to study whether the administration of dipyridamole might confer a similar protective action as IPC, presumably by elevating endogenous levels of adenosine. By using a standardized model of ischemia/reperfusion injury of rat liver,[12] we addressed this problem by comparing IPC with "pharmacologic preconditioning" by the administration of dipyridamole. Some of the results have been previously published.[13]

METHODS

Animals

The study design was approved by the animal ethics committee of Göteborg University. The experiments were conducted with Wistar rats of either sex weighing 200 to 250 g (B&K Universal, Sollentuna, Sweden). The day-night cycle was constant at 12 hours of light and dark, respectively, and the animals had free access to tap water and pellet chow. The animals were deprived of food for 12 hours before surgery but were allowed water ad libitum.

Surgical Procedures

The rats were anesthetized with pentobarbitone (30 mg/kg intraperitoneally, supplemented when needed by repeated administrations of 10 mg/kg intravenously during the course of the experiment). The right carotid artery and jugular vein were cannulated for blood pressure recording and drug administration, respectively. The liver was exposed by a midline incision. The common hepatic artery was dissected and arranged for the subsequent temporary clamping by a thin rubber band. A laser Doppler miniprobe (model 407; fiber separation = 0.25 mm) with an adhesive miniholder connected to a Periflux 4001 Master flowmeter (Perimed AB, Järfälla, Sweden) was gently placed on the surface of the liver for monitoring of peripheral liver blood flow. The incision was closed with clips. Body temperature was maintained at 37° C by radiant heat.

Evaluation of Peripheral Liver Blood Flow and Liver Cell Injury

In all groups the peripheral liver blood flow was monitored by laser-Doppler flowmetry during the study period (3 times for 60 minutes). This methodology has been used for estimations of relative changes in several organs including the liver.[14] The signal was continuously recorded and analyzed by a computer program (Perisoft, Perimed AB). Minimum and maximum blood flow, respectively, was recorded per time unit. Venous blood samples were collected on three occasions from each animal (see below), and serum alanine aminotransferase (ALT) was analyzed as an index of liver cell injury.

Experimental Protocol

The animals were assigned to one of the following four groups on termination of surgery (Fig. 1).

Group 1: Nonischemic Control rats (n = 8). In this group the common hepatic artery had only been dissected but was not subsequently clamped. These animals were monitored for a total of 180 minutes, and peripheral liver blood flow was estimated during the last 30 minutes of each 60-minute period (these last 30 minutes denoted basal, sham clamp, and post–sham clamp values). Blood samples for ALT determination were drawn at the end of each 60-minute period.

Group 2: Ischemic Control rats (n = 8). After 60 minutes of equilibration, the common hepatic artery was clamped for 60 minutes, and thereafter the liver was reperfused over 60 minutes. Peripheral liver blood flow was estimated during the last 30 minutes of each 60-minute period (these last 30 minutes denoted basal, clamp, and postclamp values). Blood samples for ALT determination were drawn at the end of each 60-minute period.

Group 3: Ischemic preconditioning (n = 11). After 35 minutes of equilibration, the liver was subjected to 10 minutes of clamping of the common hepatic artery, followed by 15 minutes of reperfusion. Thereafter the artery was clamped for 60 minutes, and the liver was then finally reperfused over 60 minutes. Peripheral liver blood flow was estimated during the 30 minutes immediately prior to IPC (denoted basal value) and then the last 30 minutes of the ischemic and reperfusion periods (these last 30 minutes denoted clamp and postclamp values, respectively). Blood samples for ALT determination were drawn at the end of each 60-minute period.

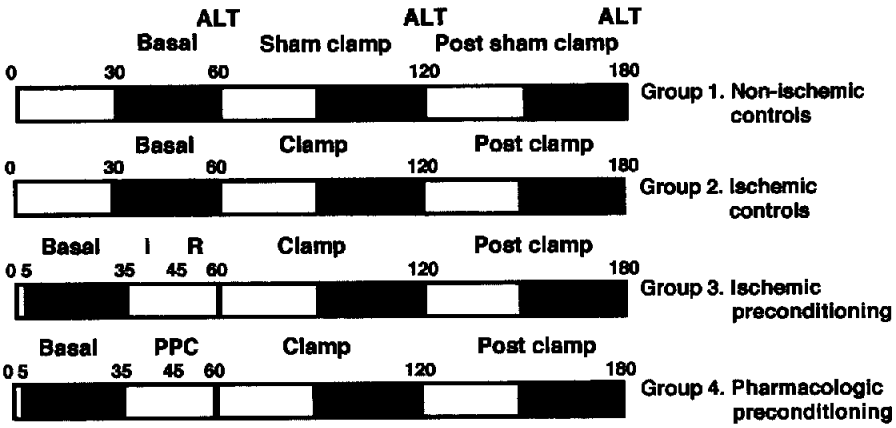


Fig. 1. Experimental design. *Group 1*, Animals were exposed to anesthesia, laparotomy, and dissection but not to subsequent clamping of the common hepatic artery; *Group 2*, After 60 minutes of equilibration, there followed 60 minutes of ischemia and thereafter 60 minutes of reperfusion; *Group 3*, After 35 minutes of equilibration, ischemic preconditioning was induced by 10 minutes of ischemia *(I)* and 15 minutes of reperfusion *(R)* before the the actual 60-minute period of ischemia, followed by 60 minutes of reperfusion; *Group 4*, After a 35 minute equilibration period, pharmacologic preconditioning *(PPC)* was accomplished by the injection of dipyridamole (0.4 mg/kg intravenously). This was followed after 25 minutes by 60 minutes of ischemia and 60 minutes of reperfusion. Peripheral liver blood flow was estimated during the last 30 minutes of each 60-minute period, except in groups 3 and 4 where instead the 30 minutes immediately prior to the preconditioning was used as the basal period (denoted by black areas). Blood samples for alanine aminotransferase *(ALT)* determination were drawn at the end of each 60-minute period.

Group 4: Pharmacologic preconditioning (n = 11). After 35 minutes of equilibration, dipyridamole was administered (0.4 mg/kg intravenously). Twenty-five minutes thereafter, the common hepatic artery was clamped for 60 minutes, and the liver was then finally reperfused over 60 minutes. Peripheral liver blood flow was estimated during the 30 minutes immediately prior to IPC (denoted basal value) and then during the last 30 minutes of the ischemic and reperfusion periods (these last 30 minutes denoted clamp and postclamp values, respectively). Blood samples for ALT determination were drawn at the end of each 60-minute period.

Bioactive Substances and Solutions

Dipyridamole (Persantin) and pentobarbitone (pentobarbital sodium) were purchased from Boehringer Ingelheim International GmbH (Ingelheim/Rhein, Germany) and from Apoteksbolaget (Umeå, Sweden), respectively. A commercially available kit from Boehringer-Mannheim (Munich, Germany) was used for the determination of serum ALT.

Statistical Analyses and Presentation of Data

Peripheral liver blood flow was expressed as percentage of change from basal value in each animal, or as arbitrary units when relevant. ALT was expressed as percentage of change from preclamp values, or as μkat/L when relevant. All data are presented as means ± standard error. Statistical analyses were performed by analysis of variance followed by the Scheffe F test for multiple comparisons. A P value <0.05 was considered statistically significant.

RESULTS

Peripheral Liver Blood Flow

In the nonischemic control rats (group 1), peripheral liver blood flow did not change significantly during the course of the experiment (Fig. 2). In group 2, clamping of the common hepatic artery resulted in a significant decrease in peripheral liver blood flow compared with basal values, and also with sham clamp values, and during the postclamp period blood flow remained significantly lower compared with basal values, and also when compared with post–sham clamp values (see Fig. 2). In groups 3 and 4 it was first determined whether IPC or pharmacologic preconditioning, respectively, resulted in any change in basal peripheral liver blood flow. Thus the 25-minute period following either intervention was compared with the 25 minutes immediately prior to these events. Because there was no significant difference between these periods (group 3, 104.5 ± 10.5 vs. 94.4 ± 9.8 [P = 0.5]; group 4, 101.2 ± 10.3 vs. 104.3 ± 13.5 [P = 0.95]; presented as arbitrary units), it may be

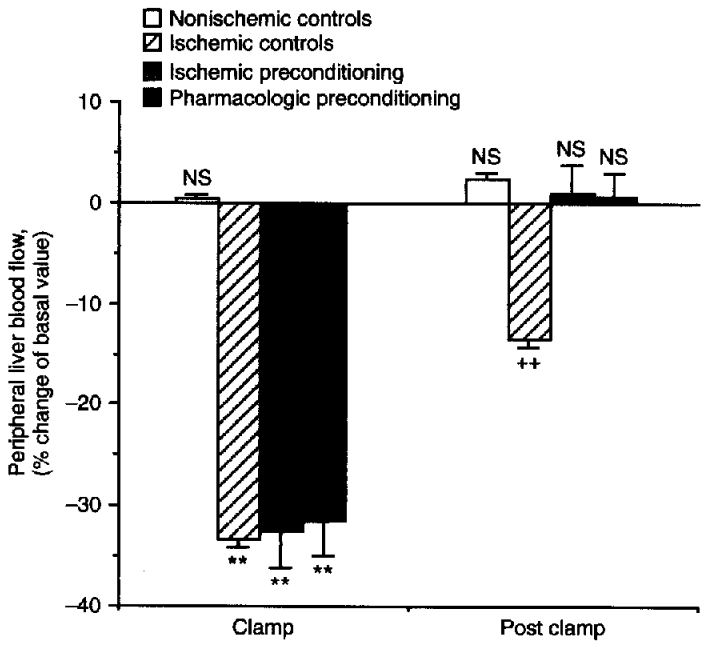


Fig. 2. Peripheral liver blood flow, expressed as percentage of change in blood flow during the basal period. NS = no significant change compared with basal value. ** = $P < 0.01$, clamp value vs. basal, and also vs. sham clamp value of the nonischemic control group. ++ = $P < 0.01$ vs. basal, and also vs. post-sham clamp value of the nonischemic control group.

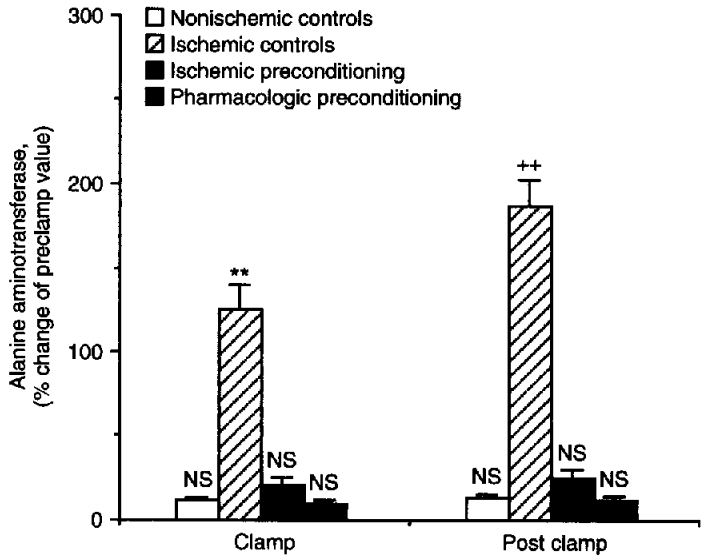


Fig. 3. Alanine aminotransferase, expressed as percentage of change in preclamp value. NS= no significant change compared with preclamp value. ** = $P < 0.01$, clamp value vs. preclamp, and also vs. sham clamp value of the nonischemic control group. ++ = $P < 0.01$ vs. preclamp, and also vs. post-sham clamp value of the nonischemic control group.

concluded that neither preconditioning treatment disturbed basal conditions. Further analyses showed that clamping of the common hepatic artery resulted in a decrease in peripheral liver blood flow to the same extent as in group 2 (see Fig. 2). After unclamping, however, blood flow returned to a level not significantly different from basal or post-sham clamp values (see Fig. 2). Thus either type of preconditioning is seemingly equipotent with regard to preventing the postclamp change in peripheral liver blood flow.

Ischemia/Reperfusion Injury

The preclamp values for ALT (μkat/L) were 0.51 ± 0.03 (group 1), 0.48 ± 0.03 (group 2), 0.65 ± 0.04 (group 3), and 0.52 ± 0.03 (group 4), respectively, which did not differ significantly. Thus the preconditioning interventions as undertaken in groups 3 and 4 did not by themselves elicit liver cell damage. There was no significant alteration of ALT values in the nonischemic control rats, whereas clamping caused a significant increase in the ischemic control rats (group 2). Such injury was, however, completely prevented by either ischemic (group 3) or pharmacologic (group 4) preconditioning (Fig. 3).

DISCUSSION

Our method of warm ischemia/reperfusion injury in the rat liver has been presented briefly in a previous

report.[12] In our view this model may offer some advantages over the model used in this species in most other laboratories (implying interruption of both arterial and portal blood flow; Pringle's maneuver), since the insult to the liver appears to be far less advanced and therefore less definite. Because the portal circulation is spared, secondary effects resulting from venous congestion in the gut are eliminated. Histopathologically, the acute as well as the long-term effects of 60 minutes of clamping of the common hepatic artery result in mild changes or none at all (unpublished findings). Moreover, biochemically (as monitored by serum levels of ALT) the extent of liver cell damage appears to be fairly limited (but significant compared to control rats) and, finally, peripheral liver blood flow, reflecting postischemic microvascular integrity, is only modestly impaired (but significant compared to control rats) on unclamping as shown by the present study and the earlier one.[12] Of importance is our finding that the deranged variables caused by clamping and unclamping of the common hepatic artery may be circumvented by ischemic or pharmacologic preconditioning procedures.[12,13]

In the current study we found that pharmacologic preconditioning by the nucleoside transport inhibitor dipyridamole was equally efficient as IPC in preventing disturbances of blood flow and cell damage. In preliminary (unpublished) experiments we found that the dosage used was optimal for pharmacologic preconditoning. In a previous report we also found that pharmacologic preconditioning with the substrate for NO synthase, L-arginine, also totally prevented the disturbance of these variables.[12] When taken together, these data could suggest a role for both adenosine and NO in IPC.

Our results with dipyridamole are in concert with those of Todo et al.,[15] which were obtained in dogs. These authors used another nucleoside transport inhibitor, viz. R75231; this compound has no effect on phosphodiesterase and prostacyclin.[16] Thus after total hepatic vascular exclusion for 2 hours, R75231 was found to improve postreperfusion hepatic blood flow and diminish liver cell damage, leading to 100% 2-week animal survival. Moreover, in a rabbit model of IPC of the heart, a potentiating action of dipyridamole on the beneficial effect of IPC was noted, which was significantly attenuated by the adenosine receptor blocker, 8-phenyltheophylline.[16,17] In a model of 10 minutes of total ischemia of the rat liver, Nakayama et al.[18] found that the closely similar antagonist, 8-sulphophenyltheophylline, abolished the effect of IPC. Likewise, it seems highly probable that the beneficial effect of dipyridamole, as noted in the current study, is due to an enhancement of available tissue adenosine. Interestingly, in the rat heart (in contrast to several other species), adenosine appears to be of only minor importance for IPC,[19] whereas in the liver this compound seemingly plays a critical role.[4,7] This discrepancy suggests that IPC is probably dependent on different mechanisms in different organs of one and the same species.

The question arises as to what is the exact relationship between adenosine and NO with regard to IPC in the rat liver. Thus, according to Peralta et al.,[4,7] the brief period of ischemia leads to a temporary accumulation of adenosine in the liver tissue, which in turn causes the induction of NO, possibly in vascular endothelial cells.[20] This view, if valid, does not, however, explain the exact mechanism behind IPC. Adenosine, either by itself or via the generation of NO, could possibly protect the liver by vasodilation, inhibition of neutrophil infiltration, and preservation of the liver microvasculature.[15] As an alternative hypothesis, Todo et al.[15] proposed that an interstitial accumulation of adenosine could lead to beneficially enhanced high-energy phosphate resynthesis in the hepatocytes. Moreover, it is interesting that NO appears to induce the production of prostaglandins.[12] Whether NO may influence the tissue levels of adenosine is not known, but adenosine triphosphate could cause the release of NO from the vascular endothelium,[21] as well as stimulate the production of prostaglandins.[22] The role of prostaglandins in (myocardial) preconditioning is, however, controversial.[23,24] Moreover, it is interesting that recent findings strongly suggest that prostaglandins could confer protection against ischemia/reperfusion injury of the liver.[25]

In conclusion, in our model of ischemia/reperfusion injury in the rat liver, we found an equally beneficial effect for ischemic and pharmacologic preconditioning. Adenosine appears to be a crucial mediator of IPC, which could have clinically important consequences.

We gratefully acknowledge Mrs. Monica Wallin for technical assistance.

REFERENCES

1. Murry CE, Jenning RB, Reimer KA. Preconditioning with ischemia: A delay in lethal cell injury in ischemic myocardium. Circulation 1986;74:1124-1136.
2. Lloris-Carsí JM, Cejalvo D, Toledo-Pereyra LH, Calvo MA, Suzuki S. Preconditioning: Effect upon lesion modulation in warm liver ischemia. Transplant Proc 1993;25:3303-3304.
3. Ishida T, Yarimizu K, Gute DC, Korthuis RJ. Mechanisms of ischemic preconditioning. Shock 1997;8:86-94.

4. Peralta C, Hotter G, Closa D, Gelpí E, Bulbena O, Roselló-Catafau J. Protective effect of preconditioning on the injury associated to hepatic ischemia-reperfusion in the rat: Role of nitric oxide and adenosine. Hepatology 1997;25:934-937.
5. Parrat JR. Protection of the heart by ischaemic preconditioning: Mechanisms and possibilities for pharmacological exploitation. Tips Rev 1994;15:19-25.
6. Peralta C, Closa D, Hotter G, Gelpí E, Prats N, Roselló-Catafau J. Liver ischemic preconditioning is mediated by the inhibitory action of nitric oxide on endothelin. Biochem Biophys Res Commun 1996;229:264-270.
7. Peralta C, Closa D, Xaus C, Gelpí E, Roselló-Catafau J, Hotter G. Hepatic preconditioning in rats is defined by a balance of adenosine and xanthine. Hepatology 1998;28:768-773.
8. Arai M, Thurman RG, Lemaster JJ. Ischemic preconditioning protects sinusoidal endothelial cells against storage/reperfusion injury by an adenosine A_2 receptor pathway. Hepatology 1998;28:405A.
9. Van Belle H, Goossens F, Wynants J. Formation and release of purine catabolites during hypoperfusion, anoxia, and ischemia. Am J Physiol 1987;252:H886-H893.
10. Knabb RM, Gidday JM, Ely SW, Rubio R, Berne RM. Effects of dipyridamole on myocardial adenosine and active hyperemia. Am J Physiol 1984;247:H804-H810.
11. Orlandi C. Pharmacology of coronary vasodilation: A brief review. J Nucl Cardiol 1996;3:S27-S30.
12. Nilsson B, Yoshida T, Delbro D, Andius S, Friman S. Pretreatment with L-arginine reduces ischemic and/or reperfusion injury of the liver. Transplant Proc 1997;29:3111-3112.
13. Nilsson B, Delbro D, Friman S. Preconditioning protects against ischemia-reperfusion injury of the liver [abstr]. Gastroenterology 1998;114:A1414.
14. Arvidsson D, Svensson H, Haglund U. Laser-Doppler flowmetry for estimating liver blood flow. Am J Physiol 1988;254: G471-G476.
15. Todo S, Zhu Y, Zhang S, Jin MB, Ishizaki N, Tanaka H, Subbotin V, Starzl TE. Attenuation of ischemic liver injury by augmentation of endogenous adenosine. Transplantation 1997;63:217-223.
16. Itoya M, Miura T, Sakamoto J, Urabe K, Iimura O. Nucleoside transport inhibitors enhance the infarct size-limiting effect of ischemic preconditioning. J Cardiovasc Pharmacol 1994;24:846-852
17. Miura T, Ogawa T, Iwamoto T, Shimamoto K, Iimura O. Dipyridamole potentiates the myocardial infarct size-limiting effect of ischemic preconditioning. Circulation 1992;86:979-985.
18. Nakayama H, Yamamoto Y, Kume M, Yamamoto H, Ozaki N, Yamaoka Y. Elevated adenosine level in the rat liver is an important factor in the effect of ischemic preconditioning to induce tolerance against ischemia-reperfusion injury. Hepatology 1997;26:340A.
19. Meldrum DR. Mechanisms of cardiac preconditioning ten years after the discovery of ischemic preconditioning. J Surg Res 1997;73:1-13.
20. Li J-M, Fenton RA, Cutler BS, Dobson JG Jr. Adenosine enhances nitric oxide production by vascular endothelial cells. Am J Physiol 1995;269:C519-C523.
21. Rubino A, Ralevic V, Burnstock G. Contribution of P1-(A2b subtype) and P2-purinoceptors to the control of vascular tone in the rat isolated mesenteric arterial bed. Br J Pharmacol 1995;115:648-652.
22. Needleman P. Stimulation of prostaglandin biosynthesis by adenine nucleotides. Profile of prostaglandin release by perfused organs. Circ Res 1974;34:455-460.
23. Miura T, Ishimoto R, Sakamoto J, Tsuchida A, Suzuki K, Ogawa T, Shimamoto K, Iimura O. Suppression of reperfusion arrhythmia by ischemic preconditioning in the rat: Is it mediated by the adenosine receptor, prostaglandin, or bradykinin receptor? Basic Res Cardiol 1995;90:240-246.
24. Arad M, Oxman T, Leor R, Rabinowitz B. Prostaglandins and the antiarrhythmic effect of preconditioning in the isolated rat heart. Mol Cell Biochem 1996;160-161:249-255.
25. Klein M, Geoghegan J, Wangemann R, Bockler D, Schmidt K, Scheele J. Preconditioning of donor livers with prostaglandin I_2 before retrieval decreases hepatocellular ischemia-reperfusion injury. Transplantation 1999;67:1128-1132.

Suppression of Gastric Acid Secretion in Patients With Gastroesophageal Reflux Disease Results in Gastric Bacterial Overgrowth and Deconjugation of Bile Acids

Jörg Theisen, M.D., Dhiren Nehra, F.R.C.S., Diane Citron, M.D., Jan Johansson, M.D., Jeffrey A. Hagen, M.D., Peter F. Crookes, M.D., Steven R. DeMeester, M.D., Cedric G. Bremner, M.D., Tom R. DeMeester, M.D., Jeffrey H. Peters, M.D.

The aim of this study was to test the hypothesis that gastric bacterial overgrowth is a side effect of acid suppression therapy in patients with gastroesophageal reflux disease (GERD) and that the bacteria-contaminated gastric milieu is responsible for an increased amount of deconjugated bile acids. Thirty patients with GERD who were treated with 40 mg of omeprazole for at least 3 months and 10 patients with GERD who were off medication for at least 2 weeks were studied. At the time of upper endoscopy, 10 ml of gastric fluid was aspirated and analyzed for bacterial growth and bile acids. Bacterial overgrowth was defined by the presence of more than 1000 bacteria/ml. Bile acids were quantified via high-performance liquid chromatography. Eleven of the 30 patients taking omeprazole had bacterial overgrowth compared to one of the 10 control patients. The median pH in the bacteria-positive patients was 5.3 compared to 2.6 in those who were free of bacteria and 3.5 in the control patients who were off medication. Bacterial overgrowth only occurred when the pH was >3.8. The ratio of conjugated to unconjugated bile acids changed from 4:1 in the patients without bacterial overgrowth to 1:3 in those with bacterial growth greater than 1000/ml. Proton pump inhibitor therapy in patients with GERD results in a high prevalence of gastric bacterial overgrowth. The presence of bacterial overgrowth markedly increases the concentration of unconjugated bile acids. These findings may have implications in the pathophysiology of gastroesophageal mucosal injury. (J GASTROINTEST SURG 2000;4:50-54.)

KEY WORDS: Bacterial overgrowth, omeprazole, deconjugation

The primary treatment of gastroesophageal reflux disease (GERD) is acid suppression therapy using proton pump inhibitors. A significant proportion of patients require chronic or lifelong therapy for continuous relief of symptoms.[1] Although acid suppression therapy can relieve the symptoms of GERD and heal esophagitis, it also allows bacterial overgrowth in the normally sterile stomach.[2-6] At a normal resting gastric pH <3, ingested bacteria are destroyed within 10 minutes. In contrast, in gastric juice with a resting pH >4, bacterial growth is feasible[7] and when excessive can cause malabsorption,[8] nosocomial pneumonia,[9] or the formation of carcinogenic N-nitroso compounds.[10]

Bile acids have been shown to be an important pathophysiologic factor promoting esophageal mucosal injury.[11-13] In the human proximal gastrointestinal tract, they occur overwhelmingly in their conjugated form, bound to the amino acids glycine and taurine. Deconjugated or free bile acids, formed in the presence of colonic bacteria, are found largely in the distal gastrointestinal tract. Deconjugated bile acids have been shown to be more toxic to gastric mucosa and squamous epithelium than their conjugated counterparts and have significantly different physiochemical properties.[14] We hypothesize that gastric bacterial overgrowth in patients with GERD receiving acid suppression therapy will influence the conjugation

From the Department of Surgery (J.T., J.J., J.A.H., P.F.C., S.R.D., C.G.B., T.R.D., and J.H.P.) and the Microbiology Research Laboratory (D.C.), University of Southern California, Los Angeles, Calif.; and University Hospital Wales (D.N.), Cardiff, U.K.
Reprint requests: Jeffrey H. Peters, M.D., Department of Surgery, University of Southern California, 1510 San Pablo St., Suite 514, Los Angeles, CA 90033-4612.

status of bile acids within the refluxed gastric juice. If this were to occur, then refluxed gastric juice containing unconjugated bile acids would be expected to be highly toxic to the esophageal mucosa. This would result in continuous mucosal injury while the patient remained symptomatically improved.

PATIENTS AND METHODS

Study Population

The study population consisted of 30 patients with symptoms of GERD and increased esophageal acid exposure on 24-hour pH monitoring. There were 12 women and 18 men who had a median age of 54.4 years (range 23 to 73 years). All patients were treated with 40 mg of omeprazole for least 3 months prior to the examination of their gastric juice. The control group consisted of 10 patients, five women and 5 men, who also had symptoms of GERD and increased esophageal acid exposure. They were prohibited from taking any acid–suppressive medications for at least 2 weeks prior to the examination of their gastric juice.

Endoscopy and Gastric Fluid Aspiration

All patients underwent upper endoscopy after an overnight fast using a disinfected endoscope. Biopsy specimens were obtained from the antrum, cardia, and esophagus. *Helicobacter pylori* infection was confirmed by a positive Giemsa stain.

At the time of endoscopy 10 ml of gastric juice was aspirated with a sterile catheter passed through the biopsy channel of the endoscope (Washing pipe, Olympus, Melville, N.Y.). Five milliliters of the aspirate was injected into a transport tube for anaerobes (Anaerobe Systems, San Jose, Calif.) and cultured within 1 hour. The remaining 5 ml was stored at −20° C and subsequently assayed for bile acids using high-performance liquid chromatography (HPLC). The pH of the aspirated fluid was assessed at the time of endoscopy with a glass probe calibrated with standard solutions of pH 1, pH 4, and pH 7.

Bacterial Cultures

Bacteria were cultured using conventional plating methodology. All isolated anaerobic and aerobic organisms were identified according to standard procedures.[15] Aliquots of 0.01 ml in a 1:100 dilution were plated to establish colonies. For the identification of anerobes and aerobes, blood agar, phenylethyl-alcohol agar, McConkey agar, *Brucella* blood agar, and *Bacteroides fragilis* bile-esculin were used. Bacterial overgrowth was defined by more than 1000 bacteria/ml.

High-Performance Liquid Chromatography

Bile acids were quantified via a recently published modified high-performance liquid chromatography (HPLC) method.[16,17] Briefly, in addition to conventional HPLC technology, a postcolumn derivation step was added to improve the sensitivity and specificity. In this step the individual bile acids were reacted with the enzyme 3-α-hydroxy steroid dehydrogenase using nicotamide adenine dinucleotide (NAD) as cofactor. This resulted in the fluorescent species NADH (nicotamide adenine dinucleotide, reduced form) as its end product allowing the bile acids to be quantified using a Jasco 821-FP fluorescence spectrophotometric detector (Ciba Corning Diagnostics, Halstead, U.K.).

Statistical Analysis

Data for pH values and bile acid concentrations are expressed as medians and interquartile ranges. The overall differences in pH values and bile acid concentrations between the three groups were assessed using the Kruskal-Wallis test. Differences between two groups were analyzed with the Mann-Whitney U test. A P value <0.05 was considered to be statistically significant.

RESULTS

Eleven (37%) of the 30 patients taking proton pump inhibitors were found to having bacterial overgrowth. This compares to only one patient in the control group not taking acid-suppressive medication. When the patients taking proton pump inhibitors were divided into patients with and without bacterial overgrowth, the median pH of the bacteria-positive patients was significantly different from those who were free of bacteria, or the control group not taking acid-suppressive medication (Fig. 1). There was no significant difference in the prevalence of antral *Helicobacter pylori* infection among the groups.

One patient in the control group not taking medication had a gastric pH of 5.7 and bacterial overgrowth. Patients in either group with a gastric pH <3.8 were free of bacterial overgrowth. Table I shows the pH and the bacteria species found in the gastric aspirates.

Median concentrations for all bile salts varied from 68 to 92 μmol/L and did not differ among the three groups (Table II). Patients with bacterial overgrowth had significantly lower concentrations of taurine and glycine conjugates and a reversed ratio of a conjugated:unconjugated (1:3) bile salts when compared to patients on proton pump inhibitors without bacterial overgrowth (3:1) and the control patients off medication (4:1) (Fig. 2). This was statistically significant (P <0.001).

Table I. Gastric pH and bacterial flora in patients with bacterial overgrowth

Medication	pH	Species (>1000/ml)
No	5.7	*Streptococcus*
Yes	3.8	Yeast, *Lactobacillus bifidus*
Yes	6.2	*E. coli*, *Streptococcus*
Yes	5.3	*Neisseria*, *Streptococcus*, *Staphylococcus*
Yes	3.9	*Streptococcus*, *Microphilis*
Yes	6.9	*Neisseria*, *Lactobacillus bifidus*, *Streptococcus*
Yes	5.9	*Streptococcus*
Yes	4.3	*E. coli*, *Aeromonas*, *Candida albicans*
Yes	5.3	*Streptococcus*, *Neisseria*, *Staphylococcus*
Yes	4.2	*Streptococcus*, *Neisseria*
Yes	7.9	*E. coli*, *Staphylococcus*, *Streptococcus*
Yes	5.5	*Streptococcus*

Table II. Median bile acid concentrations

Bile acid	No medication (n = 10)	Medication with no bacteria (n = 19)	Medication with bacterial overgrowth (n = 11)	*P* value
Taurine conjugates	21.5 [6-68]	16 [9-55]	*1.5 [0-6]	*<0.01 vs. bacteria, control
Glycine conjugates	38 [0-138]	41 [7-76]	*7 [0-13]	*<0.01 vs. bacteria, control
Unconjugates	22 [2-34]	22 [2-34]	21 [12-65]	NS
TOTAL	92.5 [0-327.5]	68 [22-154]	69 [0-256]	NS
Ratio conjugated:unconjugated	3:1	4:1	*1:3	*<0.01 vs. bacteria, control

Values for bile acid conjugates are in μmol and [interquartile ranges]; group comparison by Mann-Whitney U test (NS = not significant).

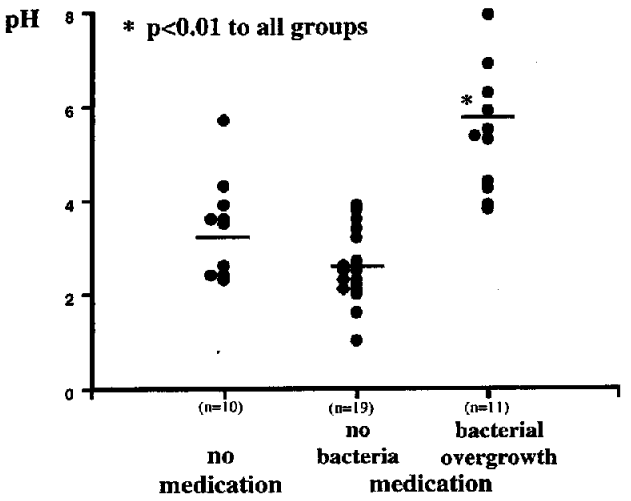


Fig. 1. pH of individual patients off acid suppression therapy (no medication), patients taking omeprazole with no bacteria, and patients taking omeprazole with bacterial overgrowth.

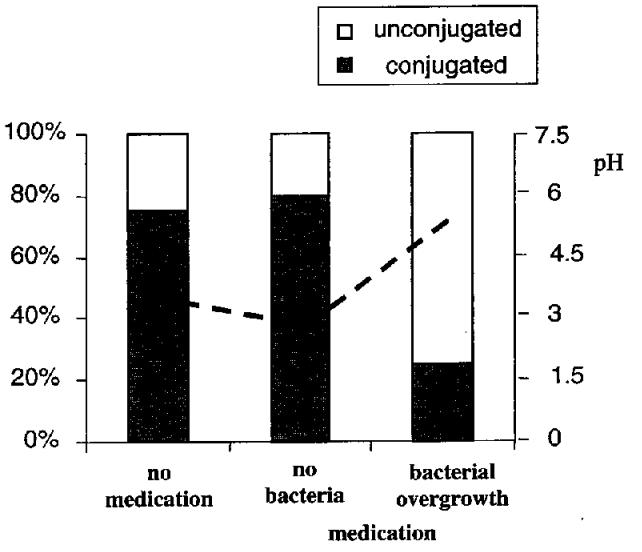


Fig. 2. Ratio of conjugated to unconjugated bile acids (bars) in relation to the median pH (hatched line) in the three groups. Off acid-suppression therapy (no medication), taking omeprazole with no bacteria, and taking omeprazole with bacterial overgrowth.

DISCUSSION

The side effects of acid suppression therapy, including the possibility of bacterial overgrowth, are well known.[2,3,6] Increased numbers of bacteria have been found in gastric aspirates of patients treated with acid suppression therapy for ulcer disease, whose pH was maintained at >4. At pH values <4, bacteria do not survive longer than 10 minutes.[7] Our results confirm this fact. The pH cut-off value for the presence of a significant number of bacteria in the present study was 3.8. No bacterial overgrowth was observed below pH 3.8.

Most bacterial species present in the aspirate of patients taking omeprazole are capable of deconjugating bile acids. Bile acid deconjugation ability has been demonstrated for *Lactobacillus*, *Streptococcus*, *Staphylococcus*, *Neisseria*, *Aeromonas*, and others. Bacteria found in our study not capable of deconjugation were *Escherichia coli* and *Candida albicans*. It is important to note that deconjugated as well as secondary bile acids have been shown to be more injurious to the gastric and esophageal mucosa than their conjugated counterparts.[18] Armstrong et al.[14] have shown, using an ex vivo gastric chamber model, that unconjugated bile acids produced greater injury (higher potential difference changes) than their conjugated counterparts. The findings were pH dependent, reflecting the pKa of the particular bile acid used.

We have shown that proton pump inhibitor therapy in patients with GERD results not only in a high prevalence of gastric bacterial overgrowth, but that the presence of bacterial overgrowth markedly increases the concentration of unconjugated bile acids, reversing the ratio of conjugated to unconjugated bile acids present in the foregut. Nehra et al.[17] have recently shown that unconjugated bile acids reflux into the esophagus. Using an improved sampling technique, they were able to show that a significant proportion of bile acids aspirated from the esophagus of patients with GERD are unconjugated. This was particularly true in the aspirates of patients with erosive esophagitis, stricture, or Barrett's esophagus.

Recent studies have been aimed at answering the question of whether bile acids have an effect on the molecular level. In vivo studies have shown that bile acids act as promoters of gastrointestinal cancer and that they enhance cell transformation in vitro. They are capable of activating protein kinase C[19] and inducing AP-1–mediated transcription.[20] Zhang et al.[21] and Theisen et al.[22] demonstrated that dihydroxy bile acids activate the transcription of the cyclooxgenase-2 gene.

For soluble bile acids to remain innocuous in a patient with chronic reflux managed by acid suppression therapy, they must remain completely ionized. This requires that the gastric pH be maintained at a level of 6 or 7, 24 hours a day, 7 days a week for the patient's lifetime. This is not only impractical but likely impossible unless very high doses of medication are used. Insufficient medication will allow the pH to drift down to 4 or 5 and cause cellular mucosal damage to occur while the patient remains relatively asymptomatic. The injury can result in mild to erosive esophagitis, ulceration, stricture, or the development of a columnar-lined esophagus with intestinal metaplasia, that is, Barrett's esophagus. The incidence of the latter has increased progressively since 1986 and initiates a sequence of mucosal changes that can ultimately lead to esophageal adenocarcinoma. It is unknown whether the movement toward cancer is due to mitogenesis secondary to chronic mucosal injury or mutagenesis from exposure to a direct mutagen. If bile salts are demonstrated to contribute to the development of malignancy, then early surgical intervention to reestablish an antireflux barrier should be encouraged.

REFERENCES

1. Sharma BK, Walt RP, Pounder RE, Gomes MD, Wood EC, Logan LH. Optimal dose of oral omeprazole for maximal 24 hour decrease of intragastric acidity. Gut 1984;25:957-964.
2. Thorens J, Froehlich F, Schwizer W, et al. Bacterial overgrowth during treatment with omeprazole compared with cimetidine: A prospective randomised double blind study. Gut 1996;39:54-59.
3. Karmeli Y, Stalnikowitz R, Eliakim R, Rahav G. Conventional dose of omeprazole alters gastric flora. Dig Dis Sci 1995;40: 2070-2073.
4. Domellof L, Reddy BS, Weisburger JH. Microflora and deconjugation of bile acids in alkaline reflux after partial gastrectomy. Am J Surg 1980;140:291-295.
5. Stockbruegger RW, Cotton PB, Menon GG, et al. Pernicious anaemia, intragastric bacterial overgrowth, and possible consequences. Scand J Gastroenterol 1984;19:355-364.
6. Freston JW. Long-term acid control and proton pump inhibitors: Interactions and safety issues in perspective. Am J Gastroenterol 1997;92:51S-57S.
7. Hill M. Normal and pathological microbial flora of the upper gastrointestinal tract. Scand J Gastroenterol (Suppl)1985; 111:1-6.
8. Sherman P, Lichtman S. Small bowel bacterial overgrowth syndrome [Review]. Dig Dis 1987;5:157-171.
9. Driks MR, Craven DE, Celli BR, et al. Nocosomial pneumonia in intubated patients given sucralfate as compared with antacids or histamine type 2 blockers. The role of gastric colonization. N Engl J Med 1987;317:1376-1382.
10. Reed PI, Smith PL, Haines K, House FR, Walters CL. Gastric juice N-nitrosamines in health and gastroduodenal disease. Lancet 1981;2:550-552.
11. Kauer WK, Peters JH, DeMeester TR, Ireland AP, Bremner CG, Hagen JA. Mixed reflux of gastric and duodenal juices is more harmful to the esophagus than gastric juice alone. The need for surgical therapy re-emphasized [discussion]. Ann Surg 1995;222:525-531.

12. Gotley DC, Morgan AP, Cooper MJ. Bile acid concentrations in the refluxate of patients with reflux oesophagitis. Br J Surg 1988;75:587-590.
13. Iftikhar SY, Ledingham S, Steele RJ, et al. Bile reflux in columnar-lined Barrett's oesophagus. Ann R Coll Surg Engl 1993;75:411-416.
14. Armstrong D, Rytina ER, Murphy GM, Dowling RH. Gastric mucosal toxicity of duodenal juice constituents in the rat. Acute studies using ex vivo rat gastric chamber model. Dig Dis Sci 1994;39:327-339.
15. Bergey DH. Bergey's Manual of Determinative Bacteriology. Baltimore: Williams & Wilkins, 1994.
16. Nehra D, Howell P, Pye JK, Beynon J. Assessment of combined bile acid and pH profiles using an automated sampling device in gastro-oesophageal reflux disease. Br J Surg 1998; 85:134-137.
17. Nehra D, Howell P, Williams CP, Pye JK, Beynon J. Toxic bile acids in gastro-oesophageal reflux disease: influence of gastric acidity. Gut 1999;44:598-602.
18. Kivilaasko E, Fromm D, Silen W. Effect of bile acids and related compounds on isolated esophageal mucosa. Surgery 1989;87:280-285.
19. Huang XP, Fan XT, Desjeux JF, Castagna M. Bile acids, non-phorbol-ester-type tumor promoters, stimulate the phosphorylation of protein kinase C substrates in human platelets and colon cell line HT29. Int J Cancer 1992;52: 444-450.
20. Hirano F, Tanada H, Makino Y, et al. Induction of the transcription factor AP-1 in cultured human colon adenocarcinoma cells following exposure to bile acids. Carcinogenesis 1996;17: 427-433.
21. Zhang F, Subbaramaiah K, Altorki N, Dannenberg AJ. Dihydroxy bile acids activate the transcription of cyclooxygenase-2. J Biol Chem 1998;273:2424-3428.
22. Theisen J, Danenberg K, DeMeester TR, et al. Effect of acid and bile salts on COX-2 gene expression on an esophageal adenocarcinoma cell line [abstr]. Proc Am Assoc Cancer Res 1999;40:505.

Clinical Outcome and Long-Term Survival Rates After Esophagectomy Are Not Determined by Age Over 70 Years

Jan Johansson, M.D., Bruno Walther, M.D., Ph.D.

Esophagectomy is considered a high-risk procedure in patients aged 70 years or older. This study evaluates the impact of two age groups (younger than 70 and 70 years or older) on clinical outcome and long-term survival rates following this procedure. This prospective study included survival analysis and clinical evaluations at 3, 6, and 12 months after esophagectomy. All esophagectomy patients undergoing gastric (n = 125), jejunal (n = 10), or colonic (n = 4) reconstructions at our institution from 1984 to 1996 were included. Fifty patients were older than 70 years, 89 were younger, and 120 of these 139 patients had tumors. The overall hospital mortality rate was 1.4% (2 of 139), both in the younger age group. All leaks from anastomoses and grafts were nonfatal, and these problems occurred in seven patients in the younger age group and two in the older group. The mean preoperative weight was 70 kg, and there was a mean weight loss of 5 kg during the first three postoperative months only but none thereafter ($P < 0.001$). This was the same for patients with benign and malignant disorders, and for those aged over or under 70 years. Between 71% and 77% of the patients experienced no dysphagia at the three evaluations during the first postoperative year. The distribution of the different grades of dysphagia was equal in the two age groups at 3-month ($P = 0.339$) and 12-month ($P = 0.669$) follow-up. The 12-year survival rate was 28% and the 5-year rate was 31%, and this was correlated to tumor stage ($P = 0.002$) but not to age over or under 70 years ($P = 0.299$). The clinical outcome was the same regardless of whether patients were over or under 70 years of age. Tumor stage but not age over 70 years was the major predictive factor for long-term survival. (J GASTROINTEST SURG 2000;4:55-62.)

KEY WORDS: Esophagectomy, survival, old age

Esophagectomy for cancer of the esophagus has long been associated with dismal survival rates because of the unfavorable biologic behavior of the tumors and the seriousness of the postoperative complications. In 1989, based on a meta-analysis of 83,783 patients reported in the literature from 1953 to 1978, Earlam and Cunha-Melo[1] calculated a 5-year survival rate of 4% for patients undergoing resection. Ten years later a 5-year survival rate of 10% was reported by Müller et al.[2] in a critical review of 1201 papers and 76,911 patients from 1980 through 1988. Modern surgical techniques together with advances in postoperative care have led to an improvement in postoperative mortality rates, but long-term survival rates for patients operated on for cancer of the esophagus are still not optimal, mainly because of an advanced tumor stage at initial presentation. Recognition of the association between adenocarcinoma of the distal esophagus with gastroesophageal reflux and Barrett's esophagus has led to the detection and treatment of cancer at an earlier stage of tumor progression,[3] and the worldwide increasing incidence of this cancer[4] has focused attention on the need to improve long-term survival rates. However, resection of esophageal tumors remains a palliative procedure in many patients, offering relief of the often severe dysphagia that led to presentation and providing an alternative to numerous office visits and/or hospitalizations for laser treatment or stent placement.[5] The question of whether the postoperative complications and out-

From the Department of Surgery, Lund University, Lund, Sweden.
Supported by the Medical faculty, Lund University, Lund, Sweden; The Crafoord Foundation, Lund, Sweden; and the Gunnar, Arvid and Elisabeth Nilsson Foundation for Treatment of Malignant Disorders, Helsingborg, Sweden.
Reprint requests: Jan Johansson, M.D., Department of Surgery, Lund University Hospital, S-221 85 Lund, Sweden. e-mail: Jan.Johansson@Kir.lu.se

come of this major surgical procedure are justified by the benefits gained in older patients remains to be addressed. The aim of this study was to assess the impact of age over 70 years on clinical outcome and to evaluate whether age, in addition to tumor stage, determined long-term survival rates.

MATERIAL AND METHODS

From 1984 through January 1996, a total of 154 patients were admitted for esophagectomy to the Department of Surgery, Lund University, Lund, Sweden. Fifteen patients underwent an exploratory operation, but their tumors were judged nonresectable. In two of them an extra-anatomic colonic bypass was performed, and the remaining 139 underwent esophagectomy. The departmental policy at our institution regarding treatment of advanced esophageal carcinoma favors palliative resection whenever possible, although stent insertion, laser therapy, and chemoradiotherapy, alone or in combination, are also offered.

All patients undergoing curative resection had an en bloc resection according to a standard technique irrespective of the substitute chosen. In 125 patients a stapled gastric tube was used as a substitute, in 10 patients a jejunal interposition graft was inserted, and in four patients a colonic interposition graft was inserted. Hospital mortality was defined as any cause of in-hospital death at the time of resection or death from any cause within 30 days of the primary resection.

Surgical Procedures

The abdomen was explored through an upper midline incision, and the dissection included removal of fat and lymph nodes in the region of the cardia, along the upper part of the abdominal aorta, around the left gastric artery, and along the celiac trunk. In creating a gastric tube, the lesser curvature of the stomach including fat and lymph nodes along the left gastric artery was removed. To minimize the risk of vascular compromise to the substitute, a phrenotomy of the hiatus was performed resulting in a space that allowed insertion of four fingers.

The gastric tube was created using a linear cutting stapler (TLC 55, Ethicon, Stockholm, Sweden or GIA, Autosuture, Stockholm, Sweden), which was inserted into the lesser curvature approximately 7 cm cranial to the pylorus. The stapler was fired multiple times, fashioning a 5 cm wide tube along the long axis of the greater curvature of the stomach. The vascular supply to the gastric tube was derived from the remaining right gastroepiploic artery and the right gastric artery. In those where a jejunal or a colonic substitute was used, the proximal part of the stomach was removed en bloc with the esophagus.

The jejunal and colonic substitutes with preserved vascular supply were harvested and a stapled side-to-side anastomosis was created between their distal ends with the posterior wall of the stomach using the TLC 55 linear cutting stapler; the remaining opening was closed with a TA 55 (Autosuture, Stockholm) RL 60, or TL 60 (Ethicon, Stockholm) stapling device.

A right posterolateral thoracotomy provided access for dissection of the thoracic esophagus and the mediastinal lymph nodes. In selected patients a cervical incision running parallel to the left sternocleidomastoid muscle was made to allow mobilization and anastomosis of the cervical esophagus. All esophageal substitutes were placed in the posterior mediastinum. Anastomoses in the apex of the right chest were stapled using a circular stapling device (Premium CEEA, Autosuture, Stockholm) and cartridges of varying sizes (25, 28, or 31 mm). A 90 mm linear stapling device (RL 90, Ethicon, Stockholm) was used to resect the remaining stomach including the gastrostomy previously used for the introduction of the circular stapler. When the stapled thoracic anastomosis was created, the largest possible cartridge was used. End-to-end anastomoses in the neck were hand sutured with a single continuous all-layer monofilament 4-0 Polydioxanone (PDS II, Ethicon, Stockholm).

A water-soluble contrast study was carried out in all patients between days 5 and 7 postoperatively, and oral intake was established on confirmation of a radiologically intact anastomosis. All patients had an appointment with a nutritionist prior to discharge from the hospital, and were prescribed supplementary carbohydrate- and protein-enriched formulas to be taken by mouth. Postoperative enteral nutrition was not routinely administered, and jejunostomy or gastrostomy catheters were not routinely placed during the operation. Preoperative chemotherapy or radiotherapy was given on a selective basis only to four patients in the younger and two in the older age group. Sixteen patients in the younger age group and seven patients in the older group received late radiotherapy or radiochemotherapy for palliation of tumor recurrence.

Follow-up

Postoperative follow-up including endoscopy was carried out at 3, 6, and 12 months, after which time the care of the patients was transferred back to the referring physician. Clinical data and routine follow-up information on all patients continued to be collected even after completion of the 12-month systematic follow-up and after all deaths were documented. A nurse who had not participated in the in-hospital care of the patients

Table I. Patient data*

	<70 years (n = 89)	>70 years (n = 50)
Age (yr)	59 (31-70)	75 (70-83)
Duration of operation (min)	600 (387-920)	540 (290-787)
Operative bleeding (ml)†	1330 (200-4800)	1240 (400-3000)
No. of blood and plasma units transfused during operations‡	2.4 (0-17)	2.5 (0-19)
Duration of chest drains (days)§	7 (0-71)	7 (5-64)
Postoperative hospital stay (days)‖	17.9 (0-83)	18.6 (9-68)

*All values are means with ranges shown in parentheses.
Student's *t* test: †$P = 0.573$; ‡$P = 0.810$; §$P = 0.240$; ‖$P = 0.782$.

conducted systematic assessment of upper gastrointestinal symptoms, and performed serial weight measurements. Symptoms of dysphagia were graded in the following manner: Mild when the dysphagia was occasional and easily tolerated; moderate when the dysphagia was sufficient to interfere with routine daily activities; and severe when the dysphagia was incapacitating, leading to an inability to perform routine activities.

Statistics

Nominal data were calculated by means of the chi-square test, when the number of the totals was no less than 30 or when the expected values were no less than five, or with the Fisher's exact test. Ordinal data were estimated by means of the Mann-Whitney U test or the chi-square test. Data from the interval or ratio scale were analyzed using parametric methods for normally distributed data (Lilliefors test, Mauchly's test of sphericity, or Box's test); otherwise, nonparametric tests were used. Student's *t* test, Mann-Whitney U test, and Kruskal-Wallis test were used to compare two or more unrelated groups. For related groups, the parametric analysis of variance (ANOVA) (repeated-measures model) or the nonparametric Friedman's test was used. A Kaplan-Meier life table was made to graphically show survival functions. The log-rank test and the Cox proportional hazards model with forward selection and Wald statistics were used for survival analysis. For checking of proportional hazards, a log minus log model was used. A *P* value of <0.05 was considered statistically significant. The SPSS statistical package 7.5 for Windows 95 basic and advanced modules (SPSS Inc., Chicago, Ill.) were used for statistical testing.

RESULTS

Sixty-two men and 27 women under the age of 70 years, and 36 men and 14 women over the age of 70 underwent an esophagectomy. In 75 of 89 patients in the under-70 group a gastric tube was used, in 10 patients a jejunal interposition graft was used, and in four a colonic interposition graft was used as the substitute conduit. All those in the over-70 group had gastric tubes placed. Data for all patients are presented in Table I. No differences were seen in the perioperative blood loss, transfusion requirements, duration of postoperative chest tube drainage, or the postoperative hospital stay between the two age groups.

Table II. Diagnoses in patients undergoing resection (N = 139)

Diagnosis*	<70 years (n = 89)	>70 years (n = 50)
Malignant disorders		
Squamous cell carcinoma	37	23
Adenocarcinoma *with* Barrett's metaplasia	19	19
Adenocarcinoma *without* Barrett's metaplasia	13	6
Other malignant lesions	3	0
TOTAL	72	48
Benign disorders		
Achalasia	6	0
Failed antireflux surgery	4	1
Severe dysplasia	2	1
Other benign lesions	5	0
TOTAL	17	2

*Malignant vs. benign disorders, age over or under 70 years, $P = 0.009$ (Fisher's exact test).

Forty-eight patients (96%) in the older group and 72 patients (81%) in the younger group were operated on because of malignancy (Fisher's exact test, $P = 0.009$) (Table II). In both groups most tumors were found in the distal esophagus, with a decreasing frequency observed further cranially (Table III). Tumor stage (chi-square, $P = 0.47$, df 3) and the presence or absence of lymph node metastases were equally distributed between the two age groups

Table III. Characteristics of the 120 patients with tumors*

	<70 years (n = 72)		>70 years (n = 48)	
Tumor site				
Proximal	6		2	
Middle	29		14	
Distal	37		31	
Multifocal	0		1	
	Lymph node metastasis	**No lymph node metastasis**	**Lymph node metastasis**	**No lymph node metastasis**
Tumor invasion				
Mucosa	0	5	0	2
Submucosa	0	10	0	5
Muscular layer	6	8	1	9
Wall penetration†	30	11	18	13

*Distribution of tumor stages (chi-square, $P = 0.47$; df = 3) and presence of lymph node metastasis (Fisher's exact test, $P = 0.23$) were equal in patients over and under 70 years of age.
†Two patients in the group over 70 years of age had no reports on the presence or absence of lymph node metastases. When survival calculations were made, these two patients were treated as having metastases.

Table IV. Postoperative complications and hospital mortality* for all patients undergoing resection (N = 139)

Complications	<70 years (n = 72)	>70 years (n = 48)
Surgical		
Anastomotic leakage		
Conservative treatment	1	2
Operation	1	0
Ulceration in the substitute		
Conservative treatment	3	0
Operation	2	0
Necrosis of membranous part of trachea and reoperation	1†	0
Postoperative bleeding and reoperation	1	1
Intestinal obstruction	2	0
Pneumothorax	0	5
Chylothorax	1	0
Vocal cord palsy	2	1
Wound infection	3	0
Negative exploration	0	1
TOTAL‡	17	10
Other		
Extremity thrombosis	2	0
Myocardial infarct/cardiac arrest	2	1
Arrhythmia/transient cardiac failure	6 (1*)	8
Pneumonia	0	2
TOTAL§	10	11
Hospital mortality	2	0

*In-hospital death at the time of resection or death within 30 days of resection.
†Indicates a lethal complication.
‡Chi-square test between groups ($P = 0.901$).
§Chi-square test between groups ($P = 0.426$).

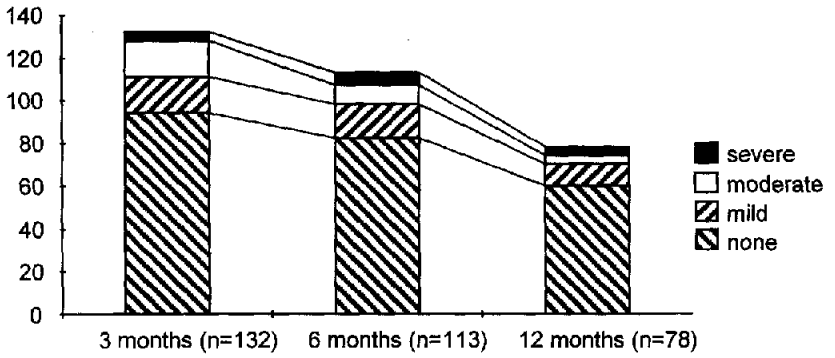


Fig. 1. Postoperative dysphagia. Number of patients undergoing esophagectomy (n = 139) and grades of dysphagia experienced during the first postoperative year. Friedman's test revealed no change in distribution of dysphagia over time (P = 0.627).

(Fisher's exact test, $P = 0.23$) (see Table III). In all resected patients, the margins were macroscopically tumor free. In 70 of 72 patients in the younger group and 47 of 48 in the older group, the resection margins were tumor free on histologic examination. Two of those who had microscopic margin involvement had tumor detected at the anastomosis on follow-up endoscopy and the third patient died of disseminated metastatic carcinoma before any gross evidence of tumor at the anastomosis became evident.

Postoperative Complications and Hospital Mortality

Nine patients had clinically or radiologically apparent leakage of the anastomosis or the substitute; none of these patients died and six were treated conservatively. Three patients in the younger group and none in the older group required surgical intervention and drainage for leaks, but there were no cases of graft failure and none of the patients required reresection. All postoperative complications are listed in Table IV. Thirty-day postoperative or hospital mortality rate was 1.4% (2 of 139); one patient with severe preexisting heart failure suffered an intractable cardiac arrhythmia during surgery for an early cancer, and one patient developed ischemic necrosis at the tracheal bifurcation that was detected on the seventh postoperative day, but which failed to heal following repair; both patients were in the under-70 age group.

Late procedure-related complications occurred in four patients. Three patients, all under the age of 70, had perforations in their substitutes and all went on to make a full recovery after corrective intervention. One of them, a patient with a jejunal interposition, had a penetrating ulcer to the heart and was admitted because of massive upper gastrointestinal bleeding. He underwent a successful reconstruction with a long Roux loop after placement of a hemostatic cardiac suture and resection of the original interposition and the remaining stomach. One patient with a gastric tube perforation and resultant fistula to the main bronchus was treated by endoscopically administered tissue glue. The third patient developed a gastropleural fistula, which was oversewn. One patient, a 71-year-old man who died at 6 months postoperatively, was found to have a mediastinal abscess on postmortem examination, which may have been procedure related. There were two cases of late intestinal obstruction that required readmission and surgery. One patient was admitted to the hospital with pneumonia, and one patient was admitted for relief of food impaction. The prevalence of minor pulmonary problems such as aspiration-induced pneumonia was difficult to assess, since most patients consult their family practitioners for these problems.

Recurrences on Follow-Up

All patients were encouraged to undergo upper gastrointestinal endoscopy at 3, 6, and 12 months after surgery, and 119 did so at 3 months, 98 at 6 months, and 83 at 12 months. Anastomotic tumor recurrence was found during endoscopy in two patients in the younger group and in five patients in the older group, two of whom had had microscopic tumor involvement of the resection margin. These recurrences were treated by laser therapy or sclerotherapy, or by stenting in one patient in the younger group and two patients in the older group. Tumor spread, locally in and around the substitute, or distant tumor dissemination, which was not responsible for obstructive symptoms, was confirmed in 29 patients in the under-70 group and in 15 patients in the older group.

Symptoms and Weight Loss

Postoperative follow-up at 3, 6, and 12 months for evaluation of symptoms revealed no dysphagia in 71%, 73%, and 77% of the patients, respectively (Fig. 1).

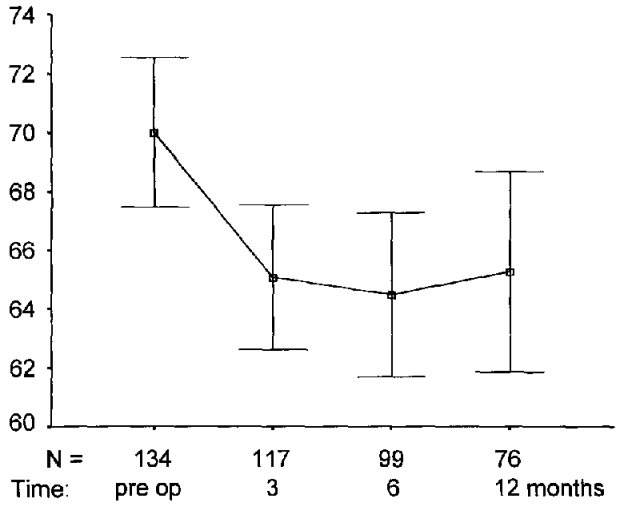
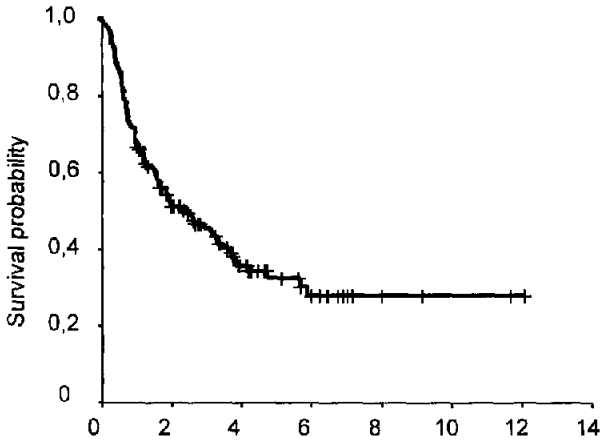


Fig. 2. Mean weight development (95% confidence interval) in kilograms in patients undergoing esophagectomy (n = 139) during the first postoperative year. Mean preoperative weight of 70 kg decreased significantly over the first postoperative year (repeated-measures ANOVA, $P < 0.001$). Weight reduction stopped after 3 months (65 kg) and further changes from 3 to 6 months (repeated-measures ANOVA, $P = 0.245$) and from 6 to 12 months (repeated-measures ANOVA, $P = 0.997$) were not statistically significant.



	0-5 yr	>5-10 yr	>10 yr
No. of patients still alive	39	13	2
No. of patients who died	83	2	0
TOTAL	122	15	2

Fig. 3. Cumulative survival in years (Kaplan-Meier) for all 139 patients undergoing esophagectomy. Censored data are marked with a plus sign.

The distribution of the various grades of dysphagia revealed no significant difference during the first postoperative year (Friedman's test, $P = 0.627$). At 6 months' follow-up, the patients over the age of 70 years had more dysphagia (moderate or severe, n = 11 of 43) than those under 70 (moderate or severe, n = 4 of 70) (Mann-Whitney U test, $P = 0.032$), but this difference was not apparent at the 3-month ($P = 0.339$) and 12-month ($P = 0.669$) follow-up examinations. In comparing those who were still alive at follow-up with those who had died during the first 15 months, there was an equal distribution of patients with different grades of dysphagia at 3 months (Mann-Whitney U test, $P = 0.742$; n = 99), 6 months (Mann-Whitney U test, $P = 0.252$; n = 82), and 12 months (Mann-Whitney U test, $P = 0.977$; n = 54).

The mean preoperative weight of 70 kg decreased significantly during the first postoperative year (repeated-measures ANOVA, $P < 0.001$) (Fig. 2). Weight loss ceased after 3 months (65 kg), and further changes from 3 to 6 months (repeated-measures ANOVA, $P = 0.245$) and from 6 to 12 months (repeated-measures ANOVA, P = 0.997) were not statistically significant. The degree of weight loss during the first postoperative year was not significantly different in those operated on for benign or malignant disorders (repeated-measures ANOVA, $P = 0.115$), or in patients younger or older than 70 (repeated-measures ANOVA, $P = 0.110$), and was not significantly influenced by the type of substitute (repeated-measures ANOVA, $P = 0.820$).

Survival Rates

All patients were followed up for at least 1 year or until the time of death. The cumulated mortality at 1 month was 2 of 139, at 3 months was 6 of 139, and at 12 months was 46 of 139. The overall survival rate was 28%, and the 5-year rate was 31% (Fig. 3). Mean survival time was 4.7 years (95% confidence interval 3.77 to 5.63). Including hospital mortality, six patients in the younger age group and four in the older group died of benign disease. One patient older than 70 years died of another type of cancer. The remaining 44 patients in the group under 70 years and the 30 in the group over 70 died of recurrent disease. Results of life-table analysis for the different tumor stages for all 139 patients are presented in Fig. 4, and for the 120 patients with tumors in the under-70 and over-70 age groups in Fig. 5. A Cox proportional hazards model was used to evaluate factors affecting long-term survival rates.

The only significant covariate for survival was tumor stage according to the TNM system of classification ($P = 0.002$). The other numeric or categoric covariates were sex, units of blood and plasma given during the operation, intraoperative bleeding, diagnoses, age over or under 70 years, type of interposition, site of anastomosis, type of anastomosis, tumor location, and perioperative adjuvant therapy ($0.083 < P < 0.982$).

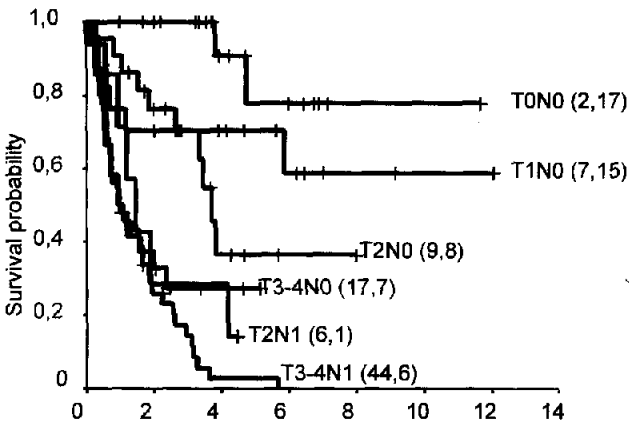


Fig. 4. Cumulative survival rates in years (Kaplan-Meier) according to tumor stage in the 139 patients by TNM classification. Censored data are marked with a plus sign. First figure in parentheses represents number of dead patients; second figure represents number of living (censored) patients at the time the evaluation was done. T0N0 = all patients with benign disorders including those who underwent resection for severe dysplasia; T1 = tumor invasion to but not through the submucosa; T2 = tumor invasion to but not through the muscular layer; T3 = tumor invasion through the muscular layer; T4 = overgrowth to surrounding organ; N0 = no regional lymph node metastasis; N1 = regional lymph node metastasis present.

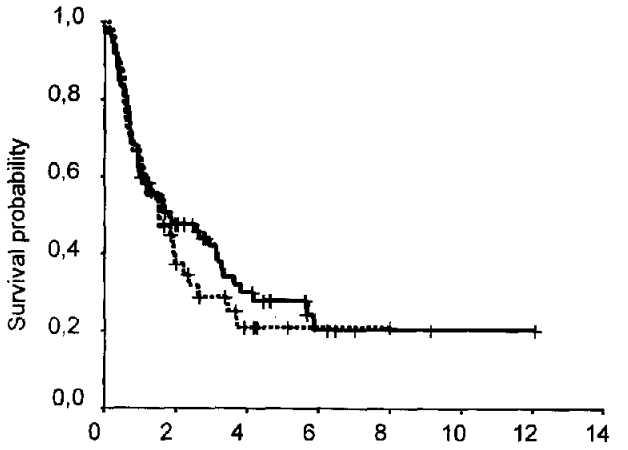


Fig. 5. Survival rates for 120 patients with tumors. No significant difference was noted between patients older than 70 years (dotted line) compared to patients younger than 70 years (continuous line) (log-rank test, $P = 0.473$).

DISCUSSION

This report focuses on the results of esophageal resection and the influence of patient characteristics, in particular age, on the outcome following surgery. The authors have not attempted to compare the outcome of different nonsurgical treatment strategies since only a few of those patients receiving curative treatment were treated by modalities other than surgery, and the number undergoing a range of alternative palliative treatments (laser, stenting, and chemoradiotherapy) without surgery was small. The patients were judged by the authors and the referring surgeons as being in acceptable physical condition for surgery, and old age in itself was not a criterion that was applied during the selection process.

We have compared the results of esophagectomy in two age groups—over 70 and under 70 years. The two groups were almost identical in terms of sex distribution, operative blood loss, transfusion requirements, duration of chest drainage, and hospital stay. The tumor site in the esophagus, degree of tumor penetration, and presence of lymph node metastases were equally distributed between the two age groups, suggesting that the indications for surgery and the stage of the tumor at presentation were the same in both groups. The majority of the patients in both groups were operated on for malignant tumors, but surgery for benign lesions was more common in the younger age group than in the older group.

There were no in-hospital deaths in the group over 70 years of age and the only two cases of 30-day or hospital mortality occurred in the younger group. This mortality rate of 1.4% is consistent with that previously reported by Akiyama et al.[6] No difference was noted when comparing the rates of all complications between the two age groups, but there were more anastomotic and graft-related complications in the younger group. The risk of postoperative cardiovascular and pulmonary problems increased with age but occurred in less than 20% of all patients.

Late complications associated with the surgical treatment were rare, but one death occurred in a patient with a healed anastomotic leak who died of an unrecognized mediastinal abscess. Late perforation of the substitute graft occurred in three patients. These perforations occurred in what was regarded as a well-vascularized part of the graft and may have resulted in association with peptic ulceration, etiologic factors that may include continued acid production in the gastric tube, and heavy alcohol intake (two of those patients had a history of alcohol abuse). In most patients in this study, a stapled gastric tube served as the esophageal substitute. The patients were not routinely given any acid-reducing medications unless they had symptoms of reflux or benign anastomotic strictures. Although duodenal alkaline reflux into the gastric tube as a potential contributor to ulcerogenesis has not been proved, we attempted to minimize alkaline reflux by performing a pyloroplasty only when it was considered essential. Despite recent reports of improved survival with adjuvant therapy,[7-9] we have chosen not to use either modality routinely since other

studies have shown increased morbidity and mortality with no improvement in long-term survival.[10-12]

One of the most objective and easily applicable assessments of satisfactory eating and drinking postoperatively is serial weight measurements. Patients with benign and malignant disorders, as well as those over and under 70 years of age, all suffered weight loss during the first 3 months, and all had approximately the same pattern of weight change during the first postoperative year. The initial weight loss is a well-recognized reaction to this type of upper alimentary tract surgery irrespective of age and indication for surgery.[5,13] The supplementary oral formulas given to the patients in this study did not prevent early postoperative weight loss, and this may suggest the need for additional support by enteral feeding.

Few studies have investigated the problem of postoperative swallowing difficulty.[13,14] Advanced dysphagia was completely relieved by esophagectomy in the vast majority of the patients irrespective of age and palliative resection. Thus this operation seems well justified in the elderly, with postoperative dysphagia being no more of a problem in older patients than in younger ones.

The 12-year overall survival rate was 28% and the 5-year rate was 31% and, as one would expect, long-term survival rates were best correlated with tumor stage. On the other hand, the histologic tumor type did not influence the long-term results, suggesting that tumor aggressiveness seems to be primarily determined by the degree of invasion. Good long-term survival rates were noted in patients with benign disease and in those with malignancies where there was no wall penetration and no lymph node metastases. The present study demonstrated no survival advantage in the under-70 age group suggesting that reasonably healthy patients older than 70 years could be considered for this type of surgery.

CONCLUSION

Esophagectomy in older patients is carried out almost exclusively for cancer, whereas in younger patients benign disorders are the more common indications for surgery. This as well as other recent studies has shown that esophagectomy in the relatively fit elderly population is a safe option,[15-17] is associated with a short postoperative hospital stay, and restores good swallowing function whether the operation is for cure or palliation. Postoperative weight fluctuations in older patients are no different from those in younger patients, and the long-term survival rate is correlated with tumor invasion but not with histologic tumor type. Older patients appear to gain as much benefit and as good an outcome after esophagectomy as younger patients while having the same overall morbidity, and we therefore conclude that esophagectomy is the preferred treatment option for both cure and palliation of esophageal cancer irrespective of age.

REFERENCES

1. Earlam R, Cunha-Melo JR. Oesophageal squamous cell carcinoma: I. A critical review of surgery. Br J Surg 1980;67:381-390.
2. Müller JM. Erasmi H, Stelzner M, Zieren U, Pichlmaier H. Surgical therapy of oesophageal carcinoma. Br J Surg 1990;77: 845-857.
3. Johansson J, Johnsson F, Walther B, Willen R, Stael von Holstein C, Zilling T. Adenocarcinoma in the distal esophagus with and without Barrett esophagus. Differences in symptoms and survival rates. Arch Surg 1996;131:708-713.
4. Pera M, Cameron AJ, Trastek VF, Carpenter HA, Zinsmeister AR. Increasing incidence of adenocarcinoma of the esophagus and esophagogastric junction. Gastroenterology 1993;104: 510-513.
5. De Leyn P, Coosemans W, Lerut T. Early and late functional results in patients with intrathoracic gastric replacement after oesophagectomy for carcinoma. Eur J Cardiothorac Surg 1992;6:79-84.
6. Akiyama H, Tsurumaru M, Watanabe G, Ono Y, Udagawa H, Suzuki M. Development of surgery for carcinoma of the esophagus. Am J Surg 1984;147:9-16.
7. Malhaire JP, Labat JP, Lozac'h P, Simon H, Lucas B, Topart P, et al. Preoperative concomitant radiochemotherapy in squamous cell carcinoma of the esophagus: Results of a study of 56 patients. Int J Radiat Oncol Biol Phys 1996;34:429-437.
8. Bates BA, Detterbeck FC, Bernard SA, Qaqish BF, Tepper JE. Concurrent radiation therapy and chemotherapy followed by esophagectomy for localized esophageal carcinoma. J Clin Oncol 1996;14:156-163.
9. Nygaard K, Hagen S, Hansen HS, Hatlevoll R, Hultborn R, Jakobsen A, et al. Pre-operative radiotherapy prolongs survival in operable esophageal carcinoma: A randomized, multicenter study of pre-operative radiotherapy and chemotherapy. The second Scandinavian trial in esophageal cancer. World J Surg 1992;16:1104-1109.
10. Chung SC, Stuart RC, Li AK. Surgical therapy for squamous-cell carcinoma of the oesophagus. Lancet 1994;343:521-524.
11. Fink U, Stein HJ, Wilke H, Roder JD, Siewert JR. Multimodal treatment for squamous cell esophageal cancer. World J Surg 1995;19:198-204.
12. Bossett J-F, Gignoux M, Triboulet J-P, Tiret E, Mantion G, Elias D, et al. Chemoradiotherapy followed by surgery compared with surgery alone in squamous cell cancer of the esophagus. N Engl J Med 1997;337:161-167.
13. Naunheim KS, Petruska PJ, Roy TS, Schlueter JM, Kim H, Baue AE. Multimodality therapy for adenocarcinoma of the esophagus. Ann Thorac Surg 1995;59:1085-1090.
14. Blazeby JM, Williams MH, Brookes ST, Alderson D, Farndon JR. Quality of life measurement in patients with oesophageal cancer. Gut 1995;37:505-508.
15. Poon RTP, Law SYK, Chu KM, Branicki FJ, Wong J. Esophagectomy for carcinoma of the esophagus in the elderly. Results of current surgical management. Ann Surg 1998;227:357-364.
16. Ellis FH, Williamson WA, Heatley GJ. Cancer of the esophagus and cardia: Does age influence treatment selection and surgical outcomes? J Am Coll Surg 1998;187:345-351.
17. Alexiou C, Beggs D, Salama FD, Brackenbury ET, Morgan WE. Surgery for esophageal cancer in elderly patients: The view from Nottingham. J Thorac Cardiovasc Surg 1998;116:545-553.

Organ-Preserving Resection of the Gastroesophageal Junction and Substitution With a Gastric Corpus Rotation Tube: An Experimental Study

Martin K. Schilling, M.D., Daniel Mettler, D.V.M., Michael Feodorovici, M.D., Markus W. Büchler, M.D.

Premalignant lesions of the gastroesophageal junction are treated conservatively or by antireflux surgical procedures. We describe a novel technique that replaces the distal esophagus after resection of the gastroesophageal junction. After resection of the gastroesophageal junction, 16 pigs were divided into two groups. In group 1 (n = 9) the gastroesophageal junction was replaced with a 3 cm wide horizontal gastric corpus tube, pedicled at the lesser curvature. In group 2 (n = 7) the tube was pedicled at the greater curvature. Tube length, volume, and compliance of the gastric remnant and blood flow in the tube (by laser Doppler flowmetry given in perfusion units [PU]) were measured before and after tube formation and 2 weeks postoperatively. Group 1 tubes were 9.5 ± 1.5 cm long and group 2 tubes were 8.2 ± 0.7 cm long. Tube formation decreased volume and compliance of the gastric remnant. After tube formation, blood flow at the tip of the tube decreased from 254 PU to 64 ± 22 PU (group 1) and 87 ± 36 PU (group 2). Volume, compliance, and blood flow returned to baseline values 2 weeks postoperatively. No anastomotic leakage was found on postmortem examination. Horizontal gastric corpus tubes might offer an alternative to replace the distal esophagus and proximal stomach after resection of premalignant lesions of the gastroesophageal junction. (J GASTROINTEST SURG 2000;4:63-69.)

KEY WORDS: Gastroesophageal reflux, Barrett's esophagus, gastric tubes

Tubular transformations of the stomach have been used since the beginning of this century to substitute for part or all of the esophagus.[1,2] The tubes most commonly used are the Kirschner-Akiyama isoperistaltic gastric tube[3,4] and the reversed anisoperistaltic Gavrilyu-Heimlich tube.[5,6] Numerous studies have addressed the vascular and geometric problems associated with replacement of the esophagus by either one of these tubes.[7-10] We recently described a new type of gastric tube that can be used to substitute for the proximal and middle esophagus.[11] Because it uses the whole gastric fundus, this new tube was longer than the Kirschner-Akiyama tube and better perfused than either the Kirschner-Akiyama or Gavrilyu-Heimlich tube.[12] Clinically fundus rotation gastroplasty has been performed safely, with an anastomotic failure rate of 6% in a Western population.[13]

As an extension of those previous studies, we describe herein the anatomic and circulatory properties of a rotation gastroplasty developed from the gastric corpus. It is intended to substitute for the distal esophagus after resection of the gastroesophageal junction with preservation of the esophagus and the distal four fifths of the stomach.

METHODS

Experiments were performed in accordance with the institutional guidelines for the use and care of laboratory animals and were approved by the local animal ethics committee. Sixteen large white pigs (22 to 26 kg) were used in this study and these pigs were divided into two groups.

For all operative procedures, anesthesia was induced with ketamine, 10 mg/kg body weight intramuscularly, followed by metomidate, 5 mg/kg body weight, and azaperone, 2 mg/kg body weight intravenously, for endotracheal intubation. Anesthesia was

From the Department of Visceral and Transplantation Surgery and the Surgical Research Unit (D.M.), University of Bern, Inselspital, Bern, Switzerland.
Reprint requests: Martin K. Schilling, M.D., Department of Visceral and Transplantation Surgery, University of Bern, Inselspital, CH-3010 Bern, Switzerland. e-mail: martin.schilling@insel.ch

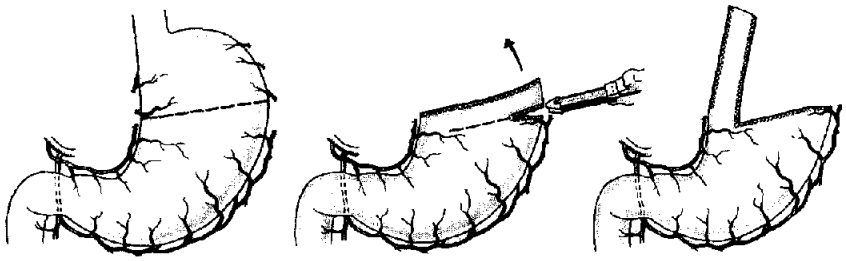


Fig. 1. Gastric corpus rotation tubes with a parenchymal pedicle at the lesser curvature (group 1). After resection of the gastroesophageal junction, a 3 cm wide tube was constructed by stapling along a line parallel to the resection line.

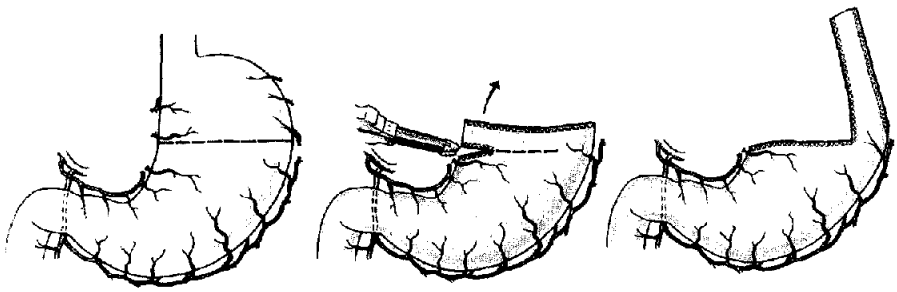


Fig. 2. Gastric corpus rotation tubes with a parenchymal pedicle at the greater curvature (group 2).

maintained with 0.50% ± 0.05% halothane (end-tidal concentration monitored with a multigas analyzer; Hellige SMU 611, Hellige, Freiburg, Germany) and 70% nitrous oxide in oxygen. Lungs were ventilated with a volume-controlled ventilator with a positive end-expiratory pressure of 3 to 4 cm H_2O (Tiberius 19, Drägerwerk, Lübeck, Germany). Tidal volume was maintained at 10 ml/kg body weight and ventilatory frequency was adjusted (13 to 18 beats/min) to maintain $PaCO_2$ at 4.5 to 5.5 kPa. A central venous catheter was inserted through the left external jugular vein after a cervical incision, and central venous pressure was kept between 8 and 12 mm Hg. Arterial pressure was monitored through a catheter placed in the left carotid artery and maintained at a mean of 60 to 70 mm Hg by infusing Ringer's lactate solution or adjusting the anesthetics.

Operative Procedures

Resection of the Esophagogastric Junction. After a midline laparotomy, the diaphragm was incised ventral to the esophagus, and the esophagus was mobilized transhiatally by blunt dissection. Stay sutures were placed on either side of the esophagus, and the esophagus was transected at the level of the inferior pulmonary veins, 4 to 5 cm proximal to the gastroesophageal junction. The proximal stomach was mobilized by transecting the short gastric arteries, followed by transection of the left gastric and left gastroepiploic artery. The proximal two fifths of the stomach was resected with a linear cutting stapler.

Tube Formation. Gastric corpus rotation tubes were formed by stapling along a line parallel to the resection line starting at the greater curvature (group 1, n = 9; Fig. 1) or the lesser curvature (group 2, n = 7; Fig. 2). The tube was designed to be slightly conical with a width of 3 cm at the tip of the tube.

Staple lines were inverted with interrupted 5-0 polydioxanon (PDS) sutures (Ethicon, Spreitenbach, Switzerland) in all except animals 1 and 2 in group 1.

Esophagogastric continuity was reestablished by a transhiatal end-to-end two-layer running suture (5-0 PDS) between the tube and the esophagus.

Animals were brought to the recovery room and allowed to take liquids starting on postoperative day 1. All animals were examined daily for signs of anorexia, fatigue, or weight loss of more than 10% and reoperated when these clinical signs developed. All other animals were reoperated after 14 days to undergo reassessment of the tubes.

Evaluation criteria were actual tube length and width and volume and compliance of the remaining gastric reservoir, measured before and after tube formation as well as 2 weeks postoperatively. In addition, blood flow was studied by laser Doppler flowmetry be-

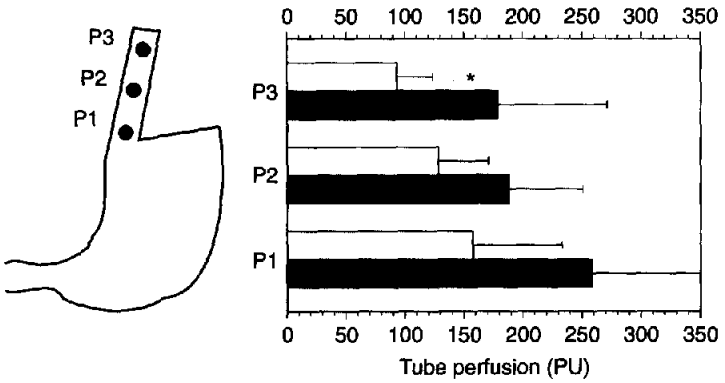


Fig. 3. Gastric tube perfusion in lesser curvature pedicled tubes (given in perfusion units *[PU]* of the mean blood flow). Blood flow was measured by laser Doppler flowmetry at points P1 to P3 before (■) and after (□) tube formation. * = $P < 0.05$ vs. P3 before tube formation (ANOVA). Correlation between tube perfusion and distance from the tubular pedicle was calculated by regression analysis (slope = −7.4 PU/cm).

fore and after tube formation as well as 2 weeks postoperatively, and tube vascularization was studied angiographically on postmortem specimens. Histologic specimens of the tube were compared with specimens of the resected stomach for signs of ischemic mucosal damage.

Gastric Volume and Gastric Compliance Measurements

Gastric volume and gastric compliance were measured as described previously.[12] Ten minutes after injection of 1 mg/kg scopolamine butyl bromide, the gastric reservoir was emptied and a clamp was placed over the pylorus. Normal saline solution was infused stepwise into the gastric reservoir through a 13-gauge needle, and the intraluminal pressure was measured hydrostatically through a second 13-gauge needle. Gastric compliance was calculated from volume-pressure regression curves of each group. Gastric compliance is the volume required to increase the intragastric pressure by 1 cm H_2O and was studied after partial gastric resection, that is, before tube formation, after tube formation, and on relaparotomy 2 weeks after the first operation.

Gastric volume is given as the volume required to bring the intragastric pressure to 20 cm H_20, also calculated from the volume-pressure regression curves.

Laser Doppler Flow Measurement

Gastric blood flow was assessed with a laser Doppler flow probe, PF 415:1 probe, and flow measurements were monitored on a Periflux 4001 Master (Perimed, Jafälla, Sweden) as previously described.[14,15] Calibration was carried out as recommended by the manufacturer. The probe was placed in a standard latex solution and at a band width of 12 kHz and a gain of 1, the flowmeter deflection was set to 25% of full scale. This level is defined as 250 perfusion units (PU). Analogue laser Doppler flow signals were digitalized with a UIM 100 recorder (Biopac Systems, Inc., Goleta, Calif.) and processed on a personal computer with the provided MP 100 WSW software (Biopac Systems, Inc.). Probe placement and analyzer operation were handled by two different investigators to avoid observer bias and variability.[16] Blood flow was recorded for at least 30 seconds after a stable signal was obtained at points 1 through 3 (Figs. 3 and 4) after gastric resection, after gastroplasty, after completion of the anastomosis, and on relaparotomy.

Gastric Tube Angiography

After 14 days, animals were reoperated. After blood flow and gastric compliance measurements, 25,000 international units of heparin was injected and stomachs were removed en bloc with the spleen and all adjacent blood vessels. Gastric and gastroepiploic arteries were cannulated on the back table. After angiography dye injection, x-ray films were obtained and macroscopically visible blood vessels running to the distal end of the tube were counted (Fig. 5).

Gastric Tube Histology

Gastric specimens were collected from the resected gastroesophageal junction as well as from the distal end of the gastric tube on relaparotomy. Specimens

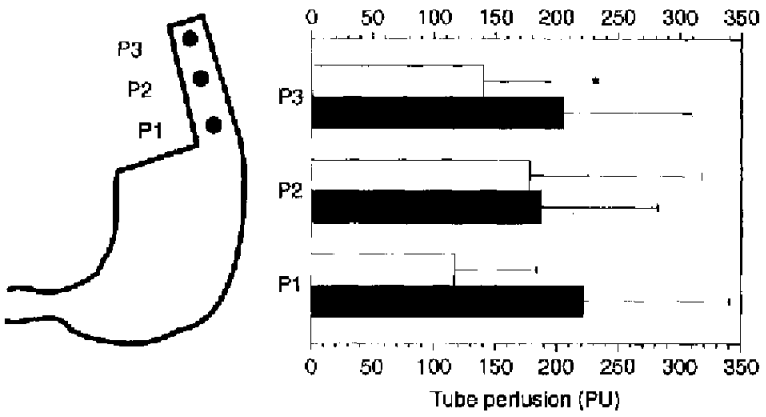


Fig. 4. Gastric tube perfusion in greater curvature pedicled tubes (given in perfusion units *[PU]* of the mean blood flow). Blood flow was measured by laser Doppler flowmetry at points P1 to P3 before (■) and after (□) tube formation. * = $P < 0.05$ vs. P3 before tube formation (ANOVA). The decline in tube perfusion with distance from the tubular pedicle was −4.2 PU/cm.

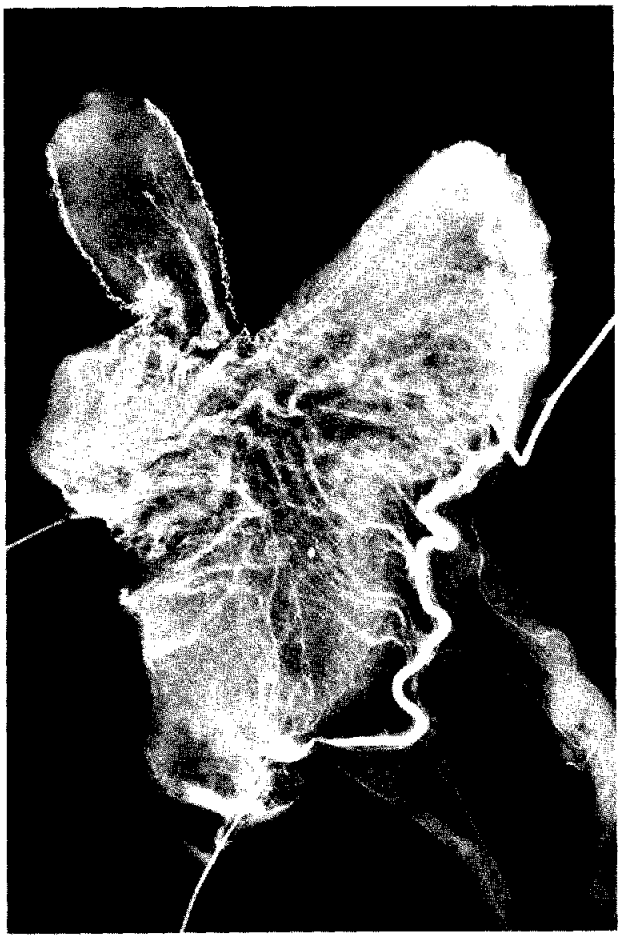


Fig. 5. Postmortem angiography of gastric tube explants for a lesser curvature pedicled gastric corpus rotation tube. There are three vessels visible in the tubes.

Table 1. Operative data for 16 pigs undergoing resection of the gastroesophageal junction and replacement with a gastric corpus rotation tube*

	Group 1 (n = 9)	Group 2 (n = 7)
Sex (M/F)	4/5	3/4
Weight (kg)	21.4 ± 3.2	22.3 ± 1.6
Operation time (min)	132 ± 32	138 ± 18
Blood loss (ml)	53 ± 15	35 ± 14†
Tube length (cm)	9.5 ± 1.5	8.2 ± 0.7
Tube width (cm)	2.8 ± 0.3	2.7 ± 0.4
Survival (14 days)	9/9	2/7
Ileus	0/9	5/7
Tube leakage	1/9	0/7
Anastomotic leakage	0/9	0/7
Vascularization	2.7 ± 0.5	3.5 ± 0.5‡

*Group 1, pedicled at the lesser curvature; group 2, pedicled at the greater curvature.
†$P < 0.05$.
‡$P < 0.01$.

were fixed in formalin and deparaffinized before routine staining with hematoxylin and eosin. Slices were evaluated for ischemic alteration in a blinded fashion as described elsewhere.[17]

Statistics

Results are given as the mean ± standard deviation (SD). Two-tailed Student's *t* test and analysis of variance (ANOVA) with Bonferroni correction for multi-

Table II. Volume and compliance of the gastric remnant after tube formation and 2 weeks postoperatively in 16 pigs undergoing resection of the gastroesophageal junction and replacement with a gastric corpus rotation tube*

	Group 1		Group 2	
	After tube formation	14 days postoperatively	After tube formation	14 days postoperatively
Volume (ml)	113	293	103	180
Compliance (ml/cm H_2O)	5.7	16.1	5.8	9.0

*Group 1, pedicled at the lesser curvature; group 2, pedicled at the greater curvature.
Volume and compliance were calculated from regression curves as previously described.[12]

ple comparisons were used to calculate differences between groups. Regression analysis was performed using SigmaPlot for Windows and InStat software on an IBM-compatible personal computer. $P < 0.05$ was considered statistically significant.

RESULTS

Operative and outcome data are summarized in Table I. Group 1 tubes were 9.5 ± 1.5 cm long and 2.8 ± 0.3 cm wide, and group 2 tubes were 8.2 ± 0.7 cm long and 2.7 ± 0.5 cm wide. This allowed for tension-free anastomosis with the esophagus at a level just below the lower pulmonary veins in all 16 animals, without further mobilization of the esophagus or the duodenum.

In animal No. 2 of group 1, a leakage of the lateral staple line was found on postmortem examination. Longer staples were used, and staple lines were inverted with interrupted 5-0 PDS sutures in all subsequent operations and no further leakage was found. No leakage at the anastomosis site was found on postmortem examination.

In group 2, one animal died of cardiac arrest during intubation for the second laparotomy 2 weeks after the first operation. Another four animals in that group had clinical signs of bowel obstruction with vomiting and abdominal tenderness and were reoperated early (on average 5 days after resection of the gastroesophageal junction). Proximal small bowel distention was found in all four animals, but no adhesions or bowel strangulations were found. Gastric volume and perfusion studies in these animals were therefore performed before the end point of 14 days.

Gastric Volume and Compliance of the Gastric Remnant

After resection of the gastroesophageal junction, the volume of the gastric remnant was 391 ± 62 ml and the compliance 11.7 ml /cm H_2O (n = 16). Tube formation reduced the volume of the gastric remnant by 71% and gastric compliance by 51% in group 1 tubes and by 74% and 50%, respectively, in group 2 tubes. Gastric volume and compliance returned to near-normal values in group 1 animals within 2 weeks postoperatively (Table II).

Blood Flow and Vascularization

After proximal gastric resection, mean blood flow along the resection line ranged between 167 ± 132 PU and 254 ± 91 PU (points P1 to P3; see Fig. 3) and compares favorably with perfusion in the normal porcine stomach.[12] After tube formation, a decline in perfusion was noted along the tube. Mean blood flow at the tip of the tube (P3) was reduced in lesser (see Fig. 3) and greater curvature (see Fig. 4) pedicled tubes, when compared to values before tube formation ($P < 0.05$). By the end of the operation (group 1, 167 ± 79 PU; group 2, 107 ± 77 PU) and 2 weeks postoperatively (group 1, 148 ± 71 PU, group 2, 154 ± 101 PU), near-normal blood flow was measured at the tip of the tubes.

On postmortem angiography, an average of 2.7 ± 0.5 branches out of the right gastric artery were visible in lesser curvature pedicled tubes and 3.2 ± 0.5 branches in greater curvature pedicled tubes ($P = 0.06$).

Macroscopic examination of the gastric tubes revealed a pink mucosa without ulcerations or signs of reflux. On histologic examination, a normal gastric mucosal architecture with no ischemic lesions or signs of gastroesophageal reflux was found. Occasional leukocytic infiltrates were seen in the submucosal layer of the gastric tubes, when compared to resected specimens of the gastroesophageal junction.

DISCUSSION

There is unequivocal agreement between surgical and medical gastroenterologists that premalignant le-

sions of the lower gastrointestinal tract should be resected either by endoscopic or open surgical procedures. Intestinal metaplasia of the gastroesophageal junction predisposes patients to a similar risk for development of malignant tumors as in villous adenomas of the colorectum. Few medical gastroenterologists, however, would favor a resective approach in patients with Barrett's esophagus and would instead recommend a nonresective or conservative approach.

One of the reasons for that observant attitude might be the extent of currently available resective procedures in the form of esophagectomies, gastrectomies, or combinations thereof.

We have described herein a simple technique of resection of the gastroesophageal junction only, which preserves most of the esophagus and a major portion of the stomach. Substitution of the distal esophagus and proximal stomach is facilitated by the use of a gastric tube that is built from the gastric corpus. It leaves an adequate gastric reservoir below the diaphragm and allows for a tension-free anastomosis between the lower esophagus and a well-perfused stomach tube. The width of the tube was selected to be 3 cm, and the length of the tube is determined by the width of the native gastric corpus. Although no data are available on the width of the human stomach, the length of the greater curvature was found to be 38 to 41 cm in Western[7,18] and 45 to 46 cm in Eastern populations.[18] In pigs of the same weight and size as those used in this study, the ratio of gastric width at the corpus to length of the greater curvature was 1:3.8,[12] which extrapolated to the human stomach could result in a gastric corpus tube with a length of 10 to 12 cm and allow for the resection of 10 to 12 cm of the gastroesophageal junction. Preservation of the right gastric or right gastroepiploic artery allowed for branches of these arteries to sufficiently supply the gastric tubes with blood. Dissection of the mural blood supply and surgical manipulation during tube formation temporarily decreased the actual perfusion of the tubes, with blood flow returning to near-baseline values at the end of the procedure. It was of interest that mean blood flow in either type of tube was well above 50 PU, which we and others[12,17] have found to be a critical perfusion limit for anastomotic healing, and histologic examination of the tubes revealed a normal gastric mucosal architecture with no ischemic lesions detectable 2 weeks postoperatively.

No anastomotic leakage occurred in either type of tube. A 1 cm long breakdown of the lateral staple line was found in a group 1 tube. Longer staples were used subsequently and staple lines were inverted with interrupted sutures with no further lateral leakage for either tube type.

As expected, tube formation from the gastric corpus decreases the volume of the gastric remnant initially. Normal food intake (as seen in group 1 animals), however, increased gastric volume and compliance to pretransformation values. In the experiments described herein, we did not study the motility of the tubes or the gastric remnant nor did we perform radiographic or scintigraphic studies to assess esophagogastroduodenal bolus transport or reflux of gastric contents into the esophagus. Earlier experimental studies by Pearson and Henderson,[19] however, indicate that if a short segment of the gastric tube is located below the diaphragm, as in our technique, no reflux will occur irrespective of additional antireflux measures. The fact that we did not see any signs of reflux in the pig, however, cannot be easily extrapolated to humans. Pigs, with a horizontal bolus transport, might have indigenous esophageal defense mechanisms against gastroesophageal reflux, so that placement of gastric corpus rotation tubes in humans might require antireflux measures. These antireflux procedures, however, could easily be applied with the neofundus, which could be wrapped around the tube in a Nissen- or Toupet-like fashion, should reflux occur in humans.

In summary, in pigs, horizontal tubes created from the gastric corpus can safely substitute for the distal esophagus after resection of the gastroesophageal junction, so that further experimental as well as clinical studies of that surgical technique seem warranted.

The excellent technical assistance of O. Beslac, H. Gisiger, and G. Vucic is gratefully acknowledged. We thank Dr. R. Vonlanthen, Department of Pathology, University of Geneva, for evaluation of the histologic specimens, and Prof. Dr. R. Berchthold for critical review of this manuscript.

REFERENCES

1. Lotheisen G. Über plastischen Ersatz der Speiseröhre, insbesondere aus dem Magen. Beitr Zur Klin Chir 1922;126:490-531.
2. Jianu A. Über Oesophagoplastik. Deutsch Zeitschr Chir 1914; 131:397-403.
3. Kirschner M. Ein neues Verfahren der Oesophagusplastik. Arch Klin Chir 1920;114:604-612.
4. Akiyama H, Hashimoto C. Resection and reconstruction for carcinoma of the thoracic oesophagus. Br J Surg 1976;63:206-209.
5. Gavrilyu D, Georgescu L. Esofagoplastie directacu material gastric. Rev Stiint Med 1951;3:33-36.
6. Heimlich HJ, Winfield JM. The use of gastric tube to replace or bypass the esophagus. Surgery 1955;37:549-559.
7. Koskas F, Gaiet B. Anatomical study of retrosternal gastric esophagoplasties. Anat Klin 1985;7:237-256.

8. Liebermann-Meffert DMI, Meier R, Siewert JR. Vascular anatomy of the gastric tube used for esophageal reconstruction. Ann Thorac Surg 1992;54:1110-1115.
9. Davydov MI, Nered SN, Kuzmichov VA, Volkov SM. Gastric esophagoplasty for esophageal carcinoma. Semin Surg Oncol 1992;8:9-15.
10. Bemelman WA, Taat CW, Slors JFM, van Lanschot JJB, Obertop H. Delayed postoperative emptying after esophageal resection is dependent on the size of the gastric substitute. J Am Coll Surg 1995;180:461-464.
11. Büchler MW, Baer HU, Seiler Ch, Schilling M. A technique for gastroplasty as substitute for the esophagus: Fundus rotation gastroplasty. J Am Coll Surg 1996;182:241-245.
12. Schilling MK, Mettler D, Redaelli C, Büchler MW. Circulatory and anatomical differences between experimental gastric tubes as esophageal replacement. World J Surg 1997;21:992-997.
13. Schilling MK, Zbären H, Baer HU, et al. First clinical experience with fundus rotation gastroplasty as substitute for the esophagus. Br J Surg 1997;84:126-129.
14. Schilling MK, Redaelli C, Maurer Ch, Friess H, Büchler MW. Gastric microcirculatory changes during gastroplasty—assessment with laser Doppler flowmetry. J Surg Res 1996;62:125-129.
15. Schilling MK, Redaelli C, Krähenbühl L, et al. Splanchnic microcirculatory changes during CO_2 laparoscopy. J Am Coll Surg 1997;184:378-382.
16. Iwao T, Toyonaga A, Ikegami M, Oho K, Sumino M. Observer agreement and variability in measuring gastric mucosal blood flow by laser Doppler flowmetry in humans. Endoscopy 1993;25:274-277.
17. Krohg-Sorensen K, Line PD, Haaland T, Horn RS, Kvernebo K. Intraoperative prediction of ischaemic injury of the bowel: A comparison of laser Doppler flowmetry and tissue oximetry to histological analysis. Eur J Vasc Surg 1992;6:518-524.
18. Goldsmith HS, Akiyama H. A comparative study of Japanese and American gastric dimensions. Ann Surg 1979;190:690-693.
19. Pearson FG, Henderson RD. Experimental and clinical studies of gastroplasty in the management of acquired short esophagus. Surg Gynecol Obstet 1973;136:737-744.

Interleukin-10 Protects Against Lethality of Intra-Abdominal Infection and Sepsis

Anthony J. Rongione, M.D., Amy M. Kusske, M.D., Karen Kwan, B.S., Stanley W. Ashley, M.D., Howard A. Reber, M.D., David W. McFadden, M.D.

The aim of this study was to determine whether interleukin-10 would alter locally derived and systemic proinflammatory cytokine expression and protect from the lethality of cecal ligation and puncture. Three groups of Sprague-Dawley rats were used. Group 1 underwent cecal manipulation. Groups 2 and 3 underwent cecal ligation and puncture. Group 2 received intraperitoneal saline injections beginning 1 hour after cecal ligation and puncture and every 3 hours thereafter for 24 hours. Group 3 received intraperitoneal interleukin-10 one hour after cecal ligation and puncture and every 3 hours thereafter. Animals were killed at 6 and 24 hours after cecal ligation and puncture or sham operation. Serum tumor necrosis factor-alpha (TNF-α) levels were determined by enzyme-linked immunosorbent assay. TNF-α messenger RNA expression was determined by reverse transcriptase–polymerase chain reaction using β-actin as the internal standard. There was a twofold increase ($P < 0.001$) in TNF-α mRNA in the liver at 6 and 24 hours after cecal ligation and puncture when compared to rats treated with interleukin-10. There was a twofold increase ($P < 0.05$) in TNF-α mRNA in the lung observed only at 24 hours after cecal ligation and puncture when compared to rats treated with interleukin-10. Serum levels of TNF-α were elevated at 6 hours in control animals, and this effect was abolished by the administration of interleukin-10. There was no difference in mortality rates at 6 hours (0% for all groups); however, at 24 hours 57% (4/7) mortality was observed in group 2 vs. 0% (0/20) in groups 1 and 3. Interleukin-10 given after the onset of cecal ligation and puncture protects against the lethality of intra-abdominal sepsis. (J GASTROINTEST SURG 2000;4:70-76.)

KEY WORDS: Interleukin-10, sepsis, tumor necrosis factor (TNF), intra-abdominal infection/abscess

Intra-abdominal infection and sepsis remain common surgical problems with high morbidity and mortality despite advances in critical care therapy and improved antibiotic regimens. Antibiotics have a primary role in therapy but are often inadequate when used alone in severely debilitated patients. These clinical results led researchers to investigate additional modes of therapy. The mortality rate of sepsis approaches 30% to 70% in most series[1,2] and is usually a result of multiorgan system failure. The multiorgan system failure does not appear to be related to the site of infection; rather, recent evidence has implicated proinflammatory cytokines released from the site of injury as the mediators of the sepsis syndrome.

The immune system is an important regulator of the response to intra-abdominal infection. More specifically, the activated macrophage and it products of inflammation are thought to direct the systemic response to this disease process. Tumor necrosis factor-alpha (TNF-α), a cytokine produced primarily by activated macrophages, is thought to be a principal mediator of the sepsis syndrome and associated multiorgan system failure.[3] Administration of TNF-α can mimic the physiologic changes seen in human sepsis, and elevated levels have been demonstrated in human

From the Departments of Surgery, UCLA and Sepulveda VA Medical Centers, Los Angeles, Calif.
Supported by Veterans Administration Merit Review Funds (H.A.R., S.W.A., and D.W.M).
Presented at the Thirty-Seventh Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, Calif., May 19-22, 1996 (poster presentation).
Reprint requests: David W. McFadden, M.D.,UCLA Medical Center, 10833 LeConte Ave., Room 72-215 CHS, Los Angeles, CA 90024

subjects with sepsis syndrome.[4,5] The study by Damas et al.[5] demonstrated a correlation between systemic levels of TNF-α and the degree of morbidity and subsequent mortality in human subjects.

Interleukin-10 (IL-10) is a 35 kd anti-inflammatory cytokine that has been shown to inhibit production of TNF-α from the macrophage.[6] Its relevance in models of sepsis and endotoxemia has recently emerged. Bean et al.[7] have shown that IL-10 protects mice against staphylococcal enterotoxin B–induced lethal shock. In separate studies by Howard et al.,[8] IL-10 protected mice from lipopolysaccharide-induced lethal endotoxemia. Furthermore, Gerard et al.[9] demonstrated that IL-10 was able to protect mice from lipopolysaccharide-induced lethal shock, presumably by preventing TNF-α production from activated macrophages.

The aim of the present study was to investigate the effects of IL-10 in an alternate model of sepsis (cecal ligation and puncture [CLP]) and to correlate the degree of severity with serum levels of TNF-α and tissue levels of TNF-α messenger RNA. The effects of IL-10 on the mortality associated with this model were also evaluated.

MATERIAL AND METHODS

Recombinant murine IL-10 was a generous gift from DNAX Research Institute (Palo Alto, Calif.). Aprotonin was obtained from Sigma Chemical (St. Louis, Mo.). Mouse TNF-α ELISA Factor-Test X Kit was obtained from Genzyme Corporation (Cambridge, Mass.). Rat TNF-α sense and antisense primers were constructed by and obtained from Midland Certified Reagent Company (Midland, Tex.). Rneasy total RNA purification kit was obtained from Qiagen Incorporated (Chatsworth, Calif.). Gene Amp thermostable rTth reverse transcriptase RNA polymerase chain reaction (PCR) kit was obtained from Perkin-Elmer (Branchburg, N.J.).

All experiments were conducted with the prior approval of the Animal Research Committee at Sepulveda VA Medical Center, Sepulveda, California. Adult female Sprague-Dawley rats, weighing 250 to 300 grams, were purchased from Harlan Sprague-Dawley Incorporated (San Diego, Calif.), fed standard laboratory chow, and allowed to acclimatize for a minimum of 1 week. Animals were anesthetized using sodium pentobarbital (40 mg/kg) given intraperitoneally and ketamine hydrochloride (30 mg/kg) given intramuscularly. CLP was performed as originally described in a review by Wichterman et al.[10] Specifically, a midline incision was made approximately 4 cm in length to expose the cecum. A 3-0 silk ligature was placed at the base of the cecum without causing bowel obstruction. The cecum was then punctured twice using an 18-gauge needle. The cecum was then gently squeezed to assure patency of the two holes as well as to express fecal material. The abdominal incision was then closed in two layers using 3-0 Prolene and 4-0 Dexon sutures, respectively. All animals were fluid resuscitated with 10 ml normal saline solution administered subcutaneously. CLP typically results in a 40% mortality rate at 24 hours in our laboratory.

Animals were allowed to recover from anesthesia and were allowed free access to laboratory chow and water. Animals were then reanesthetized at either 6 or 24 hours after CLP with intraperitoneal sodium pentobarbital. Laparotomies were performed and blood samples were immediately obtained from the inferior vena cava. Samples were collected in heparinized Vacutainers containing aprotonin (0.09 trypsin inhibitory units/ml of blood). Serum was separated and stored at −70° C until assayed. Tissue samples were obtained from liver and lung and were snap frozen in liquid nitrogen and stored for future RNA extraction.

Experimental Design

Forty-two female Sprague-Dawley rats were divided into three equal groups. Group 1 control rats underwent laparotomy and cecal manipulation alone. Groups 2 and 3 underwent laparotomy and CLP. Groups 1 and 2 received intraperitoneal injections of normal saline solution 1 hour after sham operation or CLP and every 3 hours for the duration of the experiment (100 μl/injection). To investigate the therapeutic potential of IL-10, group 3 received 150,000 units intraperitoneally of IL-10, diluted in 0.1% bovine serum albumin as a protein carrier. The IL-10 was given in an identical volume as the normal saline solution given to control rats. The dosage of IL-10 and the interval (half-life) at which it was given were based on the previous studies of Bean et al.,[7] Howard et al.,[8] and Gerard et al.[9] In these studies the dosage administered was based on the weight of the animal used, and we further extrapolated this to the rat model of CLP. The IL-10 was given 1 hour after induction of CLP and every 3 hours for the remainder of the experiment (100 μl/injection). Half of the animals were killed at 6 hours and the remaining animals were killed at 24 hours.

Serum TNF-α Assay

Serum TNF-α levels were determined using an enzyme-linked immunoabsorbant assay according to the manufacturer's instructions. All serum samples were assayed in duplicate and the results averaged at the end of the experiment.

Isolation of Total Cellular RNA and Quantitation of TNF mRNA by Differential Reverse Transcriptase-Polymerase Chain Reaction

Extraction of total cellular RNA was performed according to the commercially available method described by Qiagen Incorporated. Total cellular RNA (500 ng) was reverse transcribed according to the Gene Amp thermostable rTth reverse transcriptase RNA PCR kit protocol using primers specific for TNF-α: sense primer 5′-AGC ACG GAA AGC ATG ATC CGA GA G-3′ and antisense primer 5′-GTT GTC TTT GAG ATC CAT GCC ATT GG-3′. They were selected to span an intronic sequence to differentiate mRNA from contaminating DNA in tissue preparations; the predicted size of the amplified complementary DNA fragment for TNF-α was 374 base pairs. To correct for quantitative differences between samples and possible PCR artifacts, internal controls consisting of primers specific for β-actin mRNA were used: sense primer 5′-ATC ACC ATT GGC AAT GAG CGG TTC C-3′ and antisense primer 5′-CTC GTC ATA CTC CTG CTT GCT GAT-3′ were coamplified with each sample. The predicted size for β-actin was 329 base pairs. Briefly, 500 ng of total RNA was incubated for 15 minutes at 70° C with 5 units of rTth DNA polymerase, 300 μmol/L deoxynucleotide triphosphate, 1 mmol/L $MgCl_2$, 0.75 μmol/L antisense primers, and reverse transcriptase buffer (100 mmol/L Tris-HCl, pH 8.3, 900 mmol/L KCl) in a total volume of 20 μl. To this was added 2.5 mmol/L $MgCl_2$, 0.15 μmol/L sense primers, and chelating buffer (50% [volume/volume] glycerol, 100 mmol/L Tris-HCl, pH 8.3, 1 mol/L KCl, 0.5% [weight/volume] Tween 20, 7.5 mmol/L EGTA [ethylene glycol-bi {β-aminoethyl ether} N,N,N′,N′-tetraacetic acid] in a total volume of 80 μl). Amplification was performed on a thermal cycler (Perkin-Elmer DNA Thermal Cycler 480) for 30 cycles (denaturation, 1 minute at 95° C; annealing, 1 minute at 58° C; extension, 1 minute at 72° C). The PCR products were separated on 1.8% agarose gels (Perkin-Elmer). Photomicrographs were taken of the ethidium-bromide–stained gels. Relative mRNA levels (TNF-α mRNA/β-actin mRNA) were determined by computer-assisted densitometric scanning (Image-1, Universal Imaging Corp., Westchester, Pa.).

Statistical Analysis

Differences between groups were evaluated using paired Student's t test. Differences with $P < 0.05$ were considered significant. Results are expressed as mean ± standard error of the mean.

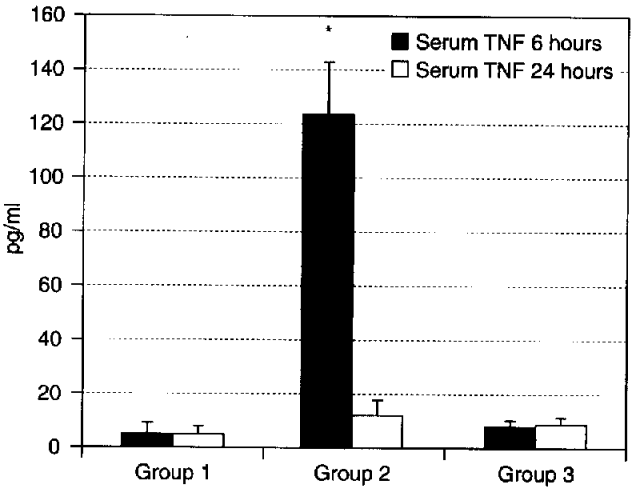


Fig. 1. Serum TNF-α levels. There was no elevation in serum TNF-α levels in sham-operated animals. Serum TNF-α levels peaked at 6 hours following CLP and returned to baseline by 24 hours after CLP. IL-10 significantly attenuated the rise in serum TNF-α. * = $P < 0.001$ vs. CLP alone.

RESULTS

Serum TNF-α

Sham-operated animals (group 1) had no detectable levels of serum TNF-α at any time point in our study. Following CLP, serum levels of TNF-α peaked by 6 hours. IL-10 given 1 hour following CLP effectively inhibited this elevation in systemic TNF-α levels ($P < 0.001$ vs. group 2). Serum levels of TNF-α had returned to baseline by 24 hours in all groups (Fig. 1).

Liver mRNA TNF-α

Tissue levels of TNF-α mRNA were not detectable in sham-operated control animals. Following CLP alone (group 2), there was approximately a twofold increase in TNF-α mRNA at 6 and 24 hours compared to animals receiving IL-10. Treatment of animals with IL-10 (group 3) was able to inhibit mRNA production of TNF-α at both time points when compared to group 2 ($P < 0.001$). To make graphic representation simpler, we arbitrarily chose a level of 100% (a level not detectable) to represent the sham-operated control animals. Results are shown in Fig. 2.

Lung mRNA TNF-α

Tissue levels of TNF-α mRNA were not detected in sham-operated control animals (group 1). Following CLP alone, animals in group 2 had a twofold in-

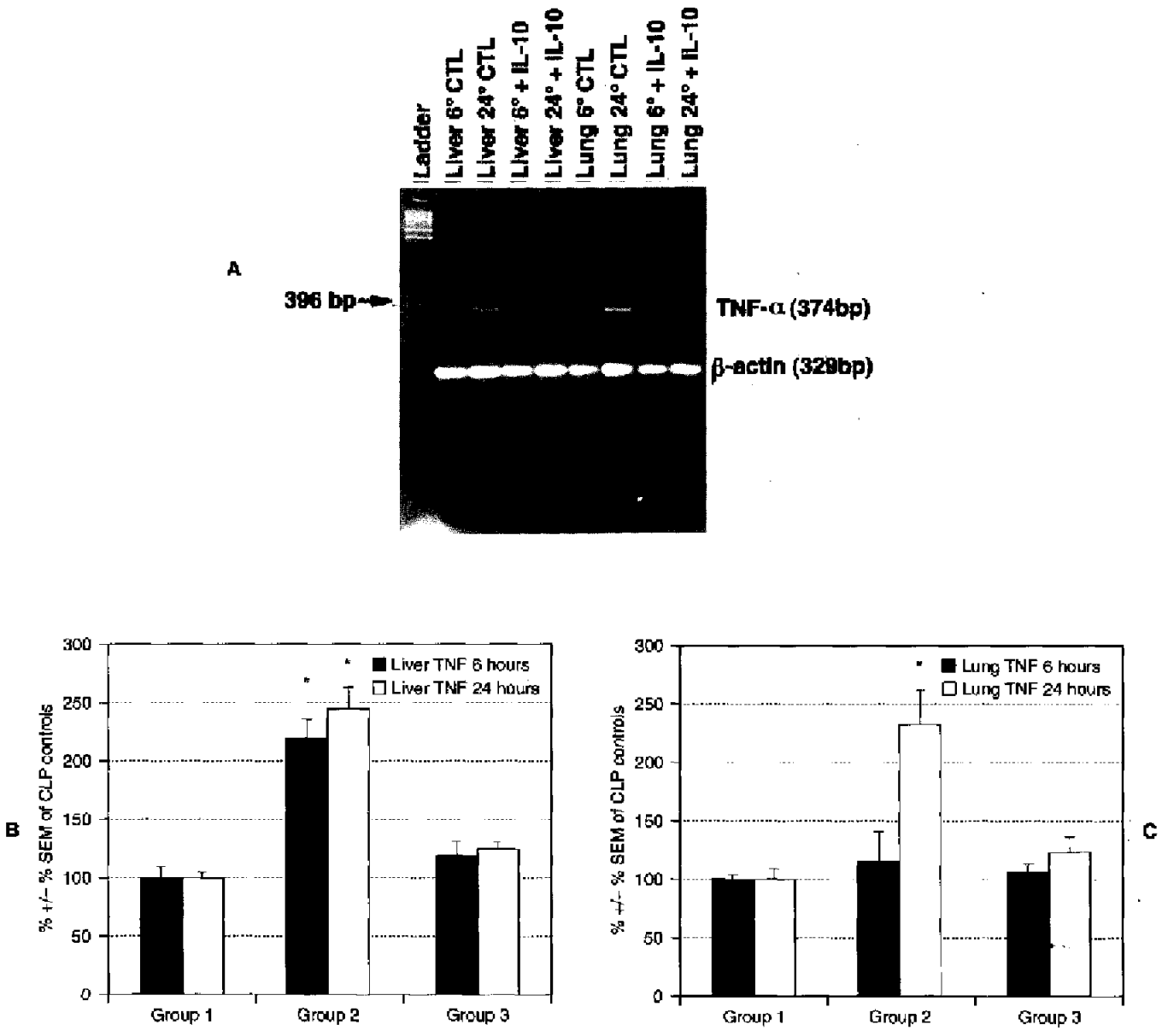


Fig. 2. A, Expression of liver and lung tissue TNF-α mRNA levels 6 and 24 hours after CLP. Liver and lung tissue levels of TNF-α mRNA were not detectable in sham-operated control animals and therefore are not pictured above. Following CLP alone (group 2), there was approximately a twofold increase in liver tissue TNF-α mRNA at 6 and 24 hours compared to animals receiving IL-10 (* = $P < 0.001$). Group 2 animals had a twofold increase in lung tissue mRNA TNF-α only at 24 hours when compared to animals receiving IL-10 (* = $P < 0.001$). β-actin was concomitantly amplified to assess RNA integrity and loading. **B** and **C,** Results of densitometric scanning. Data are expressed as percentage ± standard error of the mean of CLP control animals. CLP = cecal ligation and puncture; CTL = untreated CLP control animals; bp = base pairs.

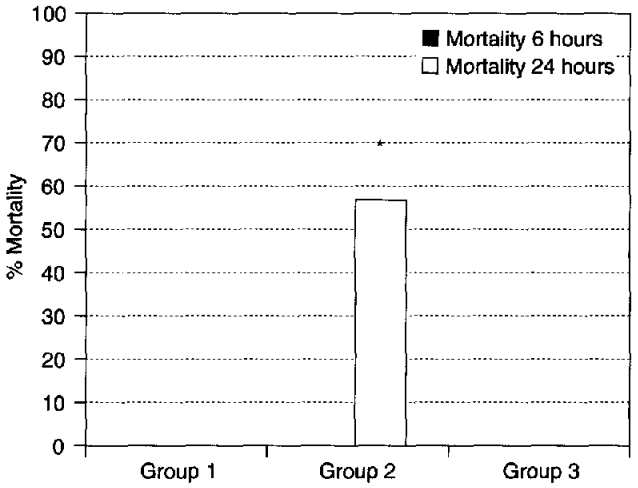


Fig. 3. Mortality rates. There were no deaths in any group at 6 hours. Mortality rates in groups 1 (sham-operated) and 3 (therapeutic IL-10) were significantly reduced (0%) when compared to group 2 (CLP alone; 57%) at 24 hours. * = $P < 0.001$.

crease in tissue mRNA TNF-α only at 24 hours when compared to animals receiving IL-10. Group 3 animals that received IL-10 had no significant elevation in mRNA TNF-α at either time point ($P < 0.001$ vs. group 2). Results are shown in Fig. 2.

Mortality

There were no deaths in sham-operated animals (group 1) or in animals that received IL-10 (group 3). Following CLP alone (group 2), there were no deaths at 6 hours, whereas at 24 hours four (57%) of seven animals had died. Results are shown in Fig. 3.

DISCUSSION

Endotoxin challenge and infection represent two types of animal models that simulate the conditions in which bacterial infection leads to sepsis. Although few animal models completely mimic the human sepsis syndrome, the literature suggests that models which permit an evolution of sepsis from a focus more closely mimic the sepsis syndrome than models that use a short-lived fulminant bolus of lipopolysaccharide or live microbes. The physiologic effects of the CLP model in the rat have been well studied and described, and more closely mimic the hemodynamic, metabolic, and inflammatory response seen in human sepsis.[10,11] In the present study we have shown that CLP invokes a systemic inflammatory response associated with serum and tissue elevations of TNF-α similar to results recently published by Hadjiminas et al.[12]

It is thought that TNF-α, a proinflammatory cytokine primarily produced by the macrophage, is a major mediator of the acute inflammatory response. Specifically TNF-α has been associated with adult respiratory distress syndrome,[13] disseminated intravascular coagulation,[14] activation of inflammatory cells, upregulation of adhesion molecules,[15] production of nitric oxide,[16] and the progression and release of other inflammatory cytokines (i.e., IL-1 and IL-6). Although the exact etiology of the multiorgan system failure caused by sepsis remains unclear and may be multifactorial, the activated macrophage and its release of cytokines, that is, TNF-α, appear to be important in the pathogenesis of the sepsis syndrome.[17] Although TNF-α may serve a protective role in the early inflammatory process, excessive tissue production may lead to multiorgan system failure and death.[18] With this in mind, several studies have attempted to address the role of TNF-α in the evolution of sepsis. For instance, some studies have shown elevated levels of serum TNF-α in patients with sepsis,[19] whereas others have demonstrated that these increased levels correlated with multiorgan system failure and death.[20,5] Furthermore, others have attempted to address therapies modulating TNF-α. Investigators have shown that neutralizing antibodies to TNF-α prevented death in mice, rabbits, and baboons following a lethal injection of endotoxin or *Escherichia coli* organisms.[21-24] In all but one of these studies, the beneficial effect of TNF-α neutralizing antibodies was only apparent if administered prior to the insult. In contrast, more recent literature has shown that inhibition of TNF activity with specific neutralizing antibodies may, in fact, be detrimental.[25-27] These and other data led us to believe that the response to treatment modalities varies with the model employed and, furthermore, TNF-α may only represent one of many mediators responsible for the evolution of sepsis.

Central to the progression of intra-abdominal infection and sepsis, the macrophage has recently emerged as a critical regulator of this inflammatory response. The production and release of proinflammatory cytokines such as IL-1, IL-6, and TNF-α by macrophages induced by CLP have clearly been demonstrated.[28-30] We therefore chose in our study to inhibit the macrophage following CLP in an attempt to inhibit locally derived and systemically released cytokine production and in doing so improve the mortality rate seen with this model. To our knowledge no current data are available that clearly demonstrate an improved survival benefit through inhibition of the

macrophage during CLP. Furthermore, no studies demonstrate whether therapeutic administration of anticytokine therapy at a time when intra-abdominal infection and sepsis were already well underway, a more clinically relevant occurrence, would be beneficial.

IL-10 has recently emerged as a potent anti-inflammatory cytokine produced primarily by macrophages. As mentioned earlier, it has been shown that IL-10 has the ability to improve outcomes in models of endotoxemia and lipopolysaccharide-stimulated sepsis. It is thought that the beneficial effect of IL-10 is through the inhibition of endotoxin and lipopolysaccharide release of proinflammatory cytokines by monocytes, macrophages, and T-helper cells. Accordingly it seemed reasonable that direct inhibition of the macrophage itself by IL-10, which would theoretically inhibit the release of its detrimental inflammatory cytokines, might be more effective than single-cytokine immunomodulatory therapy (i.e., anti–TNF-α antibodies).

Our results show that following CLP, serum levels of TNF-α are elevated early at 6 hours and return to undetectable levels by 24 hours. These findings are similar to those reported by Hadjiminas et al.[12] Administration of IL-10 one hour after CLP significantly attenuated this rise in systemically derived TNF-α.

RT-PCR analysis of TNF-α mRNA expression demonstrated a twofold increase in liver TNF-α levels at 6 and 24 hours after CLP when compared to animals that received IL-10, whereas a twofold increase in TNF-α levels in the lung was not observed until 24 hours after CLP. These findings suggest an evolution of the disease process as the liver is a more proximal organ system than the lung. These results are similar to those seen by other investigators who demonstrated that tissue TNF-α levels are elevated earlier in organ systems nearer to the site of injury.[12] These findings collectively support the concept that local production of TNF-α contributes to the progression of the sepsis syndrome. In our study we demonstrated that therapeutic administration of IL-10 clearly attenuated this local production of TNF-α in both the liver and the lung. In this study there were no deaths in any group at 6 hours; however, by 24 hours there was a 57% mortality rate in the CLP group. Therapeutic administration of IL-10 decreased the mortality rate to 0% at 24 hours. One may hypothesize that the improved mortality rate seen in this study was through inhibition of the macrophage. The current literature supports the notion that the reduction in TNF-α improves survival, a finding that is also demonstrated in our study. Further studies in our laboratory are in progress establishing mortality curves and evaluating other macrophage-derived cytokines inhibited by IL-10.

CONCLUSION

Our data appear to demonstrate the importance of the macrophage in the pathogenesis of intra-abdominal infection and sepsis. These findings suggest the possibility that agents that inhibit macrophage proinflammatory cytokine release may be beneficial early in the septic state. IL-10 may warrant further study in the treatment of overwhelming infection and sepsis as clinical trials are already in progress evaluating IL-10 as a potential therapy for other inflammatory conditions.

REFERENCES

1. Hinshaw LB, Beller-Todd BK, Archer LT. Current management of the septic shock patient: Experimental basis for treatment. Circ Shock 1982;9:543-553.
2. Zimmerman JJ, Dietrich KA. Current perspectives on septic shock. Pediatr Clin North Am 1987;34:131-163.
3. Bone RC. The pathogenesis of sepsis. Ann Intern Med 1991; 115:457-469.
4. Tracey KJ, Lowry SF, Fahey TJ, Albert JD, Fong Y, Hesse D, Beutler B, Manogue KR, Calvano S, Wei H, et al. Cachectin/tumor necrosis factor induces lethal shock and stress hormone response in the dog. Surg Gynecol Obstet 1987;164:415-422.
5. Damas P, Reuter A, Gysen P, Demonty J, Lamy M, Franchimont P. Tumor necrosis factor and interleukin-1 serum levels during severe sepsis in humans. Crit Care Med 1989;17: 975-978.
6. Moore KW, O'Garra A, de Waal Malefyt R, Vieira P, Mosmann TR. Interleukin-10. Annu Rev Immunol 1993;11:165-190.
7. Bean AGD, Freiberg A, Andrade S, Menon S, Zlotnik A. Interleukin 10 protects mice against staphylococcal enterotoxin B-induced lethal shock. Infect Immun 1993;61:4937-4939.
8. Howard M, Muchamuel T, Andrade S, Menon S. Interleukin 10 protects mice from lethal endotoxemia. J Exp Med 1993; 177:1205-1208.
9. Gerard C, Bruyns C, Marchant A, Abramowicz D, Vandenabeele P, Delvaux A, Fiers W, Goldman M, Velu T. Interleukin 10 reduces the release of tumor necrosis factor and prevents lethality in experimental endotoxemia. J Exp Med 1993;177: 547-550.
10. Wichterman KA, Baue AE, Chaudry IH. Sepsis and septic shock—A review of laboratory models and a proposal. J Surg Res 1980;29:189-201.
11. Cross AS, Opal SM, Sadoff JC, Gemski P. Choice of bacteria in animal models of sepsis. Infect Immun 1993;61:2741-2747.
12. Hadjiminas DJ, McMasters KM, Peyton JC, Cheadle WG. Tissue tumor necrosis factor mRNA expression following cecal ligation and puncture or intraperitoneal injection of endotoxin. J Surg Res 1994;56:549-555.
13. Ferrari-Baliviera E, Mealy K, Smith RJ, Wilmore DW. Tumor necrosis factor induces adult respiratory distress syndrome in rats. Arch Surg 1989;124:1400-1405.
14. Wada H, Ohiwa M, Kaneko T, Tamaki S, Tanigawa M, Takagi M, Mori Y, Shirakawi S. Plasma level tumor necrosis factor in disseminated intravascular coagulation. Am J Hematol 1991; 37:147-154.

15. Bevilaqua MP, Pober JS, Mendrick DL, Cotran RS, Gimbrone MJ. Identification of an inducible endothelial-leukocyte adhesion molecule. Proc Natl Acad Sci USA 1987;84:9238-9244.
16. Estrada C, Gomez C, Martin C, Moncada S, Gonzalez C. Nitric acid mediates tumor necrosis factor-alpha cytotoxicity in endothelial cells. Biochem Biophys Res Commun 1992; 186:475-479.
17. Pinsky MR, Vincent JL, Deviere J, Alegre M, Kahn RJ, Dupont E. Serum cytokine levels in human septic shock. Relation to multiple-system organ failure and mortality. Chest 1993;103:565-575.
18. Dinarello CA, Gelfand JA, Wolff SM. Anticytokine strategies in the treatment of the systemic inflammatory response syndrome. JAMA 1993;269:1829-1834.
19. de Groote MA, Martin MA, Densen P, Pfaller MA, Wenzel RP. Plasma tumor necrosis factor levels in patients with presumed sepsis. Results in those treated with antilipid A antibody vs placebo. JAMA 1989;262:249-251.
20. Waage A, Halstensen A, Espevik T. Association between tumor necrosis factor in serum and fatal outcome in patients with meningococcal disease. Lancet 1987;1:355-357.
21. Beutler B, Milsark IW, Cerami AC. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science 1985;229:869-871.
22. Mathison JC, Wolfson E, Ulevitch RJ. Participation of tumor necrosis factor in the mediation of gram negative bacterial lipopolysaccharide-induced injury in rabbits. J Clin Invest 1988;81:1925-1937.
23. Tracey K, Fong Y, Hesse DJ. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteremia. Nature 1987;330:662-664.
24. Hinshaw LB, Tekamp-Olson P, Chang ACK, Lee PA, Taylor FB, Murray CK Jr, Peer GT, Emerson TE, Passey RB, Kuo GC. Survival of primates in LD_{100} septic shock following therapy with antibody to tumor necrosis factor (TNFalpha). Circ Shock 1990;30:279-292.
25. Eskandari MK, Bolgos G, Miller C, Nguyen DT, DeForge LE, Remick DG. Anti-tumor necrosis factor antibody therapy fails to prevent lethality after cecal ligation and puncture or endotoxemia. J Immunol 1992;148:2724-2730.
26. Bagby GJ, Plessala KJ, Wilson LA, Thompson JJ, Nelson S. Divergent efficacy of antibody to tumor necrosis factor-α in intravascular and peritonitis models of sepsis. J Infect Dis 1991;163:83-92.
27. Evans GF, Snyder YM, Butler LD, Zuckerman SH. Differential expression of interleukin-1 and tumor necrosis factor in murine septic shock models. Circ Shock 1989;29: 279-287.
28. Furutani M, Arii S, Monden K, Adachi Y, Funaki N, Higashitsuji H, Fujita S, Mise M, Ishiguro S, Kitao T, Nakamura T, Imamura M, Tamatani T, Miyasaka M, Nakayama H, Fujita J. Immunologic activation of hepatic macrophages in septic rats: A possible mechanism of sepsis-associated liver injury. J Lab Clin Med 1994;123:430-436.
29. Ertel W, Morrison MH, Wang P, Zheng F, Ayala A, Chaudry I. The complex pattern of cytokines in sepsis. Association between prostaglandins, cachectin, and interleukins. Ann Surg 1991;214:141-148.
30. McMasters KM, Cheadle WG. Regulation of macrophage TNFα, IL-1β and Ia (1-Aα) mRNA expression during peritonitis is site dependent. J Surg Res 1993;54:426-430.

Small Bowel Transplantation Induces Adrenergic Hypersensitivity in Ileal Longitudinal Smooth Muscle in Rats

Noriya Ohtani, M.D., Bruno M. Balsiger, M.D., William J. Anding, B.A., Judith A. Duenes, B.A., Michael G. Sarr, M.D.

Our aim was to determine the effects of small bowel transplantation on contractility of longitudinal muscle in the rat ileum. Full-thickness longitudinal muscle strips from four groups of rats (naive controls, sham-operated controls, and 1 week and 8 weeks after syngeneic orthotopic small bowel transplantation) were studied in vitro. Neither baseline contractility nor response to neural blockade (tetrodotoxin) or adrenergic/cholinergic blockade differed among the groups. Although the dose response to the cholinergic agonist bethanechol and to nitric oxide did not differ among groups, the ED_{50} (negative log of concentration giving half-maximal effect) for the adrenergic agonist norepinephrine was increased 1 week and 8 weeks after transplantation, indicating a hypersensitivity response not blocked by tetrodotoxin. Nonadrenergic, noncholinergic inhibitory responses to electrical field stimulation were of greater amplitude and occurred at lesser frequencies (≤5 Hz) 1 week after small bowel transplantation, but returned to control values 8 weeks postoperatively. These inhibitory responses were blocked by the nitric oxide synthase inhibitor L-NMMA but not by methylene blue, a nonspecific inhibitor of guanylate cyclase. Small bowel transplantation induces a persistent adrenergic denervation hypersensitivity at the muscle and appears to upregulate, at least transiently, other inhibitory mechanisms mediated by neural release of nitric oxide. Small bowel transplantation does not alter muscle response to cholinergic pathways. These alterations in smooth muscle contractility may affect gut function early after clinical small bowel transplantation. (J GASTROINTEST SURG 2000;4:77-85.)

KEY WORDS: Small bowel transplantation, adrenergic nerves, denervation hypersensitivity, cholinergic receptors, adrenergic receptors

Small bowel transplantation (SBT) has been used clinically in selected patients with intestinal insufficiency.[1,2] Although most experimental emphasis has focused on the immunobiology of intestinal transplantation, little is known about the changes in enteric smooth muscle function after SBT. The physiologic changes after SBT are important because diarrhea occurs in patients after SBT, but the cause of diarrhea remains largely unknown.[1] It will be important to define the changes in smooth muscle function after SBT to help understand the pathogenesis of enteric dysfunction after SBT.

Relaxation and/or inhibition of contractile activity of the gut is mediated in part by a class of nerves within the gut wall referred to as nonadrenergic, noncholinergic (NANC) nerves because their inhibitory neurotransmitter is neither acetylcholine nor norepinephrine.[3,4] Nitric oxide (NO) appears to be one of the primary mediators of smooth muscle relaxation induced by NANC nerves.[5] NO also regulates or modulates many other gastrointestinal functions including motility patterns and secretion.[6-8] Morphologic studies have demonstrated an increase in nitrergic nerves in guinea pig ileum after extrinsic den-

From the Department of Surgery and the Gastroenterology Research Unit, Mayo Clinic and Foundation, Rochester, Minn.
Supported in part by grant DK 39337 from the National Institutes of Health, the Mayo Foundation, and the First Department of Surgery, Tohoku University, Sendai, Japan.
Parts of this work were presented at the Fortieth Annual Meeting of The Society for Surgery of the Alimentary Tract, May 16-19, 1999, and the annual meeting of the American Gastroenterological Association, May 16-19, 1999, Orlando, Fla., and published as two abstracts in *Gastroenterology* 116:A139 and A1075, 1999.
Correspondence: Michael G. Sarr, M.D., Professor of Surgery, Gastroenterology Research Unit, Mayo Clinic, 200 First Street SW, Rochester, MN 55905.

ervation[9] and in rat small intestine after SBT.[10] These studies suggest that nitrergic neurons may play an active role in NANC inhibitory neural function after SBT. We previously showed that NO had no apparent effect on spontaneous activity in ileal circular smooth muscle in rats, and NO appeared to play a minor role in NANC net inhibition of rat jejunal longitudinal smooth muscle.[11,12]

The effects of SBT on ileal longitudinal contractile activity are not understood. Motor functions of the ileum differ from other regions of the small bowel because specific types of contractions observed routinely in the ileum are observed only rarely more proximally.[13,14] Therefore our aim was to determine the changes in contractile activity, both excitatory and inhibitory, in rat ileal longitudinal muscle after isogeneic orthotopic SBT.

MATERIAL AND METHODS

Inbred male Lewis rats (Harlan Sprague-Dawley, Indianapolis, Ind.) weighing 250-300 g were used in all experiments. We specifically used inbred rats to avoid any confounding effects of *allo*transplantation (immune phenomena or need for immunosuppression). Procedures and subsequent animal care were performed according to the guidelines of the Animal Care and Use Committee of the Mayo Foundation in accordance with the guidelines of the National Institutes of Health and the Public Health Service Policy on the Human Use and Care of Laboratory Animals. General anesthesia was achieved by intraperitoneal injections of 30 to 50 mg/kg sodium pentobarbital (Ampro Pharmacy, Arcadia, Calif.). The rats were divided into the following three groups: group l, naive controls (NC) (n = 8); group 2, sham controls (SC) (n = 8); and group 3, small bowel transplantation (SBT) (n = 16). The rats in group 1 were unoperated controls, and they were used within several weeks after their delivery at our institution. The rats in group 2 were anesthetized, underwent a midline celiotomy, the bowel was manipulated bluntly, and the operative wound was closed 30 minutes later; these rats were studied 1 week after the operation. Rats in group 3 were subjected to isogeneic orthotopic SBT according to a modification of the method of Monchik and Russell,[15] as described previously,[11,12,16-21] and were studied 1 week (n = 8) and 8 weeks (n = 8) postoperatively.

Recording of Contractile Activity

The segment of the distal ileum 10 cm proximal to the ileocecal junction in NC and SC rats and 5 cm proximal to the ileal anastomosis in SBT rats was removed, cut open along the mesenteric border, and pinned in chilled modified Krebs-Ringer's bicarbonate solution (concentrations in mmol/L: NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5; $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0, calcium disodium edetate 0.26, and glucose 11.1). Full-thickness muscle strips, 2 to 3 mm wide and 8 to 10 mm long, were cut in the direction of the longitudinal muscle and suspended in separate 10 ml tissue chambers filled with modified Krebs-Ringer's bicarbonate solution. The tissue bath was maintained at 37.5° C and bubbled continuously with 95% oxygen and 5% carbon dioxide (Puritan-Bennett Corp., Lenexa, Kan.). The muscle strips were suspended vertically between a fixed point in the chamber and a noncompliant force transducer (Kulite Semiconductors Products, Inc., Leonia, N.J.) to measure isometric force. Ileal contractile activity was monitored on a strip-chart recorder (Grass 7D Polygraph, Grass Instrument Co., Quincy, Mass.) and converted to digital signals by a computerized data acquisition system (Biopac Systems, Inc., Goleta, Calif.). Digital signals displayed and stored on a personal computer were analyzed on-line using specialized software (AcqKnowledge, Biopac Systems, Inc.).

Experimental Design

After a 45-minute equilibration period in the tissue chambers, with repeated washout of the bath every 15 minutes, each strip was stretched incrementally at 8- to 10-minute intervals to its optimal length (L_0), defined as the length beyond which further stretching failed to increase the amplitude of spontaneous contractile activity. After reaching L_0, the muscle strips were allowed to equilibrate for 10 minutes, and spontaneous contractile activity was measured during this period. Changes in spontaneous contractile activity under NANC conditions were studied after a 35-minute incubation with 10^{-5} mol/L phentolamine, 5×10^{-6} mol/L propranolol, and 10^{-7} mol/L atropine in four strips per rat. Thereafter the effects on spontaneous contractile activity of exogenous NO (doses of 0, 10^{-5}, and 3×10^{-5} mol/L) alone and in the presence of 10^{-5} mol/L methylene blue (a nonspecific inhibitor of soluble guanylate cyclase) were also studied under NANC conditions in two strips per rat. The same volume of distilled water used to dissolve NO[19] was used for the control effect. Only about half of the strips responded to NO; further experiments with the NO inhibitor methylene blue were performed only in those strips showing inhibition of contractile activity to NO.

One muscle strip per rat was exposed to increasing concentrations of the cholinergic agonist bethanechol (3×10^{-8} to 3×10^{-6} mol/L) for 6 minutes before the next higher dose was evaluated. After each dose of bethanechol, the bath solution was washed and the strip was allowed to equilibrate for 3 minutes before the next higher dose was added. In separate dose-response experiments, 10^{-6} mol/L tetrodotoxin (TTX) was administered immediately after each wash, and the next higher dose of bethanechol was given after an 8- to 10-minute equilibration period. TTX blocks almost all intrinsic neural conduction by blocking sodium channels and thus allows determination of the direct effect of bethanechol on the smooth muscle itself. Similar experiments were performed on one other muscle strip using the adrenergic agonist norepinephrine (3×10^{-8} to 3×10^{-5} mol/L) added cumulatively every 6 minutes. As with bethanechol, separate experiments with norepinephrine and TTX were conducted.

All experiments examining electrical field stimulation (EFS) were performed under NANC conditions (phentolamine, propranolol, and atropine) in four strips per rat. At first, the effect of EFS (voltage, 10 volts; pulse width, 0.5 msec; duration of stimulation, 30 seconds) was evaluated at various frequencies (1, 3, 5, 7, 10, 15, and 20 Hz) as a control. The interval between the application of each stimulus was 3 to 5 minutes. Then we examined how the response to EFS was altered after administration of 5×10^{-3} mol/L N^G-monomethyl L-arginine (L-NMMA, a specific inhibitor of NO synthase), 10^{-5} mol/L methylene blue, and 10^{-6} mol/L TTX.

At the conclusion of each experiment, all strips were washed thoroughly, blotted twice, and weighed (Mettler AC 100, Mettler Instrument Corp., Greifensee, Switzerland).

Data Analysis

The integral of the force generated (g·5 min/mg tissue weight as total area under the contractile curve) was measured for 5 minutes at L_O as spontaneous basal activity. The integral of force was measured by modifications of specialized software (AcqKnowledge, Biopac Systems, Inc.). Similar measurements were made for spontaneous activity after administering TTX and under NANC conditions. The effect of L-NMMA, methylene blue, NO, and NO in the presence of methylene blue on spontaneous activity was analyzed in the same way under NANC conditions. In the NO experiments, spontaneous activity was used as the control (100%). Because NO had an inhibitory effect in only about half the strips, the response to NO was only quantitated in the responsive strips, and the effects of methylene blue were only quantitated in those same strips.

Responses to bethanechol and norepinephrine were quantitated by measuring the integral of force for the first 5 minutes after drug administration. For the procontractile dose-response curve to bethanechol, the contractile response (above basal activity) to 3×10^{-6} mol/L bethanechol was used as the 100% value and the basal activity as 0%. In contrast, for the inhibitory effect of norepinephrine, the dose-response curve was expressed by using basal activity as 100%. The negative log of the equieffective concentration that caused a 50% response (ED_{50}) was calculated directly from the dose-response curve.

In the EFS experiments, we determined the response during the first 5 seconds of the 30-second stimulation and specifically did not analyze the response immediately after stopping stimulation (off-response). The integral of force generated in response to each frequency was expressed as the percentage of spontaneous contractile activity for a mean 5-second interval calculated over the 2 minutes immediately before beginning that series of EFS. The effect of L-NMMA and methylene blue on EFS-induced inhibition was studied in similar fashion. All contractile data were standardized by milligrams of tissue weight.

Analysis of variance followed by individual Student's *t* tests was used for comparison among multiple groups. Student's *t* tests with Bonferroni's correction for multiple comparisons were used to compare the effect of NO and the effect of various drugs on spontaneous activity during EFS. All data are presented as mean ± standard error of the mean (SEM).

Drugs

Bethanechol chloride, norepinephrine bitartarate salt, DL-propranolol hydrochloride, phentolamine hydrochloride, atropine sulfate, TTX, L-NMMA, and methylene blue were purchased from Sigma Chemical Company (St. Louis, Mo.). Nitric oxide gas was purchased from Matheson Gas Production, Inc. (Joliet, Ill.).

RESULTS

Spontaneous Contractile Activity and Effect of Neural Blockade

In all groups of rats, spontaneous contractile activity was regularly present. In NC rats, basal spontaneous activity had a total contractile force of 8.4 ± 1.4 g·5 min/mg tissue. These parameters did not differ significantly in the SC, SBT-1, or SBT-8 groups

(Table I). Neither inhibiting enteric neural input within the muscle strip with TTX nor blocking adrenergic and cholinergic input to the muscle by adrenergic/cholinergic blockade (NANC conditions) significantly altered the contractile force or the pattern of spontaneous contractile activity (see Table I).

Effect of Nitric Oxide

Exogenous administration of NO (10^{-5} to 3×10^{-5} mol/L) under NANC conditions had an immediate inhibitory effect on contractile activity in all groups, but only about half the muscle strips in all groups showed this inhibitory effect (Table II). NO not only blocked spontaneous contractile activity for approximately 30 seconds to 2 minutes but also decreased basal tone. The overall effects of NO (number of strips inhibited, duration of inhibition, percentage inhibition) were similar in all groups. Methylene blue blocked the inhibitory effect of NO similarly in all groups (see Table II).

Table I. Spontaneous contractile activity of ileal longitudinal muscle strips*

Group	Baseline conditions	NANC conditions	TTX
NC	8.4 ± 1.0	8.1 ± 1.1	8.5 ± 1.1
SC	7.5 ± 1.1	7.2 ± 1.1	7.4 ± 1.0
SBT-1	6.9 ± 0.7	6.7 ± 0.7	6.5 ±0.6
SBT-8	7.1 ± 0.6	6.9 ± 0.7	6.8 ± 0.7

*Mean ± SEM, g·5 min/mg; n = 8 rats per group.

Response to Cholinergic Agonist

Bethanechol caused a dose-dependent procontractile response in all groups. There were no shifts in the dose-response curves in the SC, SBT-l, and SBT-8 groups compared to NC rats (data not shown). The ED_{50} for bethanechol did not differ among groups (Table III). In the presence of TTX, the ED_{50} for bethanechol did not change in any group nor did the ED_{50} differ among groups (see Table III).

Response to Adrenergic Agonist

Norepinephrine dose dependently exhibited an inhibitory effect in all groups. Dose-response curves to norepinephrine in both the SBT-1 and SBT-8 groups were shifted to the left compared to the NC rats; in contrast, no shift was evident in the SC group (Fig. 1, *A*). The ED_{50} for norepinephrine in SBT-1 and SBT-

Table II. Effect of nitric oxide on spontaneous contractile activity*

	Control (No NO)		NO (10^{-5} mol/L)		NO (3×10^{-5} mol/L)	
Group	Alone	With methylene blue	Alone	With methylene blue	Alone	With methylene blue
NC	2 ± 4	5 ± 4	−30 ± 9†	0 ± 7‡	−80 ± 8†	−7 ± 6‡
SC	22 ± 8	15 ± 8	−31 ± 7†	5 ± 8‡	−64 ± 3†	−4 ± 7‡
SBT-1	1 ± 7	−4 ± 4	−47 ± 5†	−8 ± 6‡	−79 ± 4†	−24 ± 3‡
SBT-8	1 ± 5	6 ± 5	−46 ± 10†	−2 ± 7‡	−75 ± 7†	−7 ± 8‡

*Percentage of spontaneous activity, mean ± SEM; n = 8 rats per group.
†P <0.01 compared to control values.
‡P <0.01 compared to NO alone.

Table III. ED_{50} for norepinephrine and bethanechol in ileum

	Norepinephrine		Bethanechol	
Group	No TTX	TTX	No TTX	TTX
NC	6.2 ± 0.2	6.4 ± 0.1	6.1 ± 0.1	6.1 ± 0.2
SC	6.3 ± 0.2	6.2 ± 0.2	6.2 ± 0.3	6.1 ± 0.2
SBT-1	7.2 ± 0.3*	7.1 ± 0.3*	6.3 ± 0.2	6.2 ± 0.2
SBT-8	6.9 ± 0.1*	6.8 ± 0.2*	6.4 ± 0.3	6.43 ± 0.3

ED_{50} = negative log of drug concentration leading to a 50% maximal response (mean ± SEM; n = 8 rats per group).
*P <0.025 compared to NC and SC groups.

8 rats was significantly greater than in NC rats (see Table III), indicating that a lesser concentration of norepinephrine was necessary to inhibit basal contractile activity by 50% and that sensitivity to norepinephrine after SBT was markedly increased compared to control values. In contrast, the ED_{50} in SC rats did not differ from that in NC rats (see Table III). Administration of TTX did not affect the dose-response curves in rats after SBT or in the neurally intact NC rats (Fig. 1, *B* and Table III).

Effect of Electrical Field Stimulation

EFS had marked effects that differed among groups. In SC rats a procontractile increase in contractile activity was observed at 1 and 3 Hz, no net effect occurred at 5 Hz, but a notable inhibition was induced at 7 Hz and reached a maximum at 15 Hz; the degree of inhibition was decreased at 20 Hz (Fig. 2, *A*). In SBT-1 rats, in contrast to SC rats, inhibition was present at all frequencies tested, the maximum occurring at 10 Hz; the degree of inhibition was less at 15 and 20 Hz (Fig. 2, *B*). In SBT-8 rats the frequency-response curves were similar to those of the SC rats (Fig. 2, *C*).

The frequency-response curves in the SC, SBT-1, and SBT-8 groups with and without methylene blue and L-NMMA are shown in Fig. 2. Methylene blue had no noticeable change in the frequency-response curve to EFS, but L-NMMA had marked effects on the frequency-response curve. In the SC group, L-NMMA blocked the inhibitory response to frequencies higher than 3 Hz and led to a procontractile response at all higher frequencies. In the SBT-1 group, a similar but less dramatic response was noted; net inhibition by EFS was blocked at all frequencies. In the SBT-8 group, L-NMMA had effects very similar to those in SC rats with abrogation of the inhibitory effect of EFS at frequencies greater than 3 Hz with a net procontractile effect.

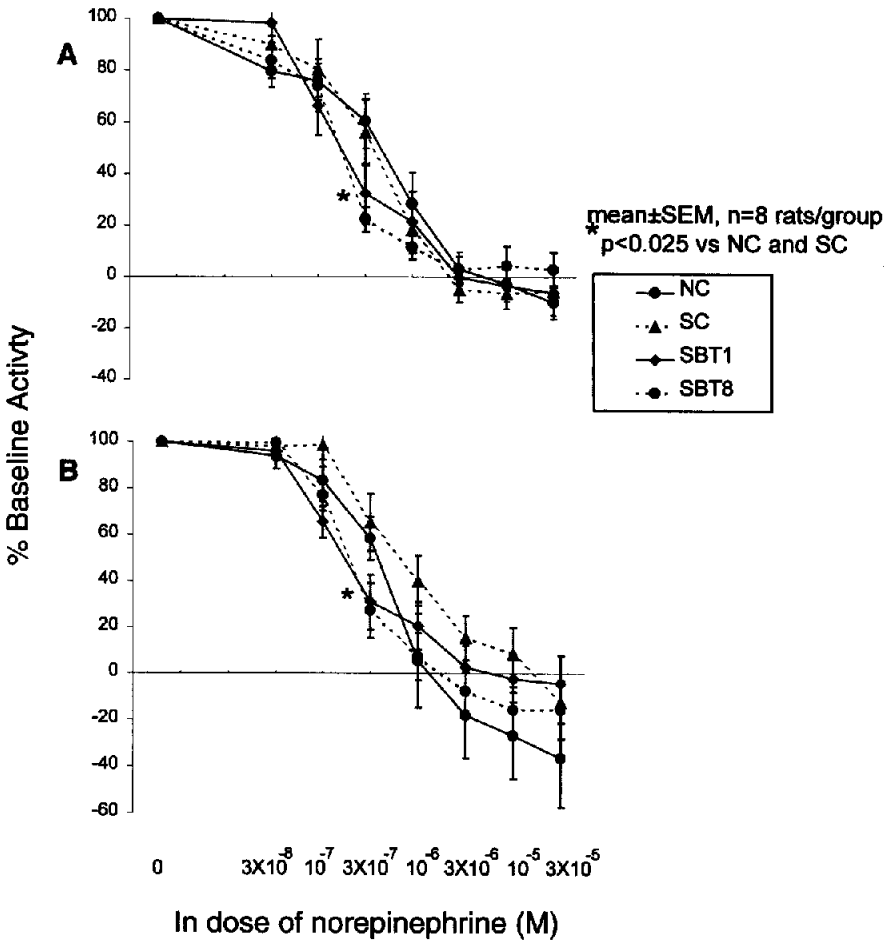


Fig. 1. Ileal response to norepinephrine. **A**, Norepinephrine. **B**, Norepinephrine plus tetrodotoxin.

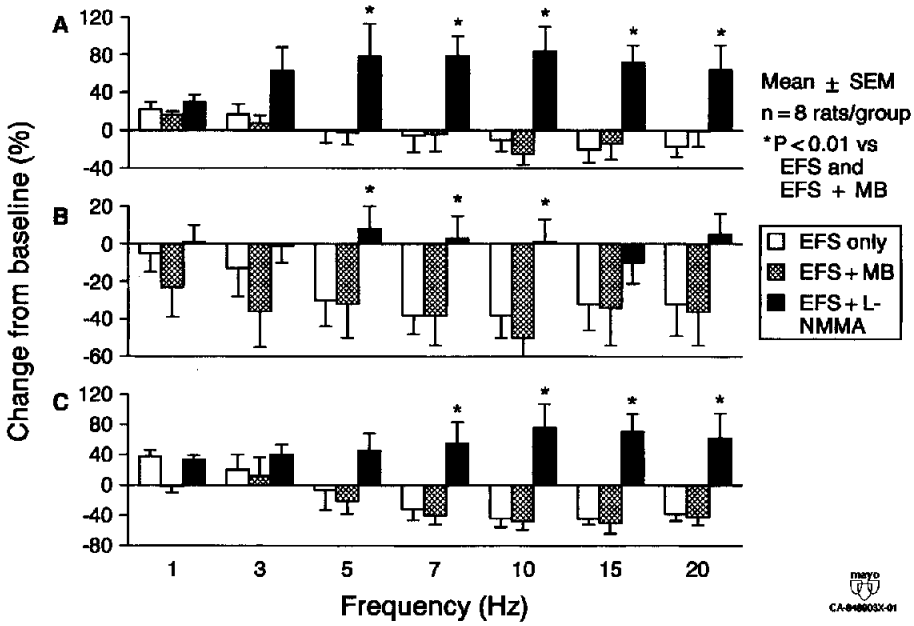


Fig. 2. Effect of electrical field stimulation *(EFS)*. MB = methylene blue; NMMA = N^G-monomethyl L-arginine. **A,** Sham controls. **B,** One week after small bowel transplantation. **C,** Eight weeks after small bowel transplantation.

DISCUSSION

This study in rat ileal longitudinal smooth muscle shows several important findings. First, SBT did not alter spontaneous activity or the response to selective (NANC conditions) or complete (TTX) inhibition of enteric neural input. Second, SBT induced an impressive adrenergic hypersensitivity but did not alter sensitivity to a cholinergic agonist. Third, exogenous NO had an inhibitory effect on rat ileal longitudinal muscle, and the NANC inhibitory effect of stimulating enteric nerves (by EFS) appears to be mediated by a methylene blue–resistant NO pathway. These alterations in smooth muscle function may have important effects on ileal motility in the transplanted gut and also on enteric function.

Previous work in our laboratory has investigated the effects of SBT, and more specifically the extrinsic denervation necessitated by SBT, on baseline contractile activity.[11,12,16-21] These studies, in conjunction with the current study, have shown marked differences in enteric neural control both of different muscle layers and of different anatomic regions of the small bowel. Spontaneous contractile activity is increased by SBT in jejunal circular muscle,[16,17] but not in ileal circular muscle,[19] jejunal longitudinal muscle,[21] or ileal longitudinal muscle in the current study. After adrenergic and cholinergic blockade (NANC conditions) or after pan-neural blockade of all enteric nerves (TTX) within the muscle strip, jejunal circular contractile activity increases markedly after SBT, ileal circular and longitudinal muscle does not change, and jejunal longitudinal muscle decreases.

These findings, in addition to differences in frequency and patterns of spontaneous contractions (circular muscle contractions occur in clusters or groups of contractions, whereas longitudinal muscle tends to contract continuously as individual contractions), lend support to the concept that both extrinsic innervation and tonic enteric neural input to the muscularis externa differ considerably not only between circular and longitudinal muscle layers but also between jejunum and ileum. Indeed, persistence of the pattern of clusters of contractions in circular muscle and individual contractions in longitudinal muscle after TTX blockade of enteric nerves suggests that the control of these contractile patterns may be myogenic, at least under the in vitro conditions of our experiments.

The physiologic importance of these findings is that circular and longitudinal muscle layers as well as jejunal and ileal anatomic regions appear to be under different neural and myogenic modulatory mechanisms, each of which may respond differently to the extrinsic denervation necessitated by SBT. Although we did not specifically investigate the effects separately of intestinal transection or the ischemia/reperfusion injury potentially necessitated by the procedure of SBT, results of our previous studies that did examine these parameters suggested that effects on spon-

taneous contractile activity before and after enteric neural blockades were mediated specifically by the extrinsic denervation accompanying SBT.

Of equally important physiologic interest, SBT induced an impressive denervation hypersensitivity to adrenergic inhibition but not to the cholinergic procontractile response in ileal longitudinal muscle. This effect appears to be mediated not via the extrinsic denervation at the myenteric plexus but rather via an effect directly on the longitudinal smooth muscle itself because TTX, which blocks all neurally mediated events in the muscle strip, did not alter this adrenergic hypersensitivity. Our previous work in rat ileal circular muscle,[19] but not jejunal circular muscle,[17] showed very similar findings. This observation is especially interesting because all extrinsic neural input from the central nervous system to gut smooth muscle is believed to occur via the intrinsic neural plexus and not by direct synapses with the muscle itself.[22,23] This finding suggests that the adrenergic hypersensitivity induced by SBT occurs primarily through effects induced at least one synapse away from the smooth muscle cell itself. Lack of the ability of TTX to alter or prevent this hypersensitivity suggests, albeit indirectly, that SBT leads to either an increase in adrenergic receptor density on the longitudinal smooth muscle cells or a change in the myogenic response[24] to norepinephrine. This latter effect might occur via an increased affinity of existing adrenergic receptors, a decrease in the enzymes that deaminate and thereby inactivate norepinephrine, or a modification of the intracellular receptor-signaling pathway; the current experimental design cannot further differentiate these possibilities. No similar effect was seen in response to a cholinergic agonist; this differs from a previous report.[25]

Finally, this study further supports the differences in control mechanisms between the circular and longitudinal muscle layers of the small bowel by the observations on the importance of NO and nitrergic mechanisms modulating NANC mechanisms of contractile inhibition. Our study showed a prominent effect of exogenous NO on inhibiting contractile activity and decreasing basal tone. Although only about half the muscle strips responded to NO, this effect was seen in all groups; we cannot explain why all muscle strips did not respond similarly. Support for a specific NO-mediated event in rat ileal longitudinal muscle comes from the inhibition of the NO effect by methylene blue, which blocks soluble guanylate cyclase,[12] the presumed intracellular pathway of a direct effect of NO on smooth muscle. This responsiveness of rat ileal longitudinal muscle is similar to our preliminary work[12] and that of Kanada et al.[26] in rat jejunal longitudinal muscle[12] but differs markedly from our work in jejunal and ileal circular muscle[11,27] in which exogenous NO had no effect on contractile activity or basal tone.

Our experiments using EFS were designed to explore the effects of SBT on NANC inhibitory mechanisms and specifically nitrergic neurons. SBT caused a marked change in EFS-induced NANC inhibition of contractile activity. Although the neurally intact control groups (SC and NC) showed a biphasic response with a procontractile response to low-frequency stimulation (1 and 3 Hz) and an inhibitory response to higher frequencies (≥7 Hz), the SBT-1 and SBT-8 groups had a prominent net inhibitory response and associated decrease in basal tone at all frequencies. These types of responses to EFS also differ from the jejunal layers[12,18] and ileal circular muscle,[11] again demonstrating regional and anatomic differences in control mechanisms. Inhibition of NO production by the addition of L-NMMA, which inhibits the enzyme that produces endogenous NO (NO synthase) blocked completely the EFS-induced inhibition, strongly suggesting that NO mediates at least the great part of the inhibitory effect induced by exciting intramural neural mechanisms by EFS. However, the inability of methylene blue to block this inhibitory effect, in contrast to the ability of methylene blue to block the inhibition induced by exogenous NO, is a very intriguing and unexpected finding. This observation suggests that the EFS-induced inhibition mediated by NO works through a methylene blue–independent pathway and thus probably not via an intracellular mechanism within the smooth muscle cell mediated by soluble guanylate cyclase. Possibilities include nitrergic effects on other intramural nerves or direct effects of NO on membrane channels[5,28] or other signaling pathways; our experimental design cannot further differentiate these or other possibilities.

In summary, SBT induces an adrenergic denervation hypersensitivity and alteration in neurally mediated NANC inhibitory mechanisms in the rat longitudinal muscle layer. These effects differ both quantitatively and qualitatively from the effects of SBT in other muscle layers and in different regions of the small bowel. These differing effects of SBT on contractility may cause marked changes in enteric function of the transplanted small bowel and may contribute in part to the enteric dysfunction that occurs after human SBT.[1]

We thank Deborah Frank for her expert assistance in the preparation of this manuscript.

REFERENCES

1. Starzl TE, Rowe MI, Todo S, Jaffe R, Tzakis A, Hoffman AL, Esquivel C, Porter KA, Venkataramanan R, Makowka L, Duquesnoy R. Transplantation of multiple abdominal viscera. JAMA 1989;261:1449-1457.
2. Starzl TE, Todo S, Tzakis A, Alessiani M, Casavilla A, Abu-Elmagd K, Fung JJ. The many faces of multivisceral transplantation. Surgery 1991;172:335-344.
3. Burnstock G, Campbell G, Rand MJ. The inhibitory innervation of the taenia of the guinea-pig caecum. J Physiol Lond 1966;182:504-526.
4. Burnstock G, Campbell G, Bennett M, Holman ME. Inhibition of the smooth muscle of the Taenia coli. Nature (London) 1963;200:581-582.
5. Sanders KM, Ward SM. Nitric oxide as a mediator of nonadrenergic noncholinergic neurotransmission. Am J Physiol 1992;262:G379-G392.
6. Toda N, Baba H, Okamura T. Role of nitric oxide in nonadrenergic, noncholinergic nerve-mediated relaxation in dog duodenal longitudinal muscle strips. Jpn J Pharmacol 1990; 53:281-284.
7. Boeckxstaens GE, Pelckmans PA, Bult H, DeMan JG, Herman AG, Van Maercke YM. Nonadrenergic noncholinergic relaxation mediated by nitric oxide in the ileocolonic junction. Eur J Pharmacol 1990;190:239-246.
8. Konturek SJ, Bilski J, Konturek PK, Cieszkowski M, Pawlik W. Role of endogenous nitric oxide in the control of canine pancreatic secretion and blood flow. Gastroenterology 1993; 104:896-902.
9. Yunker AM, Galligan JJ. Extrinsic denervation increases NADPH diaphorase staining in myenteric nerves of guinea pig ileum. Neurosci Lett 1994;167:51-54.
10. Stadelman AM, Fink JG, Mustin E, Walgenbach-Telford S, Telford GL, Koch TR. Non-adrenergic non-cholinergic (NANC) inhibitory nerves are altered in rat small intestine following transplantation. Gastroenterology 1994;106:A843.
11. Shibata C, Balsiger BM, Anding WJ, Duenes JA, Miller VM, Sarr MG. Functional changes in nonadrenergic noncholinergic inhibitory neurons in ileal circular smooth muscle after small bowel transplantation in rats. Dig Dis Sci 1998;43: 2446-2454.
12. Balsiger BM, Ohtani N, Shibata C, Sarr MG. Non-adrenergic non-cholinergic (NANC) inhibition in longitudinal smooth muscle of rat jejunum: Minor role of nitric oxide (NO). Gastroenterology 1998;114:A716.
13. Kruis W, Azpiroz F, Phillips SF. Contractile patterns and transit of fluid in canine terminal ileum. Am J Physiol 1995;249: G264-G270.
14. Fich W, Phillips SF, Hanson RB, Zinmeister AR. Rapid frequency rhythmic postprandial motility in the canine ileum. Am J Physiol 1989;257:G766-G772.
15. Monchik GJ, Russell PS. Transplantation of the small bowel in rat: Technical and immunological considerations. Surgery 1971;70:693-702.
16. Murr MM, Miller VM, Sarr MG. Contractile properties of enteric smooth muscle after small bowel transplantation in rats. Am J Surg 1996;171:212-218.
17. Murr MM, Miller VM, Sarr MG. Functional changes in enteric smooth muscle contractile activity after small bowel transplantation. Surg Forum 1995;46:196-198.
18. Murr MM, Sarr MG. Small bowel transplantation: Effects on function of nonadrenergic, noncholinergic nerves. J GASTROINTEST SURG 1997;1:439-445.
19. Shibata C, Balsiger BM, Anding WJ, Sarr MG. Adrenergic denervation-hypersensitivity in ileal smooth muscle after small bowel transplantation in rats. Dig Dis Sci 1997;42: 2213-2221.
20. Shibata C, Murr MM, Balsiger BM, Anding WJ, Sarr MG. Contractile activity of smooth muscle in rats 1 year after small bowel transplantation: Differing adaptive response of the jejunum and ileum to denervation. J GASTROINTEST SURG 1998;2:463-472.
21. Balsiger BM, Ohtani N, Shibata C, Murr MM, Anding WJ, Duenes JA, Sarr MG. Small bowel transplantation (SBT) alters non-adrenergic non-cholinergic (NANC) inhibition in longitudinal smooth muscle of the rat jejunum. Gastroenterology 1998;114:A1377.
22. Goyal RK, Hirano I. The enteric nervous system. N Engl J Med 1996;334:1106-1115.
23. Yau WM. Neurotransmitter release in the enteric nervous system. In Schultz SG, Wood JD, Rauner BB, eds. Handbook of Physiology, Section 6: The Gastrointestinal System, Vol. 1: Motility and Circulation. Bethesda: American Physiological Society, 1989, pp 403-433.
24. Fleming WW. Variable sensitivity of excitable cells: Possible mechanisms and biologic significance. Rev Neurosci 1976;2: 43-90.
25. Osinski MA, Bass P. Chronic denervation of rat jejunum results in cholinergic supersensitivity due to reduction of cholinesterase activity. J Pharmacol Exp Ther 1993;266:1684-1690.
26. Kanada A, Hata F, Suthamnatpong N, Maehara T, Ishii T, Takeuchi T, Yagasaki O. Key roles of nitric oxide and cyclic GMP in nonadrenergic and noncholinergic inhibition in rat ileum. Eur J Pharmacol 1992;216:287-292.
27. Balsiger BM, Duenes JA, Ohtani N, Shibata C, Anding WJ, Sarr MG. Neural inhibition in jejunal circular muscle after small bowel transplantation (SBT): Nitric oxide (NO) has no apparent role. Gastroenterology 1999;116:A1349.
28. Goyal RK, He XD. Evidence for NO redox form of nitric oxide as nitrergic inhibitory neurotransmitter in gut. Am J Physiol 1998;275:G1185-G1192.

Discussion

Dr. L. Traverso (Seattle, Wash.). The reproducibility of this model depends on adjusting isometric tension in your preparation; that might be difficult or it might be difficult to reproduce. When you display standard errors of the mean rather than standard deviations, it implies that the standard deviations would overlap a great deal. I am wondering why you used standard error of the mean.

Dr. M. Sarr. There are two ways to arrive at baseline conditions: one is to take a strip of muscle, hang it in a tissue chamber, and then attach a specific weight to it, such as 1 g. If you have a thicker or longer specimen, however, conditions are not the same. So we stretched the muscle as if we were trying to reproduce a Starling curve and determined its maximal tension. We studied the muscle at its optimal

length (L_0) at increasing stresses and used that as baseline. We presented the data with standard errors rather than standard deviations because it was easier to show the differences in terms of a graph. We had an "n" of eight in all groups, so the standard deviations were going to be approximately 2½ times greater than the standard error of the mean. This is statistically significant, even after a Bonferroni correction.

Dr. K. Kelly (Scottsdale, Ariz.). Did this adrenergic hypersensitivity result in any changes in bowel habits in the animals when you were observing them during the week before you conducted your experiments? Adrenergic nerves have a way of regrowing into the small bowel. Did you follow any of the animals for a time afterward, perhaps a month or so, to see whether the adrenergic hypersensitivity disappeared?

Dr. Sarr. The rats did lose some weight postoperatively, but by approximately 2 weeks they were back to their preoperative weight. Again, this was an isogeneic model so there were no immune effects and the rats did very well thereafter. The did not have prolonged diarrhea, although it is difficult to assess diarrhea in rats. They eat normally and they grow normally.

Does the hypersensitivity response to adrenergic agents persist? We have performed experiments on ileal circular muscle at 1 year and have found that it does persist. We have a group of rats we have studied, but we have not yet analyzed the data.

Various investigators have studied regrowth of extrinsic nerves to the gut at 3, 6, and 12 months postoperatively. At 6 months there is virtually none. At 12 months there is some re-enervation of the mesentery but not of the bowel wall itself. In humans, I would expect that there would be no re-enervation of the gut wall by extrinsic nerves since nerves regrow at a rate of approximately 2 cm a month but only for about 12 months, and in this case the extrinsic nerves would have to grow approximately 1 or 1½ feet.

Dr. G. Larson (Louisville, Ky.). I have a question concerning your rationale for the TTX portion of your study. Did you anticipate that there would be a difference with or without TTX? Since you used a denervated specimen, I would have thought that the nerve block by TTX would not be of any consequence.

Dr. Sarr. When the small bowl is denervated, all adrenergic nerves that go into the enteric nervous system are cut. There is nerve death back to the cell soma, so if the enteric neural system is studied for adrenergic input, none is found. There are adrenergic receptors also on smooth muscle, and our studies on the ileal circular muscle showed this hypersensitivity. We evaluated the effect of TTX and found that the effect was not blocked, which was a surprise. So when we started this experiment, we suspected that.

Nitric Oxide Pathways in Circular Muscle of the Rat Jejunum Before and After Small Bowel Transplantation

Bruno M. Balsiger, M.D., Judith A. Duenes, B.A., Noriya Ohtani, M.D., Chikashi Shibata, M.D., Gianrico Farrugia, M.D., William J. Anding, B.A., Michael G. Sarr, M.D.

Previous studies suggest that nitric oxide synthase is upregulated after small bowel transplantation which may have implications in enteric dysfunction after small bowel transplantation. The aim of this study was to determine the role of nitric oxide in nonadrenergic, noncholinergic inhibitory function after small bowel transplantation in rat jejunal circular muscle. The following four groups of rats (n = ≥8 rats per group) were studied: Neurally intact control animals; 1 week after anesthesia and sham celiotomy, and either 1 week or 8 weeks after isogeneic, orthotopic small bowel transplantation. Full-thickness jejunal circular muscle strips were evaluated under isometric conditions for spontaneous contractile activity, response to electrical field stimulation, and effects of exogenous nitric oxide and nitric oxide antagonists. Spontaneous activity did not differ among groups. Electrical field stimulation inhibited activity similarly in all groups. Exogenous nitric oxide, N^G-monomethyl L-arginine monoacetate salt (a nitric oxide synthase inhibitor), and methylene blue (cGMP antagonist) had no effect on spontaneous activity. Neither nitric oxide antagonist altered the inhibitory response to neural excitation by electrical field stimulation in any group. Nitric oxide, a known inhibitory neurotransmitter in other gut smooth muscle, has no apparent role in rat jejunal circular muscle before or after small bowel transplantation. (J GASTROINTEST SURG 2000;4:86-92.)

KEY WORDS: Motility, nitric oxide, smooth muscle, small bowel transplantation, inhibitory neurotransmitters

Although small bowel transplantation (SBT) is rapidly becoming a viable clinical option for gut failure,[1] little is known about changes in small bowel function after SBT. Understanding changes in enteric function is important because SBT is associated with multiple problems in the early and late postoperative periods including abnormalities in motility, diarrhea, and difficulty with enteral delivery of nutrition.[2] Indeed, changes in enteric function are not unexpected, because SBT obligates an extrinsic denervation of the graft, disruption of intrinsic (enteric) neural continuity of the graft with the still-innervated proximal and distal gut, an ischemia/reperfusion injury, and host-mediated immune responses to the graft.

Previous work from our laboratory has investigated the early and late effects of SBT on contractile activity of circular smooth muscle of the rat *ileum*. Although we found an impressive adrenergic hypersensitivity after SBT, spontaneous contractile activity remained unchanged.[3,4] When examined by exogenous application of nitric oxide (NO) and NO antagonists and during intrinsic neural excitation by electrical field stimulation (EFS), inhibitory neurotransmission to ileal circular muscle appeared to be independent of NO.[5] In contrast, in *jejunal* circular muscle, SBT induced an increase in spontaneous contractile activity and augmented EFS-induced nonadrenergic, noncholinergic (NANC) inhibition. Because no changes

From the Department of Surgery and the Gastroenterology Research Unit, Mayo Clinic and Foundation, Rochester, Minn.
Supported by grant DK 39337 from the National Institutes of Health, United States Public Health Service, the Mayo Foundation, the Swiss National Foundation, and the Department of Visceral and Transplantation Surgery, University of Bern, Switzerland.
Part of this study was presented at a poster session at the Fortieth Annual Meeting of The Society for Surgery of the Alimentary Tract, Orlando, Fla., May 16-19, 1999. An abstract of this work was published in *Gastroenterology* 116:A1349, 1999.
Correspondence: Michael G. Sarr, M.D., Professor of Surgery, Gastroenterology Research Unit, Mayo Clinic, 200 First Street SW, Rochester, MN 55905.

occurred in sensitivity to cholinergic or adrenergic agonists,[6] we wondered whether transplantation-related alterations in nitrergic nerves might explain these changes in contractile properties. Indeed, others have suggested that the number of nitrergic nerves is increased after extrinsic denervation and SBT,[7,8] and that NO synthase activity is upregulated by extrinsic denervation.[9] Such changes in these presumably inhibitory nitrergic nerves may have marked effects on gut motor function.

Therefore the aims of the present study were to determine the effect of NO in rat jejunal circular muscle and to identify the role of NO in EFS-induced changes in NANC inhibition before and after SBT. We hypothesized that exogenous NO would inhibit contractile activity and that changes in spontaneous contractile activity and EFS-induced NANC inhibition after SBT would be related to changes in a nitrergic mechanism with NO acting as the neurotransmitter.

MATERIAL AND METHODS

Preparation of Animals

Procedures and animal care were performed according to the guidelines of the Animal Care and Use Committee of the Mayo Foundation in accordance with the guidelines of the National Institutes of Health and the Public Health Service Policy on the Human Use and Care of Laboratory Animals.

Experimental Groups. Because we were interested in the physiologic effects of the SBT procedure and not rejection, immune suppression, or other immune phenomena, we specifically used syngeneic male Lewis rats (Harlan Sprague-Dawley, Indianapolis, Ind.) in all experiments to avoid confounding immune-related phenomena that occur after *allo*transplantation. After anesthesia was achieved by intraperitoneal sodium pentobarbital (Ampro Pharmacy, Arcadia, Calif.), orthotopic SBT was performed using standard microvascular techniques as described previously.[6] In brief, the entire jejunoileum was removed from the donor rat after flushing the intestinal lumen and infusing the graft vasculature with chilled Ringer's lactate solution. The graft was revascularized by anastomosing donor aorta to recipient aorta (end-to-side) and donor portal vein to recipient inferior vena cava (end-to-side). After resecting the recipient jejunoileum, intestinal continuity was reestablished by end-to-end jejunojejunostomy and ileoileostomy.

All rats were allowed free access to water and rat chow immediately postoperatively. Rats were studied 1 week and 8 weeks after SBT (SBT-1, n = 12; SBT-8, n = 14). Naive rats without any surgical procedure served as neurally intact controls (NC, n = 4). Also, to determine the nonspecific effects of anesthesia and celiotomy, rats 1 week after celiotomy and intestinal manipulation were used as sham-operated controls (SC, n = 8).

Recording of Contractile Activity. A segment of proximal jejunum 7 to 10 cm distal to either the ligament of Treitz or the jejunojejunostomy after SBT was removed, immersed in chilled modified Krebs-Ringer's bicarbonate solution (concentrations in mmol/L: NaCl 116.4, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 23.8, calcium disodium edetate 0.26, and glucose 11.1) and opened along the mesenteric border. Four to eight full-thickness muscle strips cut in the direction of the circular muscle layer were suspended vertically in 10 ml tissue chambers filled with modified Krebs-Ringer's bicarbonate solution; the chambers were maintained at 37.5° C and bubbled with 95% oxygen and 5% carbon dioxide (Puritan-Bennett Corp., Lenexa, Kan.). Because of the well-recognized variability between muscle strips in the same rat, identical experiments were performed on multiple muscle strips per rat. One end of the muscle strip was attached to a fixed hook, whereas the other end was connected to a noncompliant force transducer (Kulite Semiconductors Products, Inc., Leonia, N.J.) to measure isometric force.

Experimental Protocol

After an 80- to 90-minute equilibration period with intervening washout of the bath solution every 20 to 25 minutes, each strip was incrementally stretched at 12- to 15-minute intervals to its optimal length (Lo) beyond which further stretching did not increase the amplitude of spontaneous activity.[6] All subsequent experiments were performed at Lo; strips without spontaneous contractile activity or without any response to EFS were not used (less than 9% of strips).

After measuring spontaneous basal contractile activity for a 5-minute interval at Lo, atropine (10^{-7} mol/L), phentolamine (10^{-5} mol/L), and propranolol (5×10^{-6} mol/L) were added to the bath in eight chambers to induce NANC conditions, and spontaneous contractile activity under NANC conditions was measured for a 5-minute interval beginning 30 minutes later. NO in distilled water (3×10^{-6} to 3×10^{-5} mol/L) was applied directly into the chamber under NANC conditions to determine the effect of NO on spontaneous activity. NO was prepared by dissolving NO gas in distilled water previously degassed with helium according to the method reported previously.[3] Then, EFS was applied at increasing frequencies (1, 2, 3, 4, 5, 7, and 10 Hz) with a constant voltage (20 volts), pulse width (4 msec), and duration of

Table I. Spontaneous contractile activity of rat jejunal circular smooth muscle*

Group	Basal	NANC	L-NMMA	Methylene blue
SC	5.05 ± 0.55	5.79 ± 0.80	5.44 ± 1.18	6.17 ± 1.28
SBT-1	4.47 ± 0.54	4.89 ± 0.68	4.28 ± 0.65	4.19 ± 0.87
SBT-8	4.03 ± 0.40	4.50 ± 0.54	3.86 ± 0.52	4.89 ± 0.77

*Mean ± SEM, g · 5 min/wet tissue; n = ≥ 8 rats per group.

stimulation (10 seconds) to obtain a frequency-response curve. The different frequency stimulations were separated by 5 minutes to allow spontaneous activity to recover before the next stimulation. The contractile response to EFS was quantitated only during the 10-second stimulation. N^G-monomethyl-L-arginine (L-NMMA, 10^{-3} mol/L), a specific inhibitor of NO synthase, and methylene blue (10^{-5} mol/L), a nonspecific inhibitor of soluble guanylate cyclase, were administered into each of four chambers. Contractile activity was then determined for a 5-minute interval beginning 30 minutes after drug administration. EFS was repeated under the same conditions in chambers with L-NMMA and methylene blue to determine the effect of these drugs on the response to EFS. In separate experiments in control rats (data not shown), EFS was investigated in the presence of 10^{-6} mol/L tetrodotoxin which, by blocking sodium channels, inhibits almost all neural transmission; we confirmed that EFS-induced inhibition and contractile activity were tetrodotoxin sensitive and thus neurally mediated. At the conclusion of the experiments, each tissue was blotted and weighed.

Data Analysis

We quantitated total contractile activity by measuring the integral of the force generated (g · 5 min − area under the contractile curve) under each condition using specialized software (AcqKnowledge, Biopac Systems, Inc., Goleta, Calif.). Spontaneous basal activity at L_0 (4 to 8 muscle strips per rat), and the effects of NANC conditions (4 muscle strips per rat), L-NMMA (2 muscle strips per rat), and methylene blue (2 muscle strips per rat) on spontaneous activity were quantitated for 5-minute intervals. In EFS experiments (4 muscle strips per rat), we determined the response during the 10-second stimulation ("on-response") and specifically excluded the response immediately after stopping stimulation (the so-called "off-response"). The integral of force generated during the 10-second EFS at each frequency was expressed as the percentage of spontaneous contractile activity for a mean interval of 10 seconds as calculated from the 5 minutes of contractile activity measured immediately before beginning EFS. Frequency-response curves were generated. The effect of L-NMMA and methylene blue on EFS-induced inhibition was compared to the inhibition during EFS before administration of these agents in the same strip. All contractile data were standardized by milligrams of tissue wet weight.

Analysis of variance combined with Student's *t* tests were used for comparisons among multiple groups (comparison of basal spontaneous activity across groups), and separate *t* tests for paired data were used to study the effect of various drugs on spontaneous activity and during EFS. A Bonferroni correction was made for multiple comparisons whenever appropriate. All data are presented as mean ± standard error of the mean (SEM), and the n used for statistical comparisons was the number of rats (not muscle strips) per group. Since there were no differences between the NC and SC groups, we chose SC rats as controls for the SBT rats.

Drugs

DL-Propranolol hydrochloride, phentolamine hydrochloride, atropine sulfate, L-NMMA, and methylene blue were purchased from Sigma Chemical Company (St. Louis, Mo.). Nitric oxide gas was purchased from Matheson Gas Production, Inc. (Parsippany, N.J.).

RESULTS

Spontaneous Contractile Activity

No differences were noted in spontaneous basal contractile activity between groups. The effects of NANC conditions, L-NMMA, and methylene blue on spontaneous activity are shown in Table I. Induction of NANC conditions did not alter contractile activity significantly in any group (see Table I). Similarly, when evaluated under NANC conditions, L-NMMA and methylene blue had little or no effect on spontaneous contractile activity (see Table I). Exogenous administration of NO (3×10^{-6} to 3×10^{-5} mol/L) under NANC conditions also had no inhibitory effect on spontaneous phasic activity, basal tension, or total contractile activity in any group (Fig. 1).

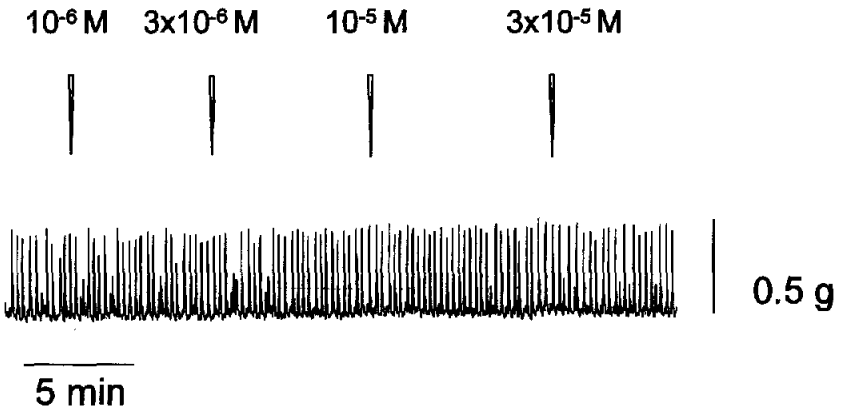


Fig. 1. Lack of effect of exogenous NO on spontaneous contractile activity in vitro under NANC conditions in rat jejunal circular muscle in the SC group. Similar lack of effect as seen in the NC and SBT groups as well.

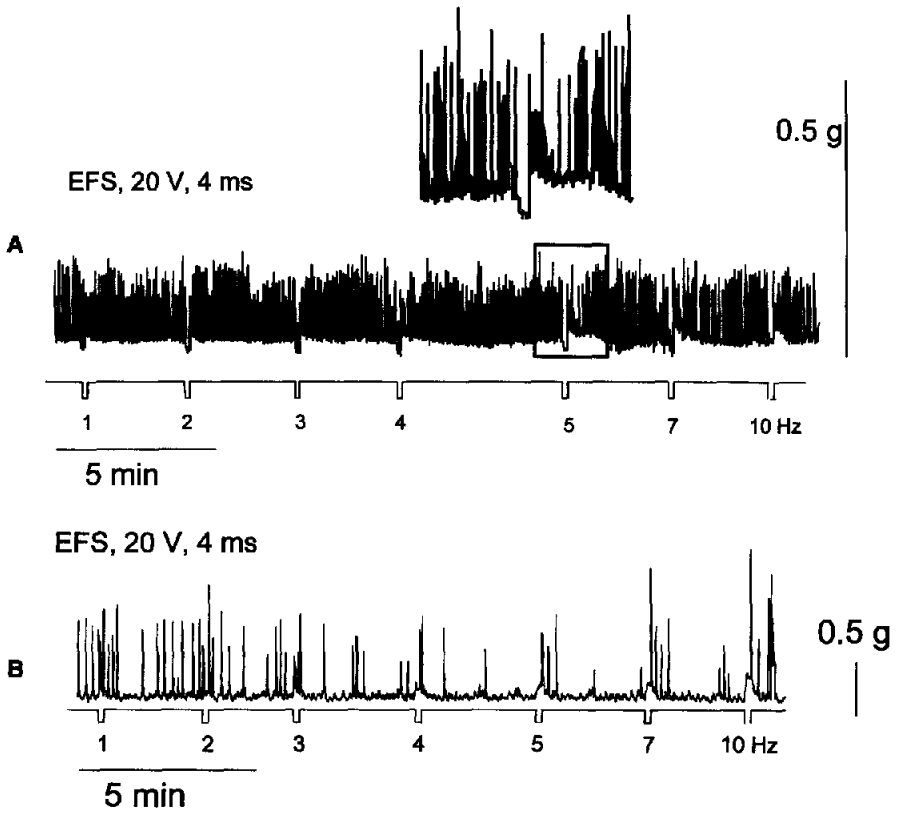


Fig. 2. Effect of electrical field stimulation on spontaneous contractile activity of rat jejunal circular muscle in vitro under NANC conditions in the SBT-1 group. **A,** Characteristic inhibitory effect at all frequencies. *Inset* represents an expansion of the segment within the box. **B,** Net contractile response. This less common pattern was seen in 9%, 10%, 47%, and 18% of strips in the NC, SC, SBT-1, and SBT-8 groups, respectively.

Effect of Electrical Field Stimulation

Fig. 2 shows two different overall patterns of responses to EFS—a primarily inhibitory one (Fig. 2, *A*) and a procontractile one (Fig. 2, *B*). The most frequently observed pattern (198 of 227 strips; Fig. 2, *A*) consisted of inhibition of phasic contractions and/or reduction in basal tension (and therefore decreasing total force) during low-frequency (1 to 7 Hz) EFS; this inhibitory effect was not apparent at 10 Hz. This pattern was present in 91%, 90%, 53%, and 82% of the strips in the NC, SC, SBT-1, and SBT-8 groups, respectively. The second pattern (Fig. 2, *B*) consisted

Table II. Effect of nitric oxide inhibitors on electrical field stimulation–induced inhibition*

Group	Condition	Frequency of electrical field stimulation (Hz)						
		1	2	3	4	5	7	10
SC	NANC†	−79 ± 12	−106 ± 11	−110 ± 10	−100 ± 8	−97 ± 9	−71 ± 12	8 ± 17
(n = 8)	L-NMMA‡	−61 ± 16	−104 ± 16	−126 ± 17	−131 ± 18	−131 ± 16	−101 ± 20	−13 ± 25
	Methylene blue‡	−129 ± 19	−140 ± 14	−139 ± 17	−134 ± 14	−138 ± 16§	−110 ± 13	−63 ± 14
SBT-1	NANC†	−50 ± 18	−98 ± 13	−89 ± 17	−91 ± 17	−72 ± 17	−31 ± 11	48 ± 18
(n = 12)	L-NMMA‡	−33 ± 17	−71 ± 11	−78 ± 12	−85 ± 12	−74 ± 9	−36 ± 110	58 ± 29
	Methylene blue‡	−85 ± 23	−125 ± 17	−125 ± 14	−129 ± 15	−115 ± 16	−73 ± 11	9 ± 20
SBT-8	NANC†	−92 ± 13	−114 ± 11	−118 ± 13	−122 ± 13	−105 ± 13	−71 ± 14	1 ± 20
(n = 14)	L-NMMA‡	−58 ± 10	−100 ± 11	−113 ± 14	−120 ± 15	−113 ± 18	−89 ± 17	−13 ± 22
	Methylene blue‡	−102 ± 11	−126 ± 10	−131 ± 10	−136 ± 12	−127 ± 12	−101 ± 15	−39 ± 25

*Mean ± SEM percent inhibition of hascline spontaneous activity (100%); n = ≥ 8 rats per group.
†Analysis of variance between groups, not significant.
‡Analysis of variance across frequencies between NANC and methylene blue or NANC and L-NMMA experiments in each group; not significant.
§Paired *t* test to NANC at same frequency (Hz); $P < 0.007$.

of EFS evoking no inhibition but rather stimulating contractile activity at all frequencies tested; this pattern occurred mostly in the SBT-1 group (47% of the strips); in 4 of the 12 rats *all* strips showed this response. Because we were interested in the inhibitory function, we focused on the former inhibitory responses. The frequency-response curves in all groups are shown in Fig. 3. In SBT-1 rats, the overall frequency-response pattern was similar to that of the SC group, but the inhibitory response at 7 Hz was smaller and the contraction at 10 Hz greater than in the SC and SBT-8 rats (see Table II). The effects of the NO antagonists L-NMMA and methylene blue on the EFS response curves in each group are shown in Table II; neither L-NMMA nor methylene blue altered the frequency-response curve to EFS in any group.

DISCUSSION

Nitric oxide has been shown to be an important inhibitory neurotransmitter in gut smooth muscle in various species.[10] Our study was designed based on the hypothesis that NO has a modulatory role before and after isogeneic SBT in circular muscle of the rat jejunum. Neither exogenous application of NO, blocking NO synthase with L-NMMA, nor inhibiting soluble guanylate cyclase with methylene blue, the presumed intracellular pathway by which NO inhibits contractile activity, had any noticeable effect on spontaneous contractile activity in rat jejunal circular muscle; these observations imply that NO does not modulate spontaneous continuous phasic activity or basal tone in this tissue. Similarly, the NO antagonists L-NMMA and methylene blue did not abrogate the EFS-induced inhibition in any group. These results suggest that under our experimental conditions, nitrergic pathways do not appear to play an important or major role in mediating long-term NANC inhibition either in normally innervated rat circular jejunal muscle or a major role in extrinsically denervated jejunum after SBT. Inasmuch as NO synthase–positive nerve fibers are abundant in the rat circular muscle layer,[8] it is possible that the role of neuronally released NO is to inhibit muscle activity under stimulated conditions or to modulate release of other neurotransmitters. NO modulation of acetylcholine release has been reported in canine colonic circular smooth muscle.[11] The data in our study were obtained under NANC conditions, which would have masked any effect of NO on acetylcholine release.

The lack of any obvious role of NO as an important inhibitory neurotransmitter in rat jejunal circular muscle is in agreement with our observations in rat ileal circular muscle where NO also did not appear to mediate

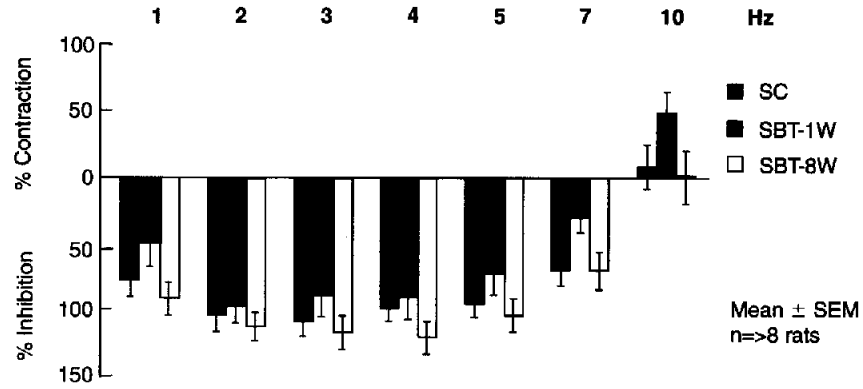


Fig. 3. Frequency-response patterns to electrical field stimulation of rat jejunal circular muscle in vitro under NANC conditions. Note only those muscle strips showing an inhibitory response are graphed; this includes 90%, 53%, and 82% of all muscle strips in the SC, SBT-1, and SBT-8 groups, respectively. No differences were noted between groups.

an important NANC rat inhibition.[5] Similar findings occurred in our preliminary work in jejunal longitudinal muscle, where the tetrodotoxin-sensitive (neurally mediated) inhibition of contractile activity induced by EFS was blocked by L-NMMA only at 1 Hz, and methylene blue had no effect on the EFS-induced inhibition.[11] Based on these observations, we conclude that NO does not appear to mediate an important NANC inhibitory function in rat jejunal and ileal circular muscle, and that other NO-independent neurotransmitters play a more prominent role in inhibitory modulation of contractile activity in this tissue.

In contrast, exogenous NO does markedly inhibit contractions in rat jejunal longitudinal muscle, and inhibition of NO synthase abrogates the EFS-induced inhibition at 1 Hz.[12] These contrasting effects suggest a differing importance and distribution of nitrergic pathways in circular and longitudinal muscle layers in the rat jejunum. Increased EFS-induced inhibition after SBT in the longitudinal but not in the circular muscle may further support this concept.[13] Several groups have suggested that extrinsic denervation and SBT induce an increase in nitrergic nerves in the myenteric plexus and a concomitant increase in their inhibitory influence in NANC inhibition. Morphologic studies have suggested that after either extrinsic denervation or SBT the number of nitrergic neurons in small bowel is increased in the rat and the guinea pig.[7,8] The increased inhibitory function in rats after extrinsic denervation appeared related to upregulation of NO synthase in the enteric nervous system.[9] Our results suggest that such neural changes after SBT have more important effects in the longitudinal versus the circular muscle in the rat jejunum.

The apparent lack of a prominent role of NO as an inhibitor in the circular jejunal muscle was surprising and unexpected because of the prominent nitrergic innervation of rat circular muscle of the gut. Effects of other NO-like products cannot be excluded. Recently different redox states of NO have been demonstrated to affect membrane potential in a different manner and may possibly have different physiologic effects.[14] Similarly, in jejunal circular muscle from humans and from dogs,[10,15] NO plays a quite prominent role in NANC inhibitory function, although other nonnitrergic inhibitory neurotransmitters are implicated as well. In view of the reported findings, we believe that other potential candidate NANC inhibitory neurotransmitters, such as carbon monoxide,[16] vasoactive intestinal polypeptide,[17] adenosine triphosphate,[18] or pituitary adenylate cyclase–activating polypeptide[19] should be evaluated in rat jejunal and ileal circular muscle. The apparent differences between the longitudinal and circular muscle layers is of considerable interest; such differences between muscle layers show the specialized function of different anatomic regions and muscle layers of the gut. In addition, the contractile patterns vary not only between muscle layers but also between anatomic regions. For instance, motor patterns in the ileum are considerably different from those in the jejunum.[20,21] Thus findings in one muscle layer within a region cannot be applied directly to other layers of gut wall in the same or distant regions. These differences in specific NANC functions in the neurally intact jejunum suggest differences in enteric and extrinsic innervation to circular and longitudinal muscle of the rat jejunum.

Our findings have potential clinical and physiologic importance. After SBT, gut function is impaired with diarrhea and abnormalities in absorptive function.[2] Previous experimental work suggested an increase in nitrergic nerves after models of extrinsic denerva-

tion.[7-9] Our experiments were specifically designed to examine nitrergic (inhibitory) pathways as well as non-nitrergic, nonadrenergic, and noncholinergic pathways that might be altered after SBT; our goal was to define mechanisms of alteration in jejunal circular muscle contractility as mediated by enteric neural dysfunction. Combined with our work in the ileum, which showed abnormalities in response of circular muscle to adrenergic agonists,[4,5] and a role of NO in jejunal longitudinal muscle,[12] the current experiments highlight differences not only in anatomic location (jejunum vs. ileum) but also in muscle layer (circular vs. longitudinal). To elucidate these physiologic phenomena and to more specifically address clinical complications related to SBT, further studies in rat and other models of isogeneic SBT are required.

We thank Deborah I. Frank for her assistance in the preparation of this manuscript.

REFERENCES

1. Kocoshis SA, Reyes J, Todo S, Starzl TE. Small intestinal transplantation for irreversible intestinal failure in children. Dig Dis Sci 1997;42:1997-2008.
2. Asfar S, Atkison P, Ghent C, Duff J, Wall W, Williams S, Seidman E, Grant D. Small bowel transplantation: A life-saving option for selected patients with intestinal failure. Dig Dis Sci 1996;41:875-883.
3. Shibata C, Balsiger MN, Anding WJ, Sarr MG. Adrenergic denervation hypersensitivity in ileal circular smooth muscle after small bowel transplantation in rats. Dig Dis Sci 1997; 42:2213-2221.
4. Shibata C, Murr MM, Balsiger B, Anding WJ, Sarr MG. Contractile activity of circular smooth muscle in rats 1 year after small bowel transplantation: Differing adaptive response of the jejunum and ileum to denervation. J GASTROINTEST SURG 1998;2:463-472.
5. Shibata C, Balsiger BM, Anding WJ, Duenes JA, Miller VM, Sarr MG. Functional changes in non-adrenergic non-cholinergic inhibitory neurons in ileal circular smooth muscle after small bowel transplantation in rats. Dig Dis Sci 1998;43:2446-2454.
6. Murr MM, Miller VM, Sarr MG. Contractile properties of enteric smooth muscle after small bowel transplantation in rats. Am J Surg 1996;171:212-218.
7. Yunker AM, Galligan JJ. Extrinsic denervation increases NADPH diaphorase staining in myenteric nerves of guinea pig ileum. Neurosci Lett 1994;167:51-54.
8. Stadelman AM, Fink JG, Mustin E, Walgenbach-Telford S, Telford GL, Koch TR. Non-adrenergic non-cholinergic (NANC) inhibitory nerves are altered in rat small intestine following transplantation [abstr]. Gastroenterology 1994; 106:A843.
9. Nakao K, Takahashi TT, Utsunomiya J, Owyang C. Extrinsic neural control of nitric oxide synthase expression in the myenteric plexus of rat jejunum. J Physiol 1998;507(part 2):549-560.
10. Sander KM, Ward JM. Nitric oxide as a mediator of non-adrenergic non-cholinergic neurotransmission. Am J Physiol 1992;262:G379-G392.
11. Rae MG, Khoyi MA, Keef KD. Modulation of cholinergic neuromuscular transmission by nitric oxide in canine colonic circular smooth muscle. Am J Physiol 1998;275:G1324-G1332.
12. Balsiger BM, Ohtani N, Shibata C, Sarr MG. Non-adrenergic non-cholinergic (NANC) inhibition in longitudinal smooth muscle of rat jejunum: Minor role of nitric oxide (NO) [abstr]. Gastroenterology 1998;114:A716.
13. Balsiger BM, Ohtani N, Shibata C, Murr MM, Anding WJ, Duenes JA, Sarr MG. Small bowel transplantation (SBT) alters non-adrenergic non-cholinergic (NANC) inhibition in longitudinal smooth muscle of the rat jejunum [abstr]. Gastroenterology 1998;114:A1377.
14. Goyal RK, He XD. Evidence of NO redox form of nitric oxide as nitrergic inhibitory neurotransmitter in gut. Am J Physiol 1998;275:G1185-G1192.
15. Stark ME, Bauer AJ, Sarr MG, Szurszewski JH. Nitric oxide mediates inhibitory nerve input in human and canine jejunum. Gastroenterology 1993;104:398-409.
16. Farrugia G, Irons WA, Rae JL, Sarr MG, Szurszewski JH. Activation of whole cell currents in isolated human jejunal circular smooth muscle cells by carbon monoxide. Am J Physiol 1993;264:G1184-G1189.
17. Suzuki M, Nokihara K, Maruse S, Kobayashi S. Vasoactive intestinal polypeptides as a chemical messenger in the neural control of the pacemaker activities of the circular muscle coat in the canine proximal colon. Ann N Y Acad Sci 1996;805: 692-696.
18. Crist JR, He XD, Goyal RK. Both ATP and the peptide VIP are inhibitory neurotransmitters in guinea-pig ileum circular muscle. J Physiol 1992;447:119-131.
19. Parkman HP, Pagano AP, Ryan JP. Dual effects of PACAP on guinea pig gallbladder muscle via PACAP-preferring and VIP/PACAP-preferring receptors. Am J Physiol 1997;272: G1433-G1438.
20. Quigley EMM, Borody TJ, Phillips SF, Wienbeck M, Tucker RL, Haddad A. Motility of the terminal ileum and ileocecal sphincter in healthy humans. Gastroenterology 1984;87:857-866.
21. Ehrlein H-J, Schemann M, Siegle M-L. Motor patterns of small intestine determined by closely spaced extraluminal transducers and videofluoroscopy. Am J Physiol 1987;253(3 part 1):G259-267.

Effect of Massive Small Bowel Resection on the Bax/Bcl-w Ratio and Enterocyte Apoptosis

Lawrence E. Stern, M.D., Richard A. Falcone, Jr., M.D., Christopher J. Kemp, B.S., Lorie A. Stuart, B.S., Christopher R. Erwin, Ph.D., Brad W. Warner, M.D.

Following small bowel resection (SBR), the remnant intestine undergoes adaptation. Enterocyte proliferation is increased and counterbalanced by increased rates of apoptosis. To elucidate a mechanism for increased enterocyte apoptosis, this study tested the hypothesis that the ratio between pro-apoptotic *Bax* and pro-survival *Bcl-w* correlates with the apoptosis that occurs following SBR. Mice (C57Bl/6; n = 76) underwent a 50% proximal SBR or sham operation. After 12 hours and 1, 2, 3, and 7 days, the ileum was removed, the apoptotic index (apoptotic bodies/crypt) was recorded, and the messenger RNA and protein for Bax and Bcl-w were quantified. The apoptotic index was equivalent in the sham and SBR mice at 12 hours; however, it was significantly elevated following SBR at every other day measured. The ratio of Bax to Bcl-w messenger RNA relative to sham operation increased after SBR at 24 hours, decreased by day 3, and returned to baseline levels by 1 week. The protein ratio showed an increase by day 1, which remained elevated through day 7. An augmented ratio of Bax to Bcl-w messenger RNA and protein corresponded with the increase in enterocyte apoptosis. Alterations in the expression ratio of these genes may play a role in establishing a new homeostatic set point between proliferation and apoptosis during adaptation. (J GASTROINTEST SURG 2000;4:93-100.)

KEY WORDS: Apoptosis, Bax, Bcl-w, intestine, short bowel syndrome

During quiescence the murine intestinal mucosa is in a constant state of renewal with progenitor crypt stem cells adding approximately 1200 new epithelial cells per day to each villus.[1] The rate of cellular production is balanced by the rate of cellular death or loss. Apoptosis, or programmed cell death, is in part responsible for achieving this homeostatic balance.[2]

Following massive small bowel resection (SBR), the remnant intestine undergoes an adaptive process characterized by increases in wet weight, protein and DNA content, villus height, crypt depth, and absorptive surface area.[3,4] Additionally, a proliferative stimulus increases crypt enterocyte mitoses resulting in augmented cellular advancement along the villus axis. These changes are consistent with an attempt to compensate for the loss of significant digestive and absorptive function.[5] Despite the need for intestinal hyperplasia, the stimuli for adaptation fail to suppress programmed cell death. In fact, indices of enterocyte crypt apoptosis are elevated following murine massive SBR.[6,7] The catalyst for increased apoptosis following resection is currently unknown and the pathways involved are poorly characterized. Understanding this process is critical, as attenuating the rate of cell death may enhance intestinal adaptation.

The Bcl-2 family of proteins plays a significant role in the regulation of several apoptotic pathways including those involved with the mitochondria. The Bcl-2 family consists of both pro-survival and pro-apoptotic gene members that share at least one of the highly conserved Bcl-2 homology domains (BH1, BH2, BH3, and BH4). Immunostaining of the enterocyte crypts has revealed moderate to intense quantities of the pro-apoptotic Bax protein, whereas

From the Division of Pediatric Surgery, Children's Hospital Medical Center, and the Department of Surgery, University of Cincinnati College of Medicine, Cincinnati, Ohio.
Supported by grants F32 DK09882 (Dr. Stern) and RO-1 DK53234 (Dr. Warner) from the National Institutes of Health and by a grant from the Children's Hospital Campaign for Children Fund, Children's Hospital Medical Center, Cincinnati, Ohio.
Presented at the Fortieth Annual Meeting of The Society for Surgery of the Alimentary Tract, Orlando, Fla., May 16-19, 1999.
Reprint requests: Brad W. Warner, M.D., Division of Pediatric Surgery, Children's Hospital Medical Center, 3333 Burnet Ave., Cincinnati, OH 45229-3039. e-mail: Brad.Warner@uc.edu

the villi have minimal Bax present.[8] Conversely Bcl-2, a major anti-apoptotic factor, has only a slight presence in the crypts with higher intensity in the absorptive areas of the small intestine.[9] Because intestinal crypts maintain a baseline level of apoptosis, it would appear necessary for an alternative pro-survival member of the Bcl-2 family, such as Bcl-w, to regulate the pro-apoptotic effects of Bax within the intestinal crypts.

Bcl-w is a 21 kdal protein, with 46% overall homology to Bcl-2 and maintenance of the S1 region, which is believed to impart prosurvival function.[10] Bcl-w has a wide tissue distribution with the highest levels identified in the brain, colon, and salivary gland.[10] In cell culture, *Bcl-w* has been demonstrated to inhibit apoptosis in a manner consistent with *Bcl-2* and *Bcl-x_L*. In *Bcl-w* knockout mice, delayed but severe apoptosis of the testes is observed despite an otherwise normal phenotype.[11] Bax is a 21 kdal protein that maintains the BH1, BH2, and BH3 domains.[12] It is predominantly located in the cytoplasm; however, on apoptotic induction it translocates to the mitochondria.[13,14] *Bax* has been shown to promote apoptosis in both cell culture and in vivo.[15,16]

The genes and pathways involved in accelerated apoptosis following SBR are likely multiple but essentially unknown. This study was designed to test the hypothesis that an increased ratio of pro-apoptotic *Bax* to pro-survival *Bcl-w* would correspond with the known increased enterocyte crypt apoptotic index following massive SBR.[17]

MATERIAL AND METHODS

Male C57Bl/6 mice (The Harlan Laboratory, Indianapolis, Ind.) weighing 25 to 29 g were housed in groups of four at 21° C on 12-hour day and night cycles (6 AM to 6 PM). Prior to experimentation, the mice acclimated to their environment for at least five days. One day prior to operation, the diet was changed from regular chow to liquid rodent diet (Micro-Stabilized Rodent Liquid Diet LAD 101/101A, Purina Mills, St. Louis, Mo.). This study was approved by the Children's Hospital Research Foundation Institutional Animal Care and Use Committee (Children's Hospital Medical Center, Cincinnati, Ohio).

Experimental Design

Mice were randomly allocated to undergo either a 50% proximal SBR or a sham operation and were then sacrificed after 12 hours or 1, 2, 3, or 7 days. At the time of death, ileal wet weight was recorded. The rate of enterocyte apoptosis at each time point was determined by histologic quantification of the crypt apoptotic index. A ribonuclease protection assay and Western blot analysis were performed at each time point to quantify *Bax* and *Bcl-w* mRNA and their respective proteins.

Operative Procedure

The techniques for SBR and sham operation have been previously presented.[18] In short, with the mice under inhaled isoflurane anesthesia and using an operating microscope, a midline abdominal incision was made. In mice undergoing sham operation, the small bowel was transected 12 cm proximal to the ileocecal valve and a reanastomosis was performed. In mice undergoing SBR, approximately 12.5 cm of proximal intestine was resected and an anastomosis performed (50% resection). After abdominal closure, the mice were resuscitated with a 3 ml intraperitoneal injection of warm 0.9% saline solution and allowed to recover in an incubator (30° C). Water was provided ad libitum for the first 24 hours. Mice from each group were then pair fed with liquid diet.

Tissue Harvest

Mice were killed by an intramuscular injection of ketamine, xylazine, and acepromazine (4:1:1 proportion) followed by cervical dislocation. Six centimeters (roughly 1 cm from the anastomosis) of ileum was excised, the luminal contents were gently expressed with cotton swabs, and the wet weight was recorded. The proximal 1 cm was immediately fixed with 10% neutral buffered formalin and used for histologic examination (see below); the remaining 5 cm was frozen in liquid nitrogen and stored at −80° C until further use.

Histologic Examination

Fixed specimens of ileum were embedded in paraffin as described previously.[18] Tissue slices (5 μm) were mounted and stained with hematoxylin and eosin. Apoptosis was quantified by scoring the number of apoptotic bodies identified within the crypts. Apoptotic bodies were defined by the presence of pyknotic nuclei, condensed chromatin, and nuclear fragmentation.[6] The apoptotic index was defined as the number of apoptotic bodies per crypt. Blinded scoring of 50 crypts per mouse was performed in duplicate by two separate investigators.

Ribonuclease Protection Assay

Individual ileum samples were thawed and homogenized (PowerGen, Fisher Scientific, Pittsburgh, Pa.). Total RNA was isolated using TRIzol reagent (Gibco

Biological Research Laboratories, Gaithersburg, Md.) following the instructions of the manufacturer.[19] The concentration of total RNA was determined spectrophotometrically at A_{260}. Samples were pooled into the appropriate sample group (i.e., postoperative day and surgical procedure) and concentration quantified. Ten micrograms of total RNA from each pooled sample was analyzed via the Multi-Probe RNase Protection Assay System using the mAPO-2 probe as described by the manufacturer (RiboQuant, Pharmingen International, San Diego, Calif.). Protected bands were exposed overnight on a storage phosphor screen (Molecular Dynamics, Sunnyvale, Calif.) and scanned on a phosphoimager (Storm 860, Molecular Dynamics). Band intensities were quantified using ImageQuant 5.0 software (Molecular Dynamics) and adjusted based on the intensities of the internal L32 and GAPDH housekeeping genes.

Western Blot Analysis

Individual ileum samples were homogenized in 5× volume of homogenization buffer (10 mmol/L Tris-HCl, pH 8.0, 0.1 mol/L EGTA, pH 8.0, 1.0 mol/L DTT, 0.1 mol/L Na_3VO_4, 5 mmol/L Na_2MoO_4, 1.0 mol/L β-glycerolphosphate, 0.1 mol/L $Na_4P_2O_7$, 1 mg/ml aprotinin, 1 mg/ml leupeptin, and 100 mmol/L PMSF) as described for the ribonuclease protection assay. Total protein was quantified by using a modified Lowry assay.[20] One hundred micrograms of protein from each sample were pooled into the appropriate group. For identification of Bax, 100 μg of protein was added to an equal volume of 2× protein sample buffer (250 mmol/L Tris-HCl, pH 6.8, 4% SDS, 10% glycerol, 0.003% bromophenol blue, and 2% BME). The samples were then run on a 10% to 20% gradient polyacrylamide gel (Page-One, Owl Separation Systems, Portsmouth, R.I.) at 4° C with standard protein running buffer (0.192 mol/L glycine, 0.025 mol/L Tris base, and 0.10% sodium dodecyl sulfate). Protein was transferred to a PVDF-Plus membrane (Micron Separations Inc., Westboro, Mass.) and after blocking in 5% nonfat milk, exposed for 1 hour at room temperature to a 1:500 dilution of mouse anti-Bax antibody (Pharmingen International). After five washings, antibody detection was accomplished by incubating the membrane for 1 hour at room temperature in a 1:15,000 dilution of horseradish peroxidase–avidin antimouse IgG (Transduction Laboratories, Lexington, Ky.) followed by use of a chemiluminescence system (Renaissance, NEN Life Science Products, Boston, Mass.) and exposure to x-ray film (Biomax ML, Eastman Kodak Co., Rochester, N.Y.). Band intensity was quantified using ImageQuant 5.0 software.

Detection of Bcl-w protein was carried out in the same manner with the following exceptions: 75 μg of protein was loaded into the gel, the membrane was exposed at 4° C overnight to a 1:500 dilution of polyclonal rabbit anti–Bcl-w (StressGen Biotechnologies Corp., Victoria, B.C.), and the secondary antibody was a 1:50,000 dilution of horseradish peroxidase–avidin goat antirabbit IgG (Calbiochem, La Jolla, Calif.).

Statistical Analysis

Results are presented as mean values (± standard error of the mean). Statistical differences were determined by analysis of variance followed by a pairwise multiple-comparison Student–Newman-Keuls method using the SigmaStat statistical package (Jandel Scientific, San Rafael, Calif.). A P value <0.05 was considered significant.

RESULTS

Adaptation was verified by increased ileal wet weight following SBR. Although no appreciable changes were noted at 12 hours, the weights were significantly elevated in the SBR groups at days 1 through 7 (Fig. 1). Samples from the sham and SBR mice were examined for histologic evidence of enterocyte crypt apoptosis. No differences between sham and SBR groups could be appreciated at 12 hours; however, at postoperative day 1 a 41.7% increase in the apoptotic index was noted in the resected animals (0.36 ± 0.02 vs. 0.51 ± 0.02; $P < 0.05$). Similar elevations in the apoptotic index of SBR mice persisted through 1 week (Fig. 2).

A ribonuclease protection assay was performed on each pooled sample and mRNA expression levels were calculated as a percentage of sham intensity. *Bax* expression following SBR increased at day 1, fell at day 3, and returned to baseline by day 7. *Bcl-w* expression following SBR showed minimal change at 12 hours, fell at day 1, increased by day 3, and returned to baseline at 1 week (Fig. 3).

The changes and ratios detected in mRNA expression implied an interaction between *Bax* and *Bcl-w*. To confirm a physiologic role, protein levels were quantified for both Bax and Bcl-w in the sham and resected animals (Fig. 4, *A*). Bax increased by day 1 and remained elevated through 1 week. Bcl-w fell by 12 hours and returned to baseline by 7 days (Fig. 4, *B*). The ratio between both the mRNA and protein showed increases favoring apoptosis. The protein ratio peaked at day 3 (≈ 3-fold), which is likely the result of the earlier rise seen in the mRNA (Fig. 5). Additionally, both the Bax and Bcl-w antibodies identi-

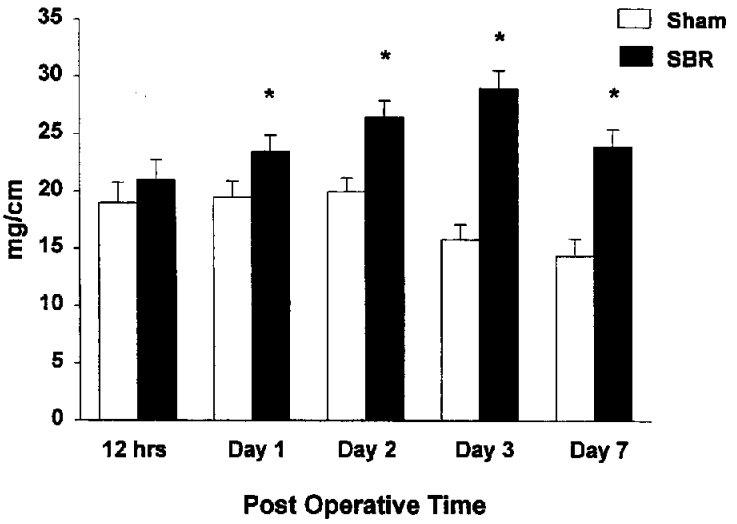


Fig. 1. Ileal wet weights (mg/cm) from mice following either sham operation (transection and reanastomosis only) or 50% proximal small bowel resection *(SBR)* were recorded at 12 hours and 1, 2, 3, and 7 days postoperatively. N = 5 to 10 per group; * = $P < 0.05$, analysis of variance.

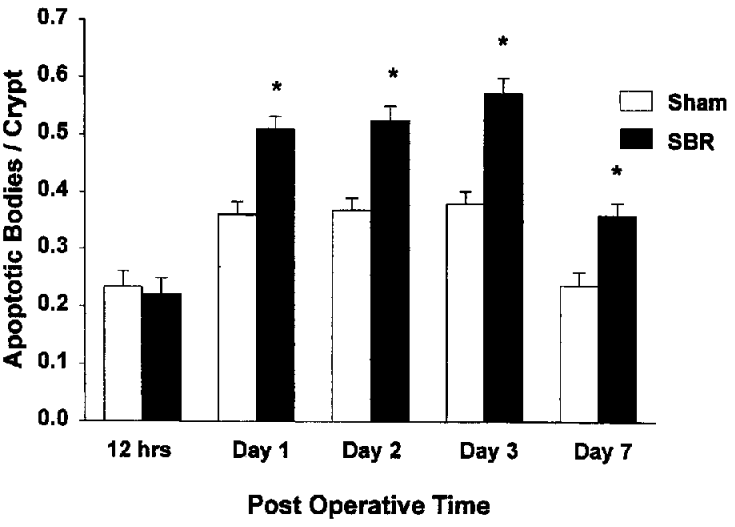


Fig. 2. Rates of apoptosis in the ileal crypts at multiple time points following either sham operation (transection and reanastomosis only) or 50% proximal small bowel resection *(SBR)* were recorded. Apoptotic index was derived by counting the number of apoptotic bodies per crypt that demonstrated characteristic abnormal morphology (pyknotic nuclei, condensed chromatin, and nuclear fragmentation). N = 5 to 10 per group; * = $P < 0.05$, analysis of variance.

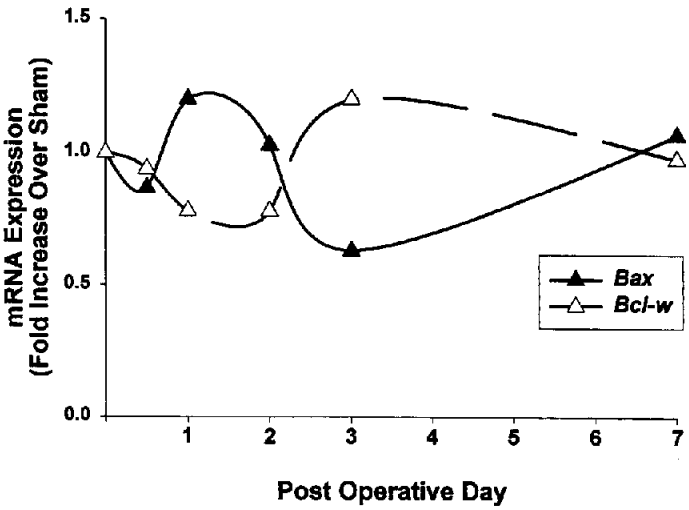


Fig. 3. Relative expression of *Bax* and *Bcl-w* mRNA expression, as determined by ribonuclease protection assay, of mice undergoing a 50% proximal small bowel resection compared to sham operation (transection and reanastomosis only). Samples are pooled with N = 5 to 10 animals per group.

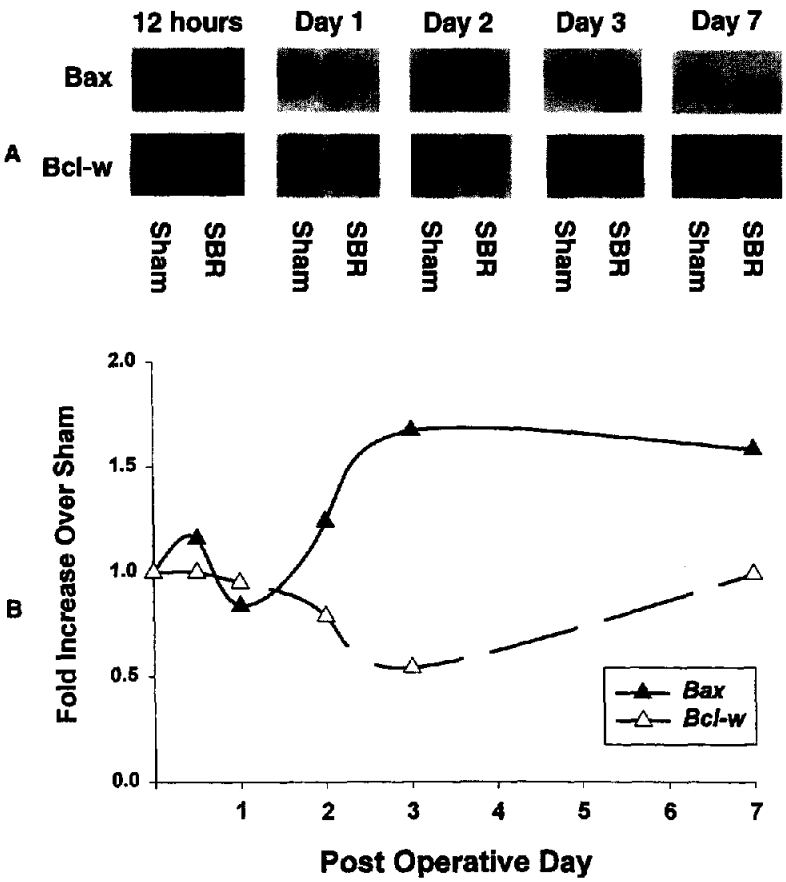


Fig. 4. A, Representative 21 kdal protein bands for Bax and Bcl-w by Western blot analysis. **B,** Relative expression of Bax and Bcl-w protein, as determined by Western blot analysis, of mice undergoing 50% proximal small bowel resection *(SBR)* compared to sham operation (transection and reanastomosis only). Samples are pooled with N = 5 to 10 animals per group.

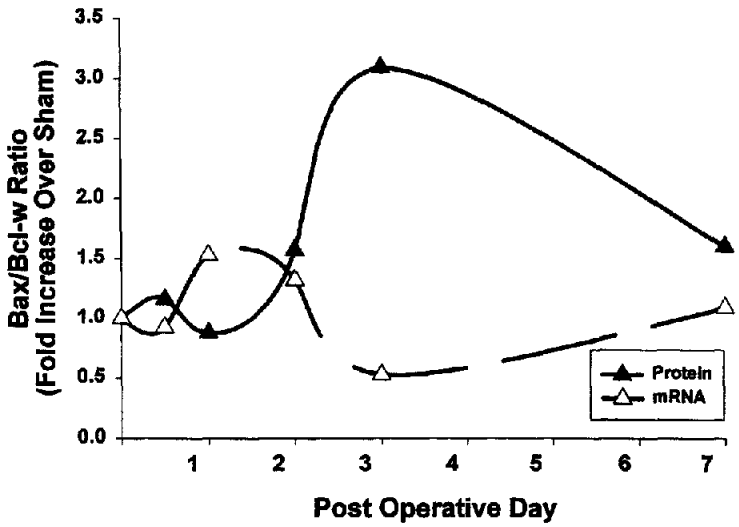


Fig. 5. Ratio of Bax to Bcl-w mRNA and protein expression, as determined by ribonuclease protection assay and Western blot analysis, of mice undergoing 50% proximal small bowel resection compared to sham operation (transection and reanastomosis only). Samples are pooled with N = 5 to 10 animals per group.

fied bands with molecular weights of approximately 38 to 43 kdal, which may be consistent with dimer formation (data not shown).

DISCUSSION

This study has shown an alteration of the mRNA and protein ratios between a pro-apoptotic gene (Bax) and a pro-survival gene (Bcl-w), which coincided with the initial increase in apoptosis following intestinal resection. This ratio favors apoptosis in the early phase of intestinal adaptation and normalizes over time. These findings appear to support an important role for the differential regulation of apoptotic proteins as coordinators of the early increase in enterocyte apoptosis that occurs after SBR.

Programmed cell death is an integral component in the maintenance of the normal small intestine mucosal architecture. The increased apoptosis following SBR has been previously documented; however, the causes and mechanisms for both basal and accelerated apoptosis are poorly understood.[6,7] Small changes in apoptosis can induce profound changes in villus and crypt architecture. The apoptotic index represents changes within the intestinal crypt, a major site of intestinal apoptosis.[2] Death of a single progenitor stem cell in the crypt may therefore result in the loss of 60 to 120 daughter cells, potentially causing a dramatic alteration of intestinal morphology.[21]

The protooncogene p53 must be considered as a candidate for apoptotic regulation following SBR, as it is an integral component of many apoptotic pathways. Studies involving p53 have demonstrated its ability to upregulate the apoptotic receptor Fas/APO-1 as well as *Bax* gene expression.[22] Additionally, irradiation-induced enterocyte apoptosis is partially prevented in p53-deficient mice.[23] Despite the numerous studies detailing the role of p53 in programmed cell death, it appears to have minimal importance in apoptotic induction following SBR. Shin et al.[24] have demonstrated that p53-null mice undergoing massive resection showed equivalent increases in apoptosis and parameters of adaptation when compared to their wild-type counterparts. It therefore appears that apoptotic induction following SBR is a p53-independent process that may involve factors downstream of p53 and/or parallel pathways of regulation.

The initial downregulation of pro-survival *Bcl-w* mRNA following SBR coincided with increased enterocyte crypt apoptosis and was followed by a mild upregulation and gradual return to baseline. This expression pattern is consistent with other physiologic systems.[25] In a previous study from this laboratory, DNAse I, an enzyme responsible for nucleotide cleavage during apoptotic induction, demonstrated increased mRNA expression and enzymatic activity 24 hours after resection, fell below control levels at 72 hours, and returned to baseline at 1 week.[26] This pattern likely coincides with the establishment of a new homeostatic set point between enterocyte proliferation and apoptosis during intestinal adaptation.

Bcl-w is a newly discovered gene located on the central portion of mouse chromosome 14 (human chromosome 14 band q11). The ability of Bcl-w to inhibit cell death following interleukin-3 deprivation or gamma-irradiation is comparable to both Bcl-2 and Bcl-x_L.[10] Bcl-w, similar to other survival proteins, may exert its prosurvival effect by directly inhibiting Apaf-1, an inducer of the caspase cascade, creating mitochondrial channels, or by antagonizing the actions of pro-apoptotic genes such as *Bax* and *Bak*.[12]

The development of a *Bcl-w*–deficient mouse demonstrated marked apoptosis in the testes on sexual maturation and failure of proper spermatogenesis.[11] Both testicular development and spermatogenesis are associated with high levels of proliferation; therefore marked acceleration of apoptosis on sexual maturation raises the possibility of a stress- or proliferation-induced requirement for the *Bcl-w* gene product.[11] The increased rates of apoptosis following SBR corresponded to the period of relative Bcl-w deficiency to Bax and is consistent with this hypothesis.

The *Bax* mRNA expression pattern following resection was essentially the inverse of *Bcl-w*. The initial increased histologic levels of apoptosis corresponded with the early increase in pro-apoptotic *Bax* transcription. The proposed mechanism(s) for Bax-induced apoptosis is multifactorial. Inducible overexpression of *Bax* in both yeast and mammalian cells triggers apoptosis, even in the absence of additional stimuli.[27] Through interaction with voltage-dependent anion channels such as the mitochondrial transition pore (PTP), Bax is able to induce changes in the mitochondrial transmembrane potential and release cytochrome c.[28] Cytochrome c release along with alterations in mitochondrial electron transport and oxidative phosphorylation can initiate apoptosis.[29] Additionally, Bax can induce release of cytochrome c in a PTP-independent manner.[30,31]

The mechanism by which the pro-apoptotic and pro-survival genes interact remains controversial. In 1993 Oltvai et al. proposed a model for apoptotic regulation in which the ratio between Bax-Bax homodimers, Bax-Bcl-2 heterodimers, and Bcl-2-Bcl-2 homodimers would determine the overall balance between apoptosis and cell survival.[1,32,33] Various pro-survival and pro-apoptotic members of the Bcl-2 family have subsequently shown the ability to dimerize with one another. Further, mutations within the binding regions of survival proteins cause failure to sup-

press apoptotic death.[27,34,35] Bcl-w immunoprecipitates with other pro-apoptotic Bcl-2 family members including Bax, Bak, Bad, and Bik, indicating heterodimerization.[36] The BH1, BH2, and BH3 domains of the pro-survival members appear necessary for homo- and heterodimerization to occur,[37,38] and is consistent with the finding that a mutation of the glycine in the Bcl-w BH1 region prevented binding to Bax.[36]

Dimerization may not be essential for all Bcl-2 protein interactions. Competitive inhibition through displacement or disruption of existing dimers is an alternative mechanism of action. Death-promoting proteins such as Bad form dimers with Bcl-x_L while simultaneously displacing Bax.[39,40] Thus Bad may initiate apoptosis by inhibiting the survival effects of Bcl-x_L or by freeing Bax and thereby causing mitochondrial-induced apoptosis. Other studies have shown the ability of pro-apoptotic genes to induce death despite failure to create heterodimers with specific pro-survival members.[16,41] These studies do not exclude the possibility of homodimerization or interactions with other Bcl-2 family members. Future studies will be necessary to delineate the role for these other family members and dimerization during adaptation.

Both *Bax* and *Bcl-w* mRNA returned to baseline levels by one week despite continued elevation of Bax protein. This may represent a temporal "lag" between changes in transcription and translation. Alternatively, it may represent an increased Bax half-life as a result of interaction with other Bcl-2 proteins. Miyashita et al.[42] have demonstrated that the half-life of Bax can be increased by overexpression of Bcl-2. The importance of alterations in half-lives has only recently been recognized; further work may provide insight into the complex regulatory mechanisms of programmed cell death. Although this study has shown an alteration of apoptotic gene expression ratios, the actual importance of each particular gene during the adaptive process remains unknown. The use of murine knockout and transgenic technology may help address these fundamental questions. Additionally, studies evaluating apoptotic receptor regulation and methods for decreasing the apoptotic rate in vivo may allow for increased levels of intestinal adaptation with associated clinical benefits.

REFERENCES

1. Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993;74:609-619.
2. Potten CS. The significance of spontaneous and induced apoptosis in the gastrointestinal tract of mice. Cancer Metastasis Rev 1992;11:179-195.
3. Vanderhoof JA, Langnas AN. Short-bowel syndrome in children and adults. Gastroenterology 1997;113:1767-1778.
4. Williamson RCN, Chir M. Intestinal adaptation. Mechanisms of control. N Engl J Med 1978;298:1444-1450.
5. Williamson RCN, Chir M. Intestinal adaptation. Structural, functional and cytokinetic changes. N Engl J Med 1978;298: 1393-1402.
6. Helmrath MA, Erwin CR, Shin CE, Warner BW. Enterocyte apoptosis is increased following small bowel resection. J GASTROINTEST SURG 1998;2:44-49.
7. Helmrath MA, Shin CE, Erwin CR, Warner BW. The EGF/EGF-receptor axis modulates enterocyte apoptosis during intestinal adaptation. J Surg Res 1998;77:17-22.
8. Krajewski S, Krajewska M, Shabaik A, Miyashita T, Wang HG, Reed JC. Immunohistochemical determination of in vivo distribution of Bax, a dominant inhibitor of Bcl-2. Am J Pathol 1994;145:1323-1336.
9. Merritt AJ, Potten CS, Watson AJ, Loh DY, Nakayama K, Hickman JA. Differential expression of bcl-2 in intestinal epithelia. Correlation with attenuation of apoptosis in colonic crypts and the incidence of colonic neoplasia. J Cell Sci 1995;108:2261-2271.
10. Gibson L, Holmgreen SP, Huang DC, et al. bcl-w, a novel member of the bcl-2 family, promotes cell survival. Oncogene 1996;13:665-675.
11. Print CG, Loveland KL, Gibson L, et al. Apoptosis regulator bcl-w is essential for spermatogenesis but appears otherwise redundant. Proc Natl Acad Sci USA 1998;95:12424-12431.
12. Adams JM, Cory S. The Bcl-2 protein family: Arbiters of cell survival. Science 1998;281:1322-1326.
13. Wood DE, Thomas A, Devi LA, et al. Bax cleavage is mediated by calpain during drug-induced apoptosis. Oncogene 1998;17:1069-1078.
14. Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol 1997;139:1281-1292.
15. Yin C, Knudson CM, Korsmeyer SJ, Van DT. Bax suppresses tumorigenesis and stimulates apoptosis in vivo. Nature 1997;385:637-640.
16. Knudson CM, Korsmeyer SJ. Bcl-2 and Bax function independently to regulate cell death. Nat Genet 1997;16:358-363.
17. Ohno T, Nakano T, Niibe Y, Tsujii H, Oka K. Bax protein expression correlates with radiation-induced apoptosis in radiation therapy for cervical carcinoma. Cancer 1998;83:103-110.
18. Helmrath MA, VanderKolk WE, Can G, Erwin CR, Warner BW. Intestinal adaptation following massive small bowel resection in the mouse. J Am Coll Surg 1996;183:441-449.
19. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate- phenol-chloroform extraction. Anal Biochem 1987;162:156-159.
20. Lowry O, Rosebrough N, Farr A, et al. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265-275.
21. Potten CS, Wilson JW, Booth C. Regulation and significance of apoptosis in the stem cells of the gastrointestinal epithelium. Stem Cells 1997;15:82-93.
22. Miyashita T, Reed JC. Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 1995;80: 293-299.
23. Merritt AJ, Allen TD, Potten CS, Hickman JA. Apoptosis in small intestinal epithelial from p53-null mice: Evidence for a delayed, p53-independent G2/M-associated cell death after gamma-irradiation. Oncogene 1997;14:2759-2766.
24. Shin CE, Falcone RA, Kemp CJ, et al. Intestinal adaptation and enterocyte apoptosis following small bowel resection is p53 independent. Am J Physiol 1999;277:G717-G724.

25. Rauch F, Polzar B, Stephan H, Zanotti S, Paddenberg R, Mannherz HG. Androgen ablation leads to an upregulation and intranuclear accumulation of deoxyribonuclease I in rat prostate epithelial cells paralleling their apoptotic elimination. J Cell Biol 1997;137:909-923.
26. Falcone RA, Stern LE, Kemp CJ, Shin CE, Erwin CR, Warner BW. Apoptosis and the pattern of DNase I expression following massive small bowel resection. J Surg Res 1999; 84:218-222.
27. St. Clair EG, Anderson SJ, Oltvai ZN. Bcl-2 counters apoptosis by Bax heterodimerization-dependent and -independent mechanisms in the T-cell lineage. J Biol Chem 1997;272: 29347-29355.
28. Narita M, Shimizu S, Ito T, et al. Bax interacts with the permeability transition pore to induce permeability transition and cytochrome c release in isolated mitochondria. Proc Natl Acad Sci USA 1998;95:14681-14686.
29. Green DR, Reed JC. Mitochondria and apoptosis. Science 1998;281:1309-1311.
30. Jurgensmeier JM, Xie Z, Deveraux Q, Ellerby L, Bredesen D, Reed JC. Bax directly induces release of cytochrome c from isolated mitochondria. Proc Natl Acad Sci USA 1998;95: 4997-5002.
31. Eskes R, Antonsson B, Osen-Sand A, et al. Bax-induced cytochrome C release from mitochondria is independent of the permeability transition pore but highly dependent on Mg2+ ions. J Cell Biol 1998;143:217-224.
32. Korsmeyer SJ, Shutter JR, Veis DJ, Merry DE, Oltvai ZN. Bcl-2/Bax: A rheostat that regulates an anti-oxidant pathway and cell death. Semin Cancer Biol 1993;4:327-332.
33. Oltvai ZN, Korsmeyer SJ. Checkpoints of dueling dimers foil death wishes. Cell 1994;79:189-192.
34. Sedlak TW, Oltvai ZN, Yang E, et al. Multiple Bcl-2 family members demonstrate selective dimerizations with Bax. Proc Natl Acad Sci USA 1995;92:7834-7838.
35. Hsu SY, Kaipia A, McGee E, Lomeli M, Hsueh AJ. Bok is a pro-apoptotic Bcl-2 protein with restricted expression in reproductive tissues and heterodimerizes with selective anti-apoptotic Bcl-2 family members. Proc Natl Acad Sci USA 1997;94:12401-12406.
36. Holmgreen SP, Huang DC, Adams JM, Cory S. Survival activity of Bcl-2 homologs Bcl-w and A1 only partially correlates with their ability to bind pro-apoptotic family members. Cell Death Differ 1999;6:525-532.
37. Chittenden T, Flemington C, Houghton AB, et al. A conserved domain in Bak, distinct from BH1 and BH2, mediates cell death and protein binding functions. EMBO J 1995; 14:5589-5596.
38. Yin XM, Oltvai ZN, Korsmeyer SJ. BH1 and BH2 domains of Bcl-2 are required for inhibition of apoptosis and heterodimerization with Bax. Nature 1994;369:321-323.
39. Kelekar A, Chang BS, Harlan JE, Fesik SW, Thompson CB. Bad is a BH3 domain-containing protein that forms an inactivating dimer with Bcl-XL. Mol Cell Biol 1997;17:7040-7046.
40. Yang E, Zha J, Jockel J, Boise LH, Thompson CB, Korsmeyer SJ. Bad, a heterodimeric partner for Bcl-XL and Bcl-2, displaces Bax and promotes cell death. Cell 1995;80:285-291.
41. Zha H, Reed JC. Heterodimerization-independent functions of cell death regulatory proteins Bax and Bcl-2 in yeast and mammalian cells. J Biol Chem 1997;272:31482-31488.
42. Miyashita T, Kitada S, Krajewski S, Horne WA, Delia D, Reed JC. Overexpression of the Bcl-2 protein increases the half-life of p21Bax. J Biol Chem 1995;270:26049-26052.

Comparison of Massive vs. Repeated Resection Leading to Short Bowel Syndrome

Jon S. Thompson, M.D.

Short bowel syndrome can result from either a single massive intestinal resection or repeated lesser resections, which might have prognostic implications. The aim of this study was to compare patient populations and outcome of short bowel syndrome caused by massive and repeated resection. The records of 95 adult patients with short bowel syndrome evaluated over a 20-year period were reviewed. Massive resection was performed in 72 patients (76%) and repeated lesser resections in 23 patients (24%). Patients undergoing massive resection were more likely to be more than 70 years of age (26% vs. 9%, $P < 0.05$). Mesenteric vascular disease was more prevalent among patients undergoing massive resection (39% vs. 9%, $P < 0.05$), whereas Crohn's disease was less prevalent (1% vs. 35%, $P < 0.05$). Distribution of remnant length, presence of the ileocecal junction, and presence of a stoma were similar. Patients undergoing massive resection were more likely to require parenteral nutrition after the first year (56% vs. 23%, $P < 0.05$). Patients with very short remnants (<60 cm) were more likely to receive parenteral nutrition after massive resection (95% vs. 60%, $P < 0.05$). Thirty-day mortality was higher after massive resection (24% vs. 4%, $P < 0.05$). However, those surviving 30 days had similar survival rates at 1 year and 5 years after massive and repeated resections. Patients undergoing massive vs. repeated resections are different with respect to age, underlying condition, and nutritional support needs. These factors may influence overall outcome in short bowel syndrome. The better nutritional prognosis of patients undergoing repeated resection given similar intestinal remnants may be related in part to enhanced intestinal adaptation. (J GASTROINTEST SURG 2000;4:101-104.)

KEY WORDS: Short bowel syndrome, intestinal adaptation

Short bowel syndrome (SBS) can result from either a single massive resection or repeated lesser resections leading to a shortened intestinal remnant. We found previously that approximately three fourths of patients with SBS had undergone a single massive resection.[1] The nature of resection can be influenced by the underlying condition necessitating resection and other patient factors. Experimental studies have suggested that multiple smaller resections will lead to an enhanced adaptive response compared to a massive resection of similar overall length.[2] Since different patient populations may be involved and the extent of resection influences the intestinal adaptive response, the manner of resection might have prognostic implications for patients with SBS. The aim of the present study was to compare patient populations and outcomes of SBS caused by massive vs. repeated lesser resection.

METHODS

This was a retrospective review of 95 consecutive adult (>18 years of age) patients undergoing treatment for SBS between 1980 and 1999 at the University of Nebraska Medical Center (University Hospital and Omaha VA Hospital). SBS was defined as an intestinal remnant less than 180 cm in length with associated malabsorption. Massive resection refers to a single intestinal resection leading to SBS. Patients undergoing repeated lesser resections underwent a mean of three resections (range 2 to 5) before developing SBS. The interval between the initial and final resections in these patients ranged from 1 to 240 months. Records were reviewed to determine patient age and sex, condition causing resection, nature of resection, status of the intestinal remnant and other digestive organs, and nutritional outcome. Patient follow-up ranged from 8 to 192 months after diagnosis of SBS,

From the Department of Surgery, University of Nebraska Medical Center, Omaha, Neb.
Presented at the Fortieth Annual Meeting of The Society for Surgery of the Alimentary Tract, Orlando, Fla., May 16-19, 1999.
Reprint requests: Jon S. Thompson, M.D., 983280 Nebraska Medical Center, Omaha, NE 68198-3280. e-mail: J Thompso@mail.unmc.edu

Table I. Patient characteristics

Patient characteristics	Massive resection	Repeated resection	Total
Age (yr)			
<30	9 (13%)	3 (13%)	12 (13%)
30-70	44 (61%)	18 (78%)	62 (65%)
≥70	19 (26%)*	2 (9%)	21 (22%)
Sex			
Male	37 (51%)	8 (35%)	45 (47%)
Female	35 (49%)	15 (65%)	50 (53%)

*P <0.05 vs. repeated resection.

Table II. Conditions causing resection

Condition	Massive resection	Repeated resection	Total
Mesenteric vascular disease	28 (39%)*	2 (9%)	30 (32%)
Cancer/irradiation	22 (31%)	8 (35%)	30 (32%)
Crohn's disease	1 (1%)*	8 (35%)	9 (9%)
Other benign disease	21 (29%)	5 (21%)	26 (27%)
TOTAL	72	23	95

*P <0.05 vs. repeated resection.

Table III. Nature of intestinal remnant

	Massive resection	Repeated resection	Total
Remnant length (cm)			
<60	27 (38%)	5 (22%)	32 (34%)
60-120	15 (20%)	5 (22%)	20 (21%)
>120	30 (42%)	13 (56%)	43 (45%)
Ileal remnant			
Yes	45 (63%)	13 (57%)	58 (61%)
No	27 (37%)	10 (43%)	37 (39%)
Colon remnant			
None	6 (8%)	3 (13%)	9 (10%)
Right	5 (7%)	2 (9%)	7 (7%)
Left	46 (64%)	15 (65%)	61 (64%)
All	15 (21%)	3 (13%)	18 (19%)
Ileocecal junction			
Present	20 (28%)	5 (22%)	25 (26%)
Absent	52 (72%)	18 (78%)	70 (74%)
Stoma			
Yes	46 (63%)	12 (52%)	57 (60%)
No	27 (37%)	11 (48%)	38 (40%)

Table IV. Nutritional prognosis and outcome

	Massive resection	Repeated resection	Total
Parenteral nutrition during the first year			
Yes	62 (78%)*	15 (56%)	77 (81%)
No	10 (22%)	8 (44%)	18 (19%)
Parenteral nutrition after the first year			
Yes	31 (70%)*	5 (25%)	36 (56%)
No	13 (30%)	15 (75%)	28 (44%)
30-day mortality			
Yes	17 (24%)*	1 (4%)	18 (19%)
No	55 (76%)	22 (96%)	77 (81%)
1-year survival			
Yes	44 (80%)	20 (91%)	64 (83%)
No	11 (20%)	2 (9%)	13 (17%)
5-year survival			
Yes	32 (68%)	17 (89%)	49 (74%)
No	15 (32%)	2 (11%)	17 (26%)

*P <0.05 vs. repeated resection.

with 66 patients (69%) being followed for more than 5 years. Statistical comparisons were made using chi-square analysis with $P < 0.05$ signifying statistical significance.

RESULTS

Massive resection was performed in 72 patients (76%) and repeated lesser resections in 23 patients (24%). Patients undergoing massive resection were more likely to be more than 70 years of age (26% vs. 9%, $P < 0.05$) but sex distribution was similar (Table I). Mesenteric vascular disease was more prevalent among patients undergoing massive resection (39% vs. 9%, $P < 0.05$) (Table II). Crohn's disease was more prevalent among those undergoing lesser resections (1% vs. 35%, $P < 0.05$).

Distribution of remnant length was comparable in the two groups with remnants less than 60 cm in 38% and 22%, respectively. The presence of an ileal remnant (63% vs. 57%), an intact ileocecal junction (28% vs. 22%), and a stoma (63% and 52%) occurred with similar frequency in both groups. Remaining colon was also similar in the two groups (Table III).

Patients undergoing massive resection were more likely to require parenteral nutrition during the first year (78% vs. 56%, $P < 0.05$) and to remain dependent on parenteral nutrition after the first year (70% vs. 25%, $P < 0.05$) (Table IV). Patients with very short remnants (<60 cm) were more likely to be on parenteral nutrition after massive resection (95% vs. 60%, $P < 0.05$).

Thirty-day mortality was higher after massive resection (24% vs. 4%, $P < 0.05$). However, those surviving 30 days had similar survival rates at 1 year (80% vs. 91%, not significant [NS]) and 5 years (68% and 89%, NS) after massive and repeated resection. Early (30-day) death occurred in 46% of patients with mesenteric vascular disease and was more common in patients more than 70 years of age (43% vs. 12%, $P < 0.05$). In the massive resection group, 13 of 17 early deaths occurred in patients with mesenteric vascular disease. Multiple organ failure (n = 9), progressive intestinal ischemia (n = 5), myocardial infarction (n = 2), and intraoperative cardiac arrest (n = 1) caused these early deaths. One patient in the repeated resection group died of a postoperative pulmonary embolus.

Intestinal procedures to treat complications or improve intestinal function were performed in significantly more patients with repeated lesser resections (44% vs. 73%, $P < 0.05$). A similar proportion of these procedures were performed within 12 months of development of SBS in the two groups (10 [42%] of 24 and 5 [31%] of 16).

DISCUSSION

An increasing number of patients are surviving with SBS.[3,4] These patients usually require specialized nutritional support. They may also become candidates for other therapeutic approaches, such as growth factor therapy, and a variety of surgical procedures including intestinal transplantation.[5,6] The nutritional and overall outcomes of these patients are influenced primarily by intestinal remnant length and function.[6] However, certain patient characteristics, for example, age and underlying condition, status of other digestive organs, and nutritional management during the period of intestinal adaptation, are other important factors.

The present study confirms our previous observation that approximately three fourths of patients develop SBS after a single massive resection. These patients differ from those undergoing repeated resections with respect to age and underlying condition necessitating resection. Patients undergoing massive resection are more elderly and more likely to have mesenteric vascular disease. Both of these factors were associated with increased risk of early postoperative death. Not unexpectedly, the 30-day mortality rate was significantly higher in this group of patients. Thus the nature of resection may influence overall outcome in the SBS because of these patient-related factors.

The manner of the intestinal resection resulting in SBS may have other important prognostic implications. Although the 1- and 5-year survival of patients surviving the initial 30 days was similar in both groups, patients undergoing repeated resections have a better nutritional prognosis given similar intestinal remnant length. This is an interesting observation because these patients were more likely to have inflammatory conditions, for example, Crohn's disease and irradiation, which might impair remnant function. Furthermore, the status of the other digestive organs was similar in the two groups. However, patients undergoing repeated resections were more likely to undergo further intestinal procedures to treat complications or improve intestinal absorption, which might account for the improved nutritional outcome.

This improved nutritional prognosis may be related in part to enhanced intestinal adaptation after repeated lesser resections. The intestinal adaptive response is primarily influenced by the extent of resection.[7] Wathen et al.[2] found, in rats, that two resections separated in time stimulated a greater proliferative response in the intestinal remnant than a single resection of similar total magnitude. The relative size of the two resections was not important, that is, 30% then 50% vs. 50% then 30%, did not influence this response. However, these resections were performed

within 7 days of each other. Patients in the present study had resections of variable lengths performed often years apart, and thus the response might be different. Furthermore, we can only speculate about possible improved adaptation since it was not directly evaluated in the present study. The reduced need for parenteral nutrition in patients undergoing repeated resection when the remnant is less than 60 cm in length suggests that differences in adaptation may be functional as well as structural.

Ostomies were created in 60% of patients in the total group with a similar percentage after massive and repeated resection (63% vs. 52%). Ward et al.[8] also formed ostomies in the majority of their patients undergoing resection for ischemia. The major issues in this setting are intestinal viability and the need to complete the operation expeditiously. When ostomies are used less frequently, anastomotic leakage and fistula frequently occur.[9] Patients with repeated lesser resection had stomas formed because of inflammation and sepsis.

Overall, patients undergoing repeated lesser resections leading to SBS have a better prognosis than those undergoing a single massive resection. Patient-related factors make a major contribution to this difference in outcome. However, experimental data support a possible difference in the adaptive response depending on the manner of resection. The improved nutritional outcome in patients undergoing repeated lesser resections suggests that this factor should be considered when deciding on treatment strategies.

REFERENCES

1. Blatchford GJ, Thompson JS, Rikkers LF. Intestinal resection in adults: Causes and consequences. Dig Surg 1989;6:57-61.
2. Wathen LM, Osborne JW, Loren DP. Effect of two intestinal resections separated by time in cell proliferation in the rat small intestine. Gastroenterology 1981;80:1535-1541.
3. Messing B, Lemann M, Landais P, et al. Prognosis of patients with nonmalignant chronic intestinal failure receiving long term home parenteral nutrition. Gastroenterology 1995;108: 1005-1010.
4. Howard L, Ament M, Fleming CR, et al. Current use and clinical outcome of home parenteral and enteral nutritional therapies in the United States. Gastroenterology 1995;109:355-365.
5. Wilmore DW, Byrne JA, Persinger RL. Short bowel syndrome: New therapeutic approaches. Curr Probl Surg 1997;34:389-444.
6. Thompson JS, Langnas AN, Pinch LW, et al. Surgical approach to short bowel syndrome. Experience in a population of 160 patients. Ann Surg 1995;222:600-607.
7. Hanson WR, Osborne JW, Sharp JG. Compensation by the residual intestine after intestinal resection in the rat. I. Influence of amount of tissue removed. Gastroenterology 1977; 72:692-700.
8. Ward D, Vernava AM, Kaminski DL, et al. Improved survival by identification of high risk non-occlusive mesenteric ischemia, aggressive re-exploration and delayed anastomosis. Am J Surg 1995;170:577-581.
9. Sitges-Serra A, Mas X, Roqueta F, et al. Mesenteric infarction: An analysis of 83 patients with prognostic studies in 44 patients undergoing a small bowel resection. Br J Surg 1988;75:544-548.

Role of Laparoscopy in the Initial Multimodality Management of Patients With Near-Obstructing Rectal Cancer

Jonathan B. Koea, M.D., Jose G. Guillem, M.D., Kevin C. Conlon, M.D., Bruce Minsky, M.D., Leonard Saltz, M.D., Alfred Cohen, M.D.

The purpose of this study was to investigate the role of diagnostic laparoscopy in the multimodality management of locally advanced, near-obstructing rectal cancer. Fourteen patients with near-obstructing adenocarcinoma of the rectum (8 men and 6 women; mean age 49 years) underwent staging laparoscopy and formation of a sigmoid loop colostomy (n = 7), transverse colostomy (n = 4), or ileostomy (n = 3). The mean operative time was 78 minutes (range 67 to 94 minutes). All patients began a regular diet on postoperative day 1 and the median time to discharge was 4 days (range 2 to 8 days). Four patients were found to have diffuse peritoneal carcinomatosis not defined on preoperative CT scan. These patients died of disease within 6 months. Ten patients with advanced, localized pelvic disease began preoperative combined-modality treatment (5040 cGy external-beam radiation therapy in conjunction with 5-fluorouracil/leucovorin) between 8 and 13 days (median 9 days) following laparoscopy, and all underwent successful resection with clear margins in a median time of 12 weeks following laparoscopy. In the initial management of patients with near-obstructing advanced rectal cancer, laparoscopy can be both therapeutic and diagnostic by clarifying the site of the primary tumor, identifying patients with unsuspected peritoneal disease, and facilitating the formation of a defunctioning stoma with minimal morbidity. This leads to the early commencement of preoperative combined-modality treatment and does not compromise the prospects of subsequent tumor resection. (J GASTROINTEST SURG 2000;4:105-108.)

KEY WORDS: Laparoscopy, rectal cancer, multimodality therapy

Five percent of patients with rectal cancer initially present with near or incomplete obstruction.[1,2] These patients tend to present at a more advanced stage (usually stage T3 or T4), have a high proportion (up to 27%) of liver or peritoneal metastases,[1] and have a worse survival rate than those with nonobstructing lesions of comparable stage.[3] We[4] and others[5] have shown that preoperative combined-modality treatment (CMT; 5040 cGy external-beam radiation therapy combined with 5-fluorouracil and leucovorin) of T3 and T4 rectal cancers is associated with improved rates of sphincter-preserving surgery, reduced local failure rates, and improved survival. However, patients who present with near-obstructing T3 or T4 rectal cancer represent a difficult clinical problem. These patients clearly benefit from preoperative CMT, but the edema resulting from pelvic radiation can convert an impending obstruction to a total obstruction, and it is recommended that a diverting stoma be used in lesions where the lumen is less than 1 cm in diameter (Minsky BD, personal communication). Furthermore, 1 to 2 weeks are required following the start of preoperative CMT for the tumor to respond. It is therefore prudent to divert the fecal stream in these patients with a proximal stoma before therapy is begun. However, laparotomy and formation of a transverse or sigmoid colostomy is associated with a mortality rate of 3% to 6%[6,7] and a prolonged

From the Colorectal Service and the Gastric and Mixed Tumor Service (K.C.C.), Departments of Surgery (J.B.K., J.G.G., and A.C.), Radiation Oncology (B.M.), and Medical Oncology (L.S.), Memorial Sloan-Kettering Cancer Center, New York, N.Y.
Supported by the Eru Pomare Fellowship from the Health Research Council of New Zealand.
Presented at the Fortieth Annual Meeting of The Society for Surgery of the Alimentary Tract, Orlando, Fla., May 16-19, 1999.
Reprint requests: Jose G. Guillem, M.D., Department of Surgery, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, N.Y. 10021. e-mail: Guillemj@mskcc.org

hospital stay ranging from 30 and 55 days.[6,8] This can result in significant morbidity and the delayed commencement of preoperative CMT.

The role of laparoscopy in cancer of the rectum remains controversial. Although it is clear that rectal tumors can be resected using a laparoscopic technique, concerns remain over the safety and oncologic wisdom of this approach, and currently it is recommended that laparoscopic colon resections be undertaken only as part of a clinical trial.[8] Similarly, the role of laparoscopic staging of rectal tumors is also unclear.[9] In almost all cases, staging of the primary lesion is based on clinical examination and endorectal ultrasound. The presence of hepatic metastases can be determined from the results of spiral CT or MRI scan. However, the presence of extrapelvic peritoneal disease, which precludes local pelvic radiation in most cases, is more difficult to define with current imaging studies. Recent experience with the staging of upper gastrointestinal cancer[10,11] suggests that diagnostic laparoscopy is the technique of choice for detecting peritoneal disease.

We report our findings in a small series of patients who presented to our institution with near-obstructing cancer of the rectum who were initially managed with diagnostic laparoscopy and formation of a stoma before preoperative CMT was begun. This technique resulted in more accurate staging of disease in that it identified patients with unsuspected peritoneal metastases and facilitated the formation of a defunctioning stoma with minimal morbidity. This resulted in early hospital discharge and early commencement of preoperative CMT.

PATIENTS AND TECHNIQUE

A series of 14 patients presenting to Memorial Sloan-Kettering Cancer Center with near-obstructing rectal cancer from 1996 to 1998 was reviewed. All patients were preoperatively staged with a spiral CT scan and clinical examination. Endorectal ultrasound could not be performed in any patient because of the size of the primary lesion and luminal narrowing. All patients presented with near-obstructing lesions defined as clinical stage T3 or T4, the presence of diarrhea, and luminal narrowing insufficient to permit the passage of a digit or sigmoidoscope but with the absence of colonic dilatation or air/fluid levels on plain abdominal x-ray films. All lesions were pathologically proved adenocarcinomas.

Laparoscopic examination was carried out with the patient in the supine position. Using an open technique, a 11.5 mm Hasson cannula was inserted into the abdominal cavity under direct vision at the umbilicus, and intra-abdominal examination was carried out using a 30-degree angled scope. A 10 mm port was placed in the right lower quadrant for purposes of peritoneal inspection and biopsy, and a 10 mm port was added in the left lower quadrant for retraction. Peritoneal adhesions were divided and a thorough systematic examination of the peritoneal cavity was carried out. In particular, the parietal peritoneum of the anterior abdominal wall and hemidiaphragms was inspected for metastatic disease as was the peritoneum over the small bowel mesentery and the omentum. All suspicious lesions were subjected to biopsy and frozen-section analysis.

Defunctioning stomas were created using either sigmoid or transverse colon or ileum. The sigmoid colon was mobilized by incising the white line of Toldt and developing the plain anterior to Gerota's fascia, gonadal vessels, and ureter until sufficient length was obtained to exteriorize the apex of the sigmoid loop through a left lower quadrant site at the 10 mm port site. This port site was then enlarged to accommodate the bowel. Similarly, the most distal loop of ileum was brought through the right abdominal wall at a site fashioned at the 10 mm port site. When the transverse colon was used, a small incision was made in the left upper quadrant and a loop of distal transverse colon exteriorized. End-loop colostomies[12] and loop ileostomies[13] were matured with interrupted absorbable sutures approximating mucosa and dermis.

At the end of the procedure, all remaining port sites were closed using absorbable sutures to close the underlying fascia and subcuticular sutures to the skin. All patients were fed and tolerated a light regular diet on postoperative day 1. In-house consultations were made by specialists in radiation and medical oncology to plan multimodality therapy in the postlaparoscopy period. All patients began CMT with 5040 cGy and 5-fluorouracil–based chemotherapy according to previously published techniques.[4] Definitive surgery was undertaken approximately 4 to 7 weeks following the completion of CMT.

RESULTS

The clinical data from the 14 patients are summarized in Table I. The average age was 49 years (median age 45 years), and eight patients were male and six were female. Four patients (Nos. 1, 2, 9, and 14) had their preoperative stage changed with laparoscopy when unsuspected peritoneal metastases were discovered, and these patients did not undergo any further surgical procedures and were managed with systemic chemotherapy alone. The average total operating time for the procedure was 89 minutes (from induction of general anesthesia to extubation) and all patients ate a light regular diet on postoperative day 1.

Table I. Summary of data from patients with near-obstructing rectal cancer managed with staging laparoscopy, defunctioning stoma, and multimodality therapy

Patient	Sex	Age (yr)	Prelaparoscopy stage	Postlaparoscopy stage	Stoma	Time (min)	Day of discharge	Day started CMT	Resection	Follow-up (mo)
1	M	33	T3NxM0	T3NxM1	Sig	104	4	8	No resection	DED10
2	M	64	T3NxM0	T3NxM1	Sig	110	5	8	No resection	DED12
3	M	54	T3NxM0	T3NxM0	Sig	100	7	9	LAR	NED36
4	F	61	T4NxM0	T4NxM0	Trans	90	5	10	LAR	NED30
5	M	45	T3NxM0	T3NxM0	Sig	87	3	10	LAR	NED34
6	F	47	T4NxM0	T4NxM0	Sig	82	5	12	LAR	NED31
7	F	35	T4NxM1	T4NxM1	Sig	93	4	7	LAR	DED6
8	M	56	T4NxM0	T4NxM0	Sig	98	4	9	LAR	DOD18
9	F	80	T4NxM0	T4NxM1	Trans	97	6	9	No resection	DOD1
10	M	59	T4NxM0	T4NxM0	Ileo	78	2	8	LAR	AWD12
11	F	70	T4NxM0	T4NxM0	Ileo	63	8	11	APR	NED13
12	M	28	T3NxM0	T3NxM0	Sig	79	4	12	LAR	NED12
13	M	39	T3NxM1	T3NxM1	Sig	86	5	9	LAR	AWD12
14	M	21	T3NxM0	T3NxM1	Sig	74	3	9	No resection	AWD12

Sig = loop sigmoid colostomy; Trans = loop transverse colostomy; Ileo = loop ileostomy; ERT = days post discharge on which preoperative CMT commenced; LAR = low anterior resection; APR = abdominoperineal resection.

Patients were discharged within a median of 4 days (range 2 to 8 days). There were no surgical complications resulting from laparoscopy. A loop sigmoid colostomy was formed in eight patients, two patients were managed with a loop transverse colostomy, and three patients had an ileostomy. The choice of stoma was dictated by the surgeon. However, in general, a sigmoid colostomy was used unless the sigmoid colon and mesentery were affected by diverticular disease. In these cases of fixed sigmoid colon and foreshortened sigmoid mesentery, a transverse colostomy or loop ileostomy was created. Ten patients began preoperative CMT therapy between 8 and 13 days (median 9 days) after hospital discharge. All 10 of these patients completed CMT, and all have undergone subsequent resection with histologically negative margins (9 low anterior resections and 1 abdominoperineal resection) between 4 and 7 weeks following the completion of radiation. Two patients (Nos. 10 and 13) have had recurrent disease in the liver and two patients (Nos. 7 and 8) have died as a result of widespread metastatic disease.

DISCUSSION

This small series of patients has demonstrated that staging and therapeutic laparoscopy is useful in the management of patients with advanced rectal cancer who present with impending obstruction. Although no complications resulted from the use of the laparoscope in our patients with partial obstructions, including visceral or vascular injuries, stoma retraction, or ischemia, we recommend that great caution be used in selecting patients and carrying out the procedure. Furthermore, in patients with an established obstruction, it is our opinion that laparoscopy is not advisable and these patients are better managed with an open operation because of the increased risk of visceral injury.

Initial management with the laparoscope was associated with a shorter hospital stay in comparison to published figures ranging from 10 to 55 days for patients managed with an open operation.[7,9] Experience at this institution suggests that most patients remain in the hospital for a week following open diversion and, more important, the commencement of multimodality therapy is delayed for several weeks while the patient recuperates. All of our patients returned home within 8 days and the majority within 6 days following laparoscopic diversion. All patients tolerated a light oral diet on postoperative day 1 and most of their hospital stay was focused on learning to care for the stoma and consulting with radiation and medical oncology services. Patients initially managed with the laparoscope were able to begin CMT between 1 and 2 weeks following hospital discharge, and all completed CMT with no complications. Nine of the 10 patients were managed with sphincter-preserving surgery consistent with our previous results.[4]

Diagnostic laparoscopy in our patients with locally advanced rectal cancer resulted in improved clinical staging in 4 of 14 patients in whom unsuspected peritoneal disease was discovered. This was not diagnosed on preoperative spiral CT scan and is consistent with the results of laparoscopic staging in upper gastroin-

testinal cancer.[10] Up to 25% of patients with locally advanced rectal cancer will have extrapelvic disease,[4] and accurate identification will help select those who will benefit most from radiation and chemotherapy directed against local and systemic disease. In comparison to minilaparotomy, diagnostic laparoscopy permits a more thorough and systematic examination of all parts of the abdominal cavity.

No differences in operative time (see Table I) or long-term morbidity were apparent between the different stomas used in this series. In 10 cases a loop sigmoid colostomy was fashioned as this is a technically straightforward and accepted method of fecal diversion in locally advanced rectal cancer.[1] Loop transverse colostomies were fashioned in two patients where the sigmoid mesentery was foreshortened and loop ileostomies were created in two patients in whom there were extensive right upper quadrant adhesions following surgery making mobilization of the transverse colon difficult.

This series demonstrates that diagnostic and therapeutic laparoscopy is useful in the initial management of patients with near-obstructing, locally advanced rectal cancer. We were able to more accurately stage patients with unsuspected peritoneal disease and enroll them in more appropriate trials of systemic chemotherapy. Furthermore, creation of a defunctioning stoma was accomplished in all cases with no complications, early resumption of oral intake, hospital discharge, and commencement of preoperative CMT. Given the importance of preoperative CMT in the management of locally advanced rectal cancer, initial staging and therapeutic laparoscopy should become a therapeutic option in the management of near-obstructing lesions.

REFERENCES

1. Phillips RK, Hittinger R, Fry JS, Fielding LP. Malignant large bowel obstruction. Br J Surg 1985;72:296-302.
2. Aldridge MC, Phillips RK, Hittinger R, Fry JS, Fielding LP. Influence of tumour site on presentation, management and subsequent outcome in large bowel cancer. Br J Surg 1986; 73:663-670.
3. Serpell JW, McDermott FT, Katrivessis H, Hughes ESR. Obstructing carcinomas of the colon. Br J Surg 1989;76:965-969.
4. Grann A, Minsky BD, Cohen A, Saltz L, Guillem J, Paty PB, et al. Preliminary results of preoperative 5-fluorouracil, low-dose leucovorin and concurrent radiation therapy for clinically resectable T3 rectal cancer. Dis Colon Rectum 1997;40:515-522.
5. Gunderson LL, Nelson H, Martensen JA, Cha S, Haddock M, Devine R, Fieck JM, Wolfe B, Dozios R, O'Connell MJ. Locally advanced primary colorectal cancer: Intraoperative and external beam radiation ± 5-FU. Int J Radiat Oncol Biol Phys 1997;37:601-614.
6. Sjodahl R, Franzen T, Nystrom PO. Primary versus staged resection for acute obstructing colorectal carcinoma. Br J Surg 1992;79:685-688.
7. Gutman M, Kaplan O, Skornick Y, Greif F, Kahn P, Rozin RR. Proximal colostomy: Still an effective emergency measure in obstructing carcinoma of the large bowel. J Surg Oncol 1989;41:210-212.
8. Milsom JW, Seon-Hahn K. Laparoscopic versus open surgery for colorectal carcinoma. World J Surg 1997;21:702-705.
9. Northover JM. Staging and management of colorectal cancer. World J Surg 1997;21:672-677.
10. Burke EC, Karpeh MS, Conlon KC, Brennan MF. Laparoscopy in the management of gastric adenocarcinoma. Ann Surg 1997;225:262-267.
11. Conlon KC, Dougherty E, Klimstra DG, Coit DG, Turnbull ADM, Brennan MF. The value of minimal access surgery in the staging of patients with potentially resectable peripancreatic malignancy. Ann Surg 1996;223:134-140.
12. Prasad ML, Pearl RK, Abcarian H. End-loop colostomy. Surg Gynecol Obstet 1984;158:380-382.
13. Veidenheimer MC. Loop ileostomy. In Braasch C, Sedgwick K, Veidenheimer M, Ellis H, eds. Atlas of Abdominal Surgery. Philadelphia: WB Saunders, 1991, pp 109-111.

MDM2/p53 Protein Expression in the Development of Colorectal Adenocarcinoma

Ghada Abdel-Fattah, M.D., Ph.D., Boris Yoffe, M.D., Bhuvaneswari Krishnan, M.D., Vladimir Khaoustov, M.D., Ph.D., Kamal Itani, M.D.

The murine double minutes 2 (MDM2) oncoprotein inhibits p53-mediated tumor suppression. MDM2 has been shown to be overexpressed in sarcomas and more recently was implicated in the pathogenesis of carcinomas. The purpose of this study was to determine the expression pattern of MDM2 in adenomas and colorectal adenocarcinomas and decide whether there is a correlation between MDM2 and p53 protein status. Paraffin-embedded tissues from 52 colorectal cancer (CRC) specimens and their adjacent normal tissue (N-CRC) were studied. In addition, 56 sporadic adenomas were investigated for the immunohistochemical expression of MDM2 and p53 proteins. Immunoreactivity of p53 indicating p53 gene mutation (p53+) was significantly higher in CRC (44%) compared to adenomas (23.2%) ($P < 0.01$). None of the N-CRC specimens expressed the immunoreactive p53 protein. MDM2 overexpression (MDM2+) was similar in adenomas (30.3%) and CRC (25%), but only 2 (3.8%) of 52 N-CRC specimens showed overexpression of MDM2. In most cases MDM2 expression was associated with negative p53 expression (wild-type p53) in both adenomas ($r = 0.59$, $P < 0.001$) and CRC ($r = 0.69$, $P < 0.0001$). No correlation was found between MDM2, p53 expression, and either the histologic grade, nodal stage or morphology of the tumors. There is greater p53 mutation in CRC compared to adenomas and N-CRC. The data indicate that MDM2 is overexpressed in CRC and is significantly associated with wild-type p53 compared to N-CRC specimens from the same patient. The MDM2 expression pattern is similar in adenomas and CRC, which may suggest that MDM2 overexpression is an early event in the progression of CRC. (J GASTROINTEST SURG 2000;4:109-114.)

KEY WORDS: *mdm2* oncogene, p53, colorectal cancer

Colorectal cancer (CRC) is the second leading cause of cancer related-death in the United States. It accounts for 14% of all cancer-related deaths in both males and females,[3,4] with 134,000 newly diagnosed cases per year.[5] CRC is a disease of multiple genetic alterations and is further classified as hereditary, familial, or sporadic colon cancer. Although germ line gene mutations are more common in inherited forms of CRC, somatic genetic alterations are more frequently seen in sporadic cancer. Furthermore, in the defined genetic model of the adenoma-carcinoma sequence of progression to CRC,[6] the precise molecular mechanisms by which these alterations occur are not yet clear. In this study we investigated sporadic colon cancer. In this category, p53 gene alterations are encountered in more than 50% of the tumors. The p53 alterations could occur either as a result of gene mutations or they could be functionally inactivated by interaction with cellular and viral proteins.[7] One of the cellular proteins is an oncoprotein known as murine double minutes 2 (MDM2), which binds to the transactivation domain of p53 via its p53-binding motif located on the N-terminal domain. This protein-protein interaction leads to a complex formation and inhibition of the p53 tumor suppressor functions. The *mdm2* gene was first cloned as a gene amplified in the form of double minutes in a spontaneously transformed derivative of mouse tumorigenic cell line.[8]

The gene encoding the MDM2 protein is located on chromosome 12q13-14, in close proximity to the

From the DeBakey Department of Surgery (G.A.-F. and K.I.), Departments of Medicine (B.Y. and V.K.) and Pathology (B.K.), Houston VA Medical Center and Baylor College of Medicine, Houston, Tex.
Supported by a grant from The Methodist Hospital Foundation, Houston, Tex.
Presented at the Fortieth Annual Meeting of The Society for Surgery of the Alimentary Tract, Orlando, Fla., May 16-19, 1999.
Reprint requests: Kamal Itani, M.D., Department of Surgery, VAMC-112A, Houston, TX 77030. e-mail: kitani@bcm.tmc.edu

cell cycle regulatory protein cyclin-dependent kinase 4 (CDK4). MDM2 has been shown to be amplified or overexpressed in a variety of sarcomas and carcinomas but has not been fully investigated in CRC.[9,10] Therefore, in this study, we investigated sporadic CRC and hypothesized that in CRC, MDM2 inhibits p53-mediated growth arrest and apoptosis. This leads to uncontrolled proliferation and malignant transformation. The aim of this study was to investigate the expression pattern of MDM2 and p53 proteins, and to determine whether MDM2 protein is overexpressed in patients diagnosed with adenoma or CRC and their correlation to the clinical and pathologic characteristics of these patients. Our second objective was to determine the correlation between MDM2 overexpression and wild-type p53.

MATERIAL AND METHODS

Between 1994 and 1995, fifty-two CRC specimens and 56 adenomas that were formalin fixed and embedded in paraffin were retrieved from the pathology department at the Houston Veterans Administration Medical Center. The protocols were approved by the institutional human research review board. The morphology, histology, and differentiation for each specimen were reviewed, and the stage of disease in all patients with CRC was determined by reviewing the medical records. The control normal tissue was selected from the distal free margin of the normal colonic epithelium adjacent to the CRC (N-CRC). The N-CRC was embedded in a separate cassette. Sections 4 μm thick were prepared and deparaffinized with xylene three times (3 minutes for each wash) and dehydrated using several graded alcohol concentrations, and phosphate-buffered saline rinses.[11] Antigen retrieval was performed by microwave heating of the preheated 0.1 mol/L sodium citrate buffer for 5 minutes. After blocking the nonspecific binding with normal goat serum, specimens were incubated for 30 minutes at room temperature with mouse monoclonal antibody (p53, clone BP53-12-1, Biogenex, San Ramon, Calif.) or for 2 hours at 37° C with mouse monoclonal antibody MDM2 raised against the N-terminal domain (Clone IF2, Oncogene, Cambridge, Mass.), at a dilution of 2:100 for p53 and 1:10 for MDM2. The specimens were then incubated with biotinylated antimouse immunoglobulin (IgG), alkaline phosphatase–conjugated streptavidin, and a chromogenic substrate solution (naphthol phosphate substrate and fast red chromogen) according to the manufacturer's protocols.[9] Positive staining for MDM2 protein and p53 was of the intranuclear type. Assessment of the protein status was done by counting the cells under a light microscope (high-power resolution 100 to 400×) to determine the percentage of nuclear staining. Any specimen showing more than 5% staining for either p53 or MDM2 was considered positive for that stain. In cases of questionable positive results due to intensity or counts, the test was repeated. If doubt persisted, the specimens were considered to be negative for that stain. The expression of both proteins was correlated to the clinical characteristics of each patient and the pathologic features of the tumor.

The chi-square test was used to determine differences in staining between N-CRC, CRC, and adenomas. Linear regression analysis was used to determine the correlation between MDM2 and p53 expression. Correlation between clinical status, pathologic status, and MDM2 and p53 staining was determined by means of Fisher's exact test and chi-square tests. Significance for all statistical tests was set at $P < 0.05$.

RESULTS

Among all of the normal colonic tissues adjacent to the tumors (N-CRC), only two N-CRC specimens (3.8%) were positive for MDM2 (Table I). The rest of the N-CRC specimens stained negative for MDM2 (Fig. 1, *A*).

Among the 56 adenoma specimens investigated, 17 (30%) stained positive for MDM2 protein (Table I). A representative example of positive staining for MDM2 in adenomas is shown in Fig. 1, *B*. The rest of the 39 adenoma specimens were negative for MDM2 expression.

Among the 52 CRC specimens investigated, 13 (25%) stained positive for MDM2 protein (see Table I). An example of positive staining for MDM2 in CRC is shown in Fig. 1, *C*.

None of the normal colonic tissues adjacent to the tumor expressed p53 protein (Fig. 1, *D*). Among the 56 adenoma specimens investigated, 13 (23.2%) stained positive for p53 protein (see Table I). A representative example of positive staining for p53 in adenomas is shown in Fig. 1, *E*. The rest of the 39 adenoma specimens were negative for p53 expression, indicating wild-type 53 in these specimens, which cannot be visualized by immunohistochemical analysis.

Among the 52 CRC specimens investigated, 23 (44%) stained positive for p53 protein (see Table I). An example of positive staining for p53 in CRC is shown in Fig. 1, *F*. The positive expression of p53 protein in CRC (44%; see Table I) was significantly higher compared to that in adenomas (23.2%; see Table I). However, the positive expression of MDM2 protein was similar in adenomas (30%) and CRC (25%) (see Table I). The correlation between the expression of MDM2 and p53 in adenomas showed a weak but significant association between MDM2 overexpression and negative p53 expression in adenomas (Fig. 2, *A*) ($r = 0.59$, $P < 0.001$). The correlation

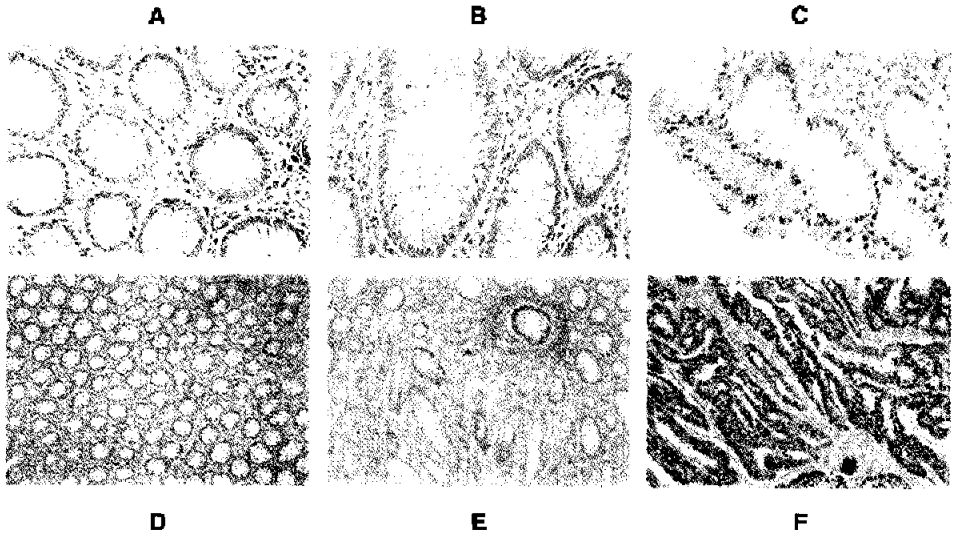


Fig. 1. Light microscopic appearance of MDM2- and p53-stained colonic tissue. **A,** Negative immunohistochemical expression of MDM2 protein in N-CRC (×400). **B,** Immunohistochemical expression of MDM2 protein in colorectal adenoma showing red nuclear localization within the tumor (×400). **C,** Immunohistochemical expression of MDM2 protein in CRC showing red nuclear localization (×400). **D,** Negative immunohistochemical staining for p53 protein in N-CRC (×100). **E,** Immunohistochemical expression of p53 protein in adenoma showing positive red nuclear staining of p53 protein (×100). **F,** Immunohistochemical expression of p53 protein in CRC showing red nuclear staining of the tumor (×100).

Table I. MDM2 and p53 expression in colonic tissue as determined by immunohistochemistry

IHC	Adenoma (n = 56)	N-CRC (n = 52)	CRC (n = 52)
MDM2+	17 (30%)	2 (3.8%)*	13 (25%)
p53+	13 (23.2%)	0 (0%)*	23 (44%)†

IHC = immunohistochemistry; CRC = colorectal cancer; N-CRC = normal colonic epithelium adjacent to the CRC.
*P <0.05 N-CRC vs. adenoma and CRC (chi-square test).
†P <0.01 CRC vs. adenoma and N-CRC (chi-square test).

between the expression of MDM2 and p53 in CRC showed a weak but significant association between MDM2 overexpression and negative p53 expression in CRC ($r = 0.69$, P <0.001), indicating that MDM2 overexpression is only partially associated with wild-type p53 (Fig. 2, *B*).

No correlation was found between MDM2, p53 expression and either the histologic grade, Dukes' stage, or morphology of the tumors (Table II).

DISCUSSION

The oncogenic role of MDM2 protein was previously demonstrated in several tumors. The *mdm2* gene, located on chromosome 12q13-14, was found to be amplified in human sarcomas,[15] chronic lymphocytic leukemia,[15] soft tissue tumors,[16] and gliomas.[14] In addition, MDM2 protein was shown to be overexpressed in breast carcinomas.[17] Both *mdm2* gene amplification and protein overexpression are important events in tumor progression.[16,17] MDM2 has been described as an inhibitor of p53 function[18] and more recently was implicated in promoting p53 degradation.[19]

MDM2 protein expression was significantly elevated in colorectal adenomas and CRC compared to normal colonic mucosa adjacent to the tumor (see Table I). However, the positive expression of MDM2 was similar in adenomas and adenocarcinomas suggesting that MDM2 overexpression is an early event during the progression from adenoma to CRC.

Under normal conditions, wild-type or normal p53 protein has a short half-life and is undetectable by immunohistochemical analysis,[7,20,21] as compared to most p53 mutations, which have prolonged half-lives and stain positively by immunohistochemistry.[7,20,22,23] In addition, MDM2, which is upregulated only by wild-type p53,[24] promotes the rapid degradation of

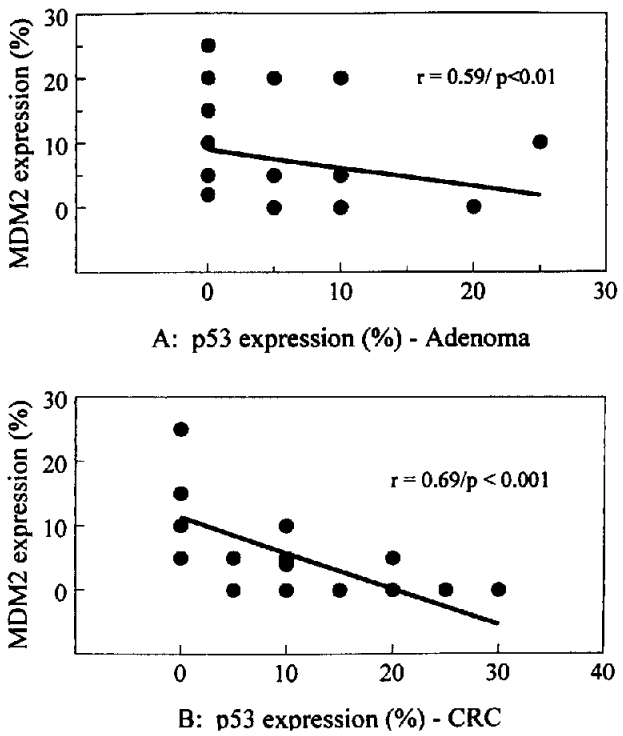


Fig. 2. Correlation between MDM2 overexpression and p53 status **(A)** in colorectal adenoma as determined by linear regression analysis ($r = 0.59$, $P < 0.001$) and **(B)** in CRC ($r = 0.69$, $P < 0.0001$).

Table II. Clinicopathologic characteristics in relation to MDM2 and p53 protein expression

	MDM2+	MDM2−	p53+	p53−
Adenoma				
Morphology				
Tubular	8	23	8	25
Villous	2	4	2	5
Tubulovillous	7	8	3	9
Colorectal adenocarcinoma				
Dukes' stage				
B	7	23	12	19
C	6	14	11	10
Differentiation				
Good	3	10	6	6
Moderate	5	18	10	12
Poor	5	9	7	8

None of these clinicopathologic characteristics reached statistical significance in relation to MDM2 and p53 protein expression ($P > 0.2$, Fisher's exact test and chi-square test).

that protein.[25] Therefore lack of staining of wild-type p53 can be due to either a short half-life or rapid degradation and can only be determined by additional in vitro studies. We found that mutated p53 protein accumulation was significantly higher in CRC compared to adenomas, which indicates that p53 mutation is a late event in the progression of the adenoma-carcinoma sequence.

The correlation between MDM2 expression and p53 status suggests that MDM2 overexpression is associated with the presence of wild-type p53 in adenomas (Fig. 2, *A*) and CRC (Fig. 2, *B*). However, the

r value of the slope of the regression line in both adenomas ($r = 0.59$, $P < 0.01$) and CRC ($r = 0.69$, $P < 0.001$) suggests that the correlation is only partial, which indicates that MDM2 overexpression is only partially accounted for by the presence of wild-type p53. This finding suggests that MDM2 oncoprotein has an additional effect on tumorigenesis, which is not accounted for by the interaction with wild-type p53. Interestingly, this result is in agreement with the current notion that MDM2 may serve as an oncogene by more than one mechanism.[19,26] The first mechanism described is the interaction with p53 protein via binding of MDM2 to the transactivation domain of p53.[18] The second mechanism is through p53-independent mechanisms possibly via upregulating of the cell cycle regulatory proteins.[27,28]

Several recent studies have shown that the p53-independent pathway can involve the interaction of MDM2 with CDK4,[29] retinoblastoma protein (pRB),[27,28] and the cyclin inhibitors p19 ARF[30,31] and p14.[32] Our results indicate that MDM2 overexpression is an early event during the transformation from adenoma to adenocarcinoma. This intriguing observation merits further investigation to characterize the molecular mechanisms involved in the progression of CRC.

CONCLUSION

Our results suggest that MDM2 protein is overexpressed in colorectal adenomas and adenocarcinomas compared to the normal colonic mucosa adjacent to the tumors. Our data suggest that MDM2 is correlated to wild-type p53. In addition, the MDM2 overexpression pattern was similar in adenomas and CRC, which indicates that MDM2 overexpression is an early event during the progression of colorectal cancer. p53 abnormal protein expression is higher in CRC compared to both adenomas and N-CRC.

REFERENCES

1. American Cancer Society: Cancer Facts and Figures, 1996. Atlanta: American Cancer Society (ACS), 1996. Publication No. 5008-96.
2. Greenberger NJ. Updates in gastroenterology. Ann Intern Med 1998;129:309-316.
3. Ransohoff DF. Colorectal cancer in digestive disease in the United States: Epidemiology and impact. In Everhart JE, ed. 1994, NIH Publication No. 94-1447 pp 207-223.
4. Boring CC, Squire TS, Tong T, Montgomery S. Cancer Statistics, 1994. CA Cancer J Clin 1994;44:7-26.
5. Agency for Health Care Policy and Research (AHCPR). Research Activities 1997;200:15-18.
6. Fearon ER, Vogelstein B. A genetic model of colorectal tumorigenesis. Cell 1990;61:759-767.
7. Wang XW, Harris CC. p53 tumor-suppressor gene: Clues to molecular carcinogenesis. J Cell Physiol 1997;173:247-255.
8. Cahilly-Snyder L, Yang-Feng T, Franke U, George DL. Molecular analysis and chromosomal mapping of amplified genes isolated from transformed mouse 3T3 cell line. Somat Cell Mol Genet 1987;13:235-244.
9. Valassiadou KE, Stefanaki K, Tzardi M, Datseris G, Georgoulias V, Mellissas J, Tsiftsis DD, Delides G, Kana Varos P. Immunohistochemical expression of p53, mdm2, and wafl/p21 proteins in colorectal adenocarcinomas. Anticancer Res 1997;17:2571-2576.
10. Hao XP, Gunther T, Roessner A, Price AB, Talbott IC: Expression of mdm2 and p53 in epithelial neoplasms of the colorectum. Mol Pathol 1998;51:26-29.
11. Genta RM, Bleyzer I, Cate TR, Tandon AK, Yoffe B. In situ hybridization and immunohistochemical analysis of cytomegalovirus-associated ileal perforation. Gastroenterology 1993;104:1822-1827.
12. Oliner JD, Kinzler KW, Melzer PS, George D, Vogelstein B. Amplification of gene encoding a p53 associated protein in human sarcomas. Nature 1992;358:80-83.
13. Merup M, Juliusson G, Xu XH, Jasson M, Stellan B, Rasool O, Roijer E, Stenman G, Gahrton G, Einhorn S. Amplification of multiple regions of chromosome 12, including 12q13-15, in chronic lymphocytic leukemia. Eur J Hematol 1997;58:174-180.
14. Nilbert M, Rydholm A, Millen H, Mitelman F, Mandahl N. MDM2 gene amplification correlates with ring chromosomes in soft tissue tumors. Genes Chromosomes Cancer 1994;9:261-265.
15. Ladanyi M, Lewis R, Rhanwar S, Gerald W, Huvos A, Healey J. MDM2 and CDK4 gene amplification in Ewing's sarcoma. J Pathol 1995;53:16-18.
16. Nakayama T, Toguchida J, Wadayama B, Kanoe H, Kotoura Y, Sasaki M. MDM2 gene amplification in bone and soft-tissue tumors: Association with tumor progression in differentiated adipose-tissue tumors. Int J Cancer 1995;64:342-346.
17. Giannikaki E, Kouvidou C, Tzardi M, Stefanaki K, Koutsoubi K, Gregoriou M, Zois E, Kakolyris S. p53 protein expression in breast carcinoma. Cooperative study with wild type p53 induced proteins MDM2 and p21/WAF1. Anticancer Res 1997;17:2123-2127.
18. Momand J, Zambetti GP, Olson DC, George D, Levine AL. The *mdm2* oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell 1992;69:1237-1245.
19. Piette J, Neel H, Marechal V. Mdm2: Keeping p53 under control. Oncogene 1997;15:1001-1010.
20. Higashyama M, Doi O, Ykokouchi H, Kasugal T, Ishiguro S, Takami K, Nakayama T, Nishisho I. MDM2 gene amplification and expression in non-small cell lung cancer: Immunohistochemical expression of its protein is a favorable prognostic marker in patients without p53 accumulation. Br J Cancer 1997;75:1302-1308.
21. Drager BJ, Kushima M, Goebell P, Jax TW, Gerharz CD, Bultel H, Schulz WA, Ebert T, Ackermann R. P53 and MDM2 in the development and progression of bladder cancer. Eur Urol 1997;32:487-493.
22. Barnes DM, Hanby AM, Gillett CE, Mohamed S, Hogson S, Bobrow LG, Leigh IM, Purkis T, MacGeoch C, Spurr NK, Bartek J, Vojtesek B, Picksley SM, Lane DP. Abnormal expression of wild type p53 protein in normal cells of cancer family patient. Lancet 1992;340:259-263.

23. Grimm M-O, Jurgens B, Schulz WA, Makri D, Schmitz-Drager BJ. Inactivation of tumor suppressor genes and deregulation of the c-myc gene in urothelial cancer cell lines. Urol Res 1995;23:293-300.
24. Haupt Y, Maya R, Kazaz A, Oren M. Mdm2 promotes rapid degradation of p53. Nature 1997;387:296-299.
25. Barak Y, Juven T, Haffner R, Oren M. MDM2 expression is induced by wild type p53 activity. EMBO J 1993;12:461-468.
26. Juven T, Barak Y, Zauberman A, George D, Oren M. Wild type p53 can mediate sequence specific transactivation of an internal promoter within the *mdm2* gene. Oncogene 1993;8: 3411-3416.
27. Khatib Z, Matsushime H, Valentine M, Shapiro D, Sherr C, Look A. Coamplification of the CDK4 gene with MDM2 and GL1 in human sarcomas. Cancer Res 1993;53:5535-5541.
28. DeCaprio JA, Furukawa YK, Ajchenbaum F, Griffin JF, Livingstone DM. The retinoblastoma susceptibility gene produce becomes phosphorylated in multistages during cell cycle entry and progression. Proc Natl Acad Sci 1992;89: 1795-1798.
29. Zhang Y, Xiong Y, Yarbrough Y. ARF-promotes MDM2 degradation and stabilizes p53: ARF-locus deletion impairs both RB and p53 tumor suppressor pathways. Cell 1998;92: 725-734.
30. Palmero I, Pantoja C, Serrano M. $P19^{ARF}$ links the tumour suppressor p53 to Ras. Nature 1998;395:125-126.
31. Pomerantz J, Schreiber-Agus N, Liegeois NJ, Silverman A, Alland L, Chin L, Potes J, Chen K, Orlow I, Lee HW, Cordon-Cardo C, DePinho RA. The INK4a tumor suppressor gene product, p19 Arf, interacts with MDM2 and neutralizes MDM2's inhibition of p53. Cell 1998; 92:713-723.
32. Bates S, Phillips AC, Clark P, Stott F, Peters G, Ludwig R, Vousden K. $P14^{ARF}$ links the tumour suppressors RB and p53. Nature 1998;395:124-125.