Standard vs. Radical Pancreaticoduodenectomy for Periampullary Adenocarcinoma: A Prospective, Randomized Trial Evaluating Quality of Life in Pancreaticoduodenectomy Survivors

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This study was designed to assess the health-related quality of life (QOL) of patients who had been randomly assigned to either standard or radical pancreaticoduodenectomy for periampullary adenocarcinoma. Pancreaticoduodenectomy has been performed in increasing numbers for periampullary adenocarcinoma. The appropriate extent of resection (standard vs. radical [extended]) remains controversial, particularly as concerns survival benefit. Past reports comparing standard vs. radical resection have suggested that the more extensive resection is attended by negative functional outcomes (diarrhea and weight loss) and poorer QOL, diminishing the impact of any possible survival advantage of the radical resection. A prospective, randomized single-institution trial comparing standard pancreaticoduodenectomy (pylorus preservation preferred) to radical pancreaticoduodenectomy (including distal gastrectomy and retroperitoneal lymphadenectomy) evaluated 299 patients with periampullary adenocarcinoma between April 1996 and June 2001. A standard Functional Assessment of Cancer Therapy-Hepatobiliary (FACT-Hep) QOL survey designed for hepatobiliary cancer was sent to 150 of these patients surviving pancreaticoduodenectomy. QOL and functional status were assessed via a series of subscale scores for physical, social, emotional, and functional well-being. A total of 105 QOL surveys (70%) were returned and analyzed, with 55 of the patients having been randomized to the standard group and 50 to the radical group. The patients were evaluated at a mean of 2.2 years after pancreaticoduodenectomy. The two groups were statistically similar with regard to multiple parameters including age at operation (64.6 years), race, intraoperative blood transfusions, pathologic diagnosis and staging, and perioperative complications. The radical group had a significantly higher percentage of men (66% vs. 44%; $P = 0.02$), a longer operative time (369 minutes vs. 327 minutes; $P < 0.001$), and a longer postoperative length of hospital stay (13.6 days vs. 10.1 days; $P < 0.01$). The FACT-Hep total QOL scores were similar between the standard and radical groups: 143.5 vs. 147.3, respectively. Additionally, the individual FACT-G subscale scores evaluating physical (22.1 vs. 23.3), social (24.5 vs. 24.4), emotional (19.2 vs. 19.6), and functional well-being (20.6 vs. 22.4) were comparable between the standard and radical groups. Subgroup analyses based on pathologic diagnosis (pancreatic, ampullary, distal bile duct, etc.) failed to reveal any differences in QOL assessment between the standard and radical pancreaticoduodenectomy groups. Finally, QOL measures were similar when comparing time since operation (<2 years' follow-up vs. >2 years' follow-up) and age (≤65 years vs. >65 years). This is the largest report comparing QOL assessment in survivors of pancreaticoduodenectomy randomized between standard and radical resection. These data demonstrate no differences in long-term QOL between standard and radical resection. These results imply that no negative long-term QOL measures are associated with radical pancreaticoduodenectomy (as performed in this study) for periampullary adenocarcinoma. (J GASTROINTEST SURG 2003;7:1–11.) © 2003 The Society for Surgery of the Alimentary Tract, Inc.

KEY WORDS: Pancreaticoduodenectomy, quality of life, periampullary adenocarcinoma

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (oral presentation).

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Supported in part by grants from the National Institutes of Health (R01-CA56130 and P50-CA62924).

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Published by Elsevier Science Inc.
1091-255X/03/$—see front matter
PII: S1091-255X(02)00187-7

The concept of health-related quality of life (QOL) has gained importance in the surgical literature.[1] Particularly as it relates to surgical patients, QOL seeks to measure the impact of a disease process and/or an operative procedure on the physical, psychological, and social aspects of an individual's life and sense of well-being.[2] This article will address QOL in patients with periampullary adenocarcinoma, specifically focusing on survivors of pancreaticoduodenectomy.

Worldwide, pancreaticoduodenectomy is being applied in increasing numbers to patients with adenocarcinoma originating in the pancreas or periampullary region.[3–5] One of the unresolved issues in the management of these patients concerns the extent of soft tissue and lymphatic clearance appropriate to achieve optimal survival.[6,7] Although some investigators have argued that extended resections can be performed safely, and are perhaps associated with improved survival,[8–10] the experience of others is that the more extensive operations (typically performed with retroperitoneal lymphadenectomy and circumferential clearance of the celiac and superior mesenteric arteries) can be associated with increased postoperative morbidity, without improved survival.[11–13] Furthermore, it is conceivable that any small survival benefit derived from extended resection may be offset by measurable reductions in patient functional outcome and QOL that may be associated with the more extensive procedure. Examples of such potential adverse findings after radical resection include postoperative diarrhea, weight loss, and nutritional disturbances, adverse findings that have been shown to be present in some patients after radical or extended resection.[14,15]

To date, several small studies of less than 50 patients[16–20] and one large study of 192 patients[21] have evaluated some aspect of QOL in pancreaticoduodenectomy survivors. In general, pancreaticoduodenectomy survivors without evidence of recurrence have been found to have near-normal QOL in multiple domains, with subtle variation from study to study. However, there has been no formal QOL assessment of patients with periampullary adenocarcinoma who have survived pancreaticoduodenectomy, comparing patients treated via standard or radical (extended) resection. This study was designed to assess the QOL of pancreaticoduodenectomy survivors who had been randomly assigned to either standard or radical resection for periampullary adenocarcinoma.

PATIENTS AND METHODS

This study was approved by the Joint Committee on Clinical Investigation of The Johns Hopkins University School of Medicine. Informed consent was obtained preoperatively from all participating patients. Two previous reports have detailed the recruitment, surgical technique, postoperative management, pathologic review, data collection, and statistical analyses used for these patients.[12,13] In brief, between April 1996 and June 2001, patients with presumed periampullary adenocarcinoma were recruited into this study prior to surgery, with the anticipation of performing a pancreaticoduodenectomy. Specific exclusion criteria included the following: (1) preoperative chemotherapy or chemoradiation therapy; (2) presence of gross tumor left behind at the conclusion of the standard pancreaticoduodenal resection; (3) pathology revealing tumor other than adenocarcinoma primary to the periampullary region; or (4) absence of informed consent. Consenting patients were randomized intraoperatively (n = 299), after completion of a standard, margin-negative pancreaticoduodenal resection, using a computer-generated random number pattern. The randomization was between two procedures: (1) standard pancreaticoduodenal resection or (2) radical (extended) pancreaticoduodenal resection. For patients randomized to standard resection, the resection was complete at randomization, because these patients had just undergone a standard resection. Patients randomized to radical (extended) resection underwent additional resection, as described under "Protocol."

Protocol

For the standard resection, preservation of the pylorus was preferred, and lymph node groups resected en bloc included the anterior pancreaticoduodenal lymph nodes, the posterior pancreaticoduodenal lymph nodes, nodes along the right lateral aspect of the superior mesenteric artery and vein, and nodes in the lower hepatoduodenal ligament. For the standard resection, if preservation of the pylorus could not be performed because of duodenal cuff ischemia or because of an inadequate local duodenal margin, then a distal gastrectomy varying from 10% to 40% was performed.

For the radical (extended) resection (Fig. 1), the standard resection was extended to include (1) a 30% to 40% distal gastrectomy and (2) a retroperitoneal lymph node dissection extending from the portal vein to below the third portion of the duodenum in the vertical axis, and from the right renal hilum to the left lateral border of the aorta in the horizontal axis.

In both the standard and radical operations, the uncinate process of the pancreas was removed from underneath the superior mesenteric vein, flush with

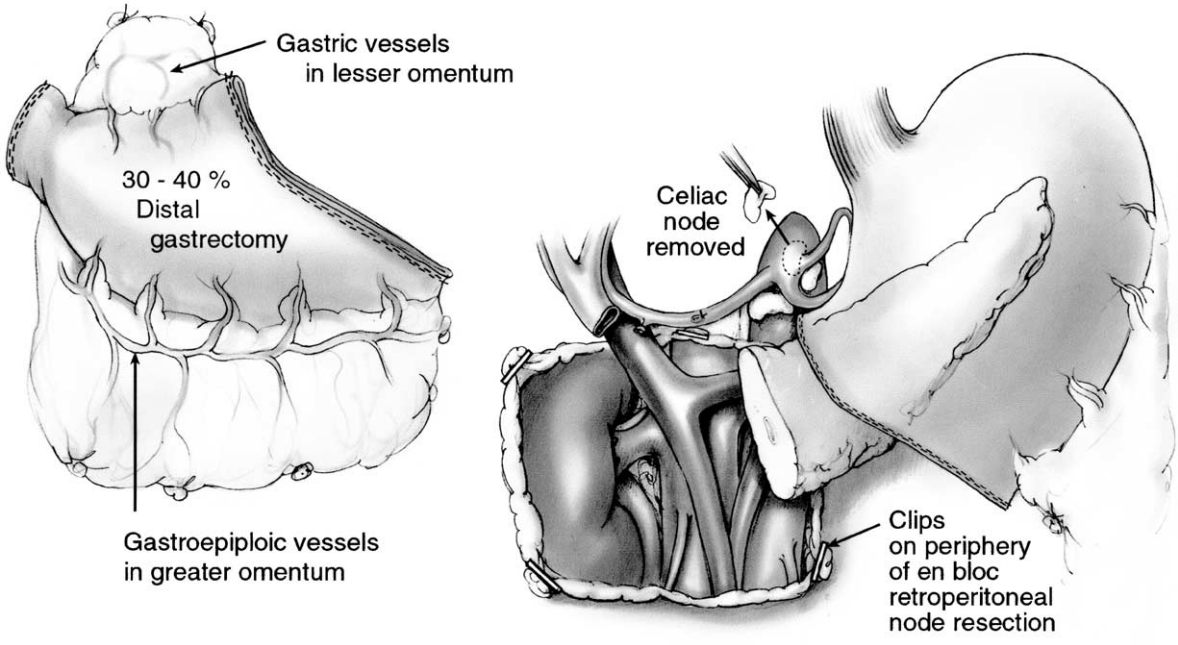

Fig. 1. Illustration depicting the components of the radical procedure. *Left*, The 30% to 40% distal gastrectomy specimen, which includes the pylorus and 1 to 2 cm cuff of the duodenum. *Right*, The retained stomach, pancreatic body and tail, and an overview of the retroperitoneal dissection. The retroperitoneum is dissected from the hilum of the right kidney to the left lateral border of the aorta in the horizontal axis. In the vertical axis, the dissection extends from the level of the portal vein to below the level of the third portion of the duodenum. Titanium clips have been placed to mark the extent of the retroperitoneal dissection. A celiac node is removed for histologic analysis. (From Yeo CJ, Cameron JL, Sohn TA, et al. Pancreaticoduodenectomy with or without extended retroperitoneal lymphadenectomy for periampullary adenocarcinoma: Comparison of morbidity and mortality and short-term outcome. Ann Surg 1999;229:613–624.)

the superior mesenteric artery. The pancreatic resections were performed favoring partial pancreatectomy, leaving in place the remnant pancreatic body and tail. Most reconstructions were performed to a single retrocolic jejunal limb, with a proximal pancreaticojejunostomy, downstream hepaticojejunostomy, and further downstream duodeno- or gastrojejunostomy.

Postoperative Management

Patients were managed using an institutionally generated critical pathway, with all patients receiving perioperative antibiotics for less than 24 hours and histamine H_2 receptor antagonists, and most receiving erythromycin lactobionate.[22] Drains left in the area of the pancreatic and bile duct anastomoses were usually removed between postoperative days 5 and 8. All patients were evaluated postoperatively by medical oncology and radiation oncology consultants, who discussed recommendations for adjuvant chemoradiation therapy.[23–25]

Pathologic Review

All pancreaticoduodenal specimens were reviewed to determine the site of the primary tumor, resected lymph node status, margin status, and overall pathologic staging.[26]

Quality-of-Life Assessment

A standard Functional Assessment of Cancer Therapy-Hepatobiliary QOL survey (FACT-Hep) designed for adults with hepatobiliary cancer was sent by mail to the 150 patients surviving pancreaticoduodenectomy. The remaining 149 patients had either died of disease or did not have accurate contact information. The FACT-Hep self-reporting scale is comprised of the FACT-G core (27 general items; version 4), designed for adults with various cancer diagnoses, combined with the FACT-Hep subscale, which includes 18 additional items specific for hepatobiliary cancer. The FACT-G explores the domains of physical well-being, social/family well-being, emotional well-being, and functional well-

being. The four domains are formulated in separate subscales that make up a series of 27 Likert-type items. Patients are asked to respond to each item on a scale of 0 to 4, with 0 meaning "not at all" and 4 meaning "very much." The possible range of scores is 0 to 108. The FACT-G has undergone appropriate testing of reliability and validity, has been compared to the 36-item Short-Form Health Survey (SF-36), and has been found to be a reliable, valid, and user-friendly instrument in the older adult population with cancer.[27,28]

The FACT-Hep 45-item questionnaire has recently been demonstrated to have high internal consistency, excellent test-retest validity, and evidence of convergent and divergent validity.[29] The FACT-Hep subscale uses a question structure similar to the FACT-G, yielding a possible range of scores of 0 to 72. Total FACT-Hep scores range from 0 to 180.

In addition, data concerning changes in patient body weight, pancreatic enzyme use, and stool patterns were assessed via separate questionnaires completed by 45 randomized patients immediately preoperatively and approximately 6 months postoperatively.

Study End Points

This report addresses postoperative QOL assessment and functional outcomes with the use of a subgroup of pancreaticoduodenectomy survivors. Multiple additional end points are evaluable from this study including postoperative survival, perioperative morbidity, and perioperative mortality. These end points have been addressed in two previous publications.[12,13]

Data Collection

Perioperative data were collected prospectively from all patients. QOL assessments were evaluated using FACT-Hep patient self-reported questionnaires, with the assessor being blinded to the allocation groups (i.e., standard vs. radical resection). Similarly, the separate questionnaires assessing body weight, enzyme use, and stool patterns were tabulated with the assessor being blinded to the allocation groups.

Statistical Analyses

Comparability of the standard and radical groups was verified using Student's paired and unpaired *t* tests and chi-square statistics. Results are reported as mean ± standard deviation. Significance was accepted at the 5% level.

RESULTS

The study population consisted of 105 patients who returned the QOL surveys, out of the 150 pancreaticoduodenectomy survivors to whom QOL surveys were sent (yield = 70%). Of the 105 patients who returned the QOL surveys, 55 had been randomized to the standard pancreaticoduodenectomy group, whereas 50 had been randomized to the radical pancreaticoduodenectomy group (including distal gastrectomy and retroperitoneal lymphadenectomy). The demographics of the patients are presented in Table 1. The mean patient age and racial distribution were similar in the standard and radical groups (mean age 64.6 years; 92% white). There were significantly more men in the radical group (66%) as compared to the standard group (44%; $P = 0.02$). The mean time from operation to completion of the QOL survey was 768 ± 509 days in the standard group (approximately 2.1 years), and was somewhat longer at 869 ± 548 days in the radical group (approximately 2.4 years; $P = 0.33$).

Table 1. Patient demographics

	Standard (n = 55)	Radical (n = 50)	*P* value
Age (yr)			
Mean	64.4 ± 10.5	64.8 ± 9.3	0.85
Sex			
Male	24 (44%)	33 (66%)	0.02
Female	31 (56%)	17 (34%)	
Race			
White	51 (93%)	46 (92%)	0.76
Black	2 (35%)	1 (2%)	
Other	2 (3.5%)	3 (6%)	
Mean time from operation to completion of QOL assessment (days)	768 ± 409	869 ± 548	0.33

The perioperative factors and pathologic findings are presented in Table 2. Eighty-seven percent of patients in the standard group underwent pylorus preservation, whereas all patients in the radical group were assigned to pylorus resection with distal gastrectomy ($P < 0.001$). The two groups were comparable with respect to intraoperative blood loss and units of red blood cells transfused. The mean operative time was significantly longer in the radical group (369 ± 57 minutes) as compared to the standard group (327 ± 47 minutes), because of the additional time needed to perform the distal gastrectomy and retroperitoneal lymphadenectomy ($P < 0.001$). The final pathologic findings (site of tumor origin) in the resected specimens did not differ significantly between the two groups. Nearly half of all patients had pancreatic adenocarcinoma, followed in descending order by ampullary adenocarcinoma, distal bile duct adenocarcinoma, duodenal adenocarcinoma, intraductal papillary mucinous neoplasm (IPMN) of the pancreas with invasive adenocarcinoma, and islet cell tumor. Seventy-four percent of patients had lymph node involvement in the resection specimen, representing stage III disease. Three percent of patients underwent vein resection as part of the pancreaticoduodenal resection.

Table 3 depicts the postoperative complications and hospital course in the 105 patients who survived their pancreaticoduodenectomies and completed the QOL survey. Several postoperative complications were observed significantly more frequently in the group of patients treated via radical resection, as compared to the group undergoing standard resection. These included pancreatic fistula (16% vs. 2%), wound infection (12% vs. 2%), and intra abdominal abscess (8% vs. 0%; all successfully drained percutaneously). The mean postoperative length of hospital stay was 10.1 days in the standard group and significantly longer (13.6 days) in the radical group ($P < 0.01$).

The overall QOL scores from the FACT-Hep scales are presented in Table 4. The highest possible scores for the FACT-G physical, social, emotional, and functional subscales (total 27 items) are 28, 28, 24, and 28, respectively, yielding a total highest possible FACT-G score of 108. The highest possible score for the 18-item FACT-Hep subscale is 72. By combining the FACT-G and FACT-Hep subscale scores, the highest possible score is 180. As can be seen in Table 4, there are no significant differences in the results of any subscale score, or the total FACT-Hep scores, when the standard and radical groups were compared.

Table 5 depicts the total FACT-Hep scores for various patient subgroups. The subgroups included different pathologic diagnoses (pancreatic, ampullary, or distal bile duct adenocarcinoma), age at operation (≤65 years vs. >65 years), sex, and length of time between the operation and QOL assessment (<2 years vs. >2 years). There were no significant differences in the FACT-Hep scores in any of these subgroups, when the standard and radical groups were compared. Although the differences did not achieve significance, there were trends that favored improved QOL scores in the radical group for women and for patients more than 2 years after operation.

Table 2. Perioperative factors and pathologic findings

	Standard (n = 55)	Radical (n = 50)	*P* value
Type of resection			
Pylorus-preserving	48 (87%)	0 (0%)	<0.001
Classic	7 (13%)*	50 (100%)	
Mean intraoperative blood loss (ml)	693 ± 382	802 ± 459	0.23
Mean red blood cells transfused intraoperatively (units)	0.50 ± 0.95	0.37 ± 0.71	0.48
Mean operative time (min)	327 ± 47	369 ± 57	<0.001
Site of tumor origin			
Pancreas	25 (45%)	23 (46%)	0.72
Ampullary	21 (38%)	10 (20%)	
Distal bile duct	5 (9%)	8 (16%)	
Duodenal	2 (4%)	7 (14%)	
IPMN with adenocarcinoma	2 (4%)	0 (0%)	
Islet cell	0 (0%)	2 (4%)	

IPMN = intraductal papillary mucinous neoplasm.

*These seven patients were randomized to standard resection but underwent distal gastrectomy because of duodenal cuff ischemia or tumor involvement of the proximal duodenum. These patients did not undergo retroperitoneal lymph node dissection. For statistical purposes, they are analyzed on an "intent-to-treat" basis, as having undergone standard resection.

Table 3. Postoperative complications and hospital course

	Standard (n = 55)	Radical (n = 50)	*P* value
Early delayed gastric emptying	4 (7%)	6 (12%)	0.40
Pancreatic fistula	1 (2%)	8 (16%)	<0.01
Wound infection	1 (2%)	6 (12%)	0.03
Bile leak	1 (2%)	4 (8%)	0.13
Intra-abdominal abscess	0 (0%)	4 (8%)	0.03
Cardiac event	3 (5%)	1 (2%)	0.43
Mean postoperative length of hospital stay (days)	10.1 ± 4.2	13.6 ± 8.2	<0.01

Table 6 shows the data from a subgroup of 45 patients who completed separate questionnaires immediately preoperatively and then 6 months postoperatively. The patients' self-reported weight fell significantly ($P < 0.001$) from 6 months before surgery until the time of surgery in both groups, and then again declined significantly at 6 months after surgery ($P < 0.001$). However, there were no differences when the standard and radical groups were compared. When assessing pancreatic enzyme usage and the number of bowel motions per day, both the use of enzymes and the number of bowel motions per day increased significantly postoperatively, but there were no differences when the standard and radical groups were compared.

DISCUSSION

Health-related QOL for patients surviving pancreaticoduodenectomy is an area fertile for investigation and analysis. With the increased use of pancreaticoduodenal resection in patients with malignant and benign periampullary disease, the number of patients who are alive and functional years after resection has increased. This study evaluated QOL and other functional outcomes in a group of patients who had periampullary adenocarcinoma and survived pancreaticoduodenectomy, who had been randomized to standard resection (pylorus-preserving pancreaticoduodenectomy) or radical resection (adding 30% to 40% distal gastrectomy and retroperitoneal lymphadenectomy). A separate report details the morbidity, mortality, and survival data from this randomized, controlled trial.[13] Briefly, patients undergoing radical resection were observed to have a significantly greater number of lymph nodes retrieved (28.5 vs. 17.0 standard; $P = 0.001$), as well as higher overall complication rates (43% vs. 29% standard; $P = 0.01$). At a mean follow-up of 24 months, there were no significant differences in 1-year, 3-year, 5-year, and median survival when the standard and radical groups were compared. The data from this study[13] suggest that the widespread use of extended resections for patients with pancreatic and periampullary adenocarcinoma will not be associated with improved long-term survival.

Table 4. Overall quality-of-life assessment

FACT-G scales* (Possible range)	Standard (n = 55)	Radical (n = 50)	*P* value
Physical well-being subscale (range 0–28)	22.1 ± 6.4	23.3 ± 5.8	0.32
Social/Family well-being subscale (range 0–28)	24.5 ± 3.8	24.4 ± 3.7	0.96
Emotional well-being subscale (range 0–24)	19.2 ± 5.0	19.6 ± 4.7	0.65
Functional well-being subscale (range 0–28)	20.6 ± 7.4	22.4 ± 6.0	0.19
Total FACT-G score (range 0–108)	86.4 ± 18.4	89.7 ± 15.4	0.32
FACT-Hep subscale† (range 0–72)	57.1 ± 10.9	57.6 ± 11.2	0.83
Total FACT-Hep score‡ (range 0–180)	143.5 ± 28.3	147.3 ± 22.5	0.45

No significant differences were noted when the standard and radical groups were compared.
*The highest possible scores for the FACT-G physical, social, and functional subscales are all 28. The highest possible score for the FACT-G emotional subscale is 24. This yields a highest possible total FACT-G score of 108.
†The highest possible score for the FACT-Hep subscale is 72.
‡The highest possible score for the total FACT-Hep score is 180.

Table 5. Total FACT-Hep Quality-of-Life score by major patient subgroups

Subgroup	Standard (n = 55)	Radical (n = 50)	*P* value
Pathology			
Pancreatic adenocarcinoma (n = 48)	143 ± 29 (25)	149 ± 24 (23)	0.24
Ampullary adenocarcinoma (n = 31)	147 ± 24 (21)	146 ± 22 (10)	0.87
Distal bile duct adenocarcinoma (n = 13)	152 ± 19 (5)	148 ± 28 (8)	0.81
Age at operation (yr)			
65 Years or under (n = 52)	144 ± 24 (29)	149 ± 23 (23)	0.71
Over 65 (n = 53)	138 ± 28 (26)	150 ± 27 (27)	0.12
Sex			
Male (n = 57)	145 ± 23 (27)	148 ± 28 (30)	0.60
Female (n = 48)	138 ± 28 (28)	152 ± 21 (20)	0.08
Time since operation			
Less than 2 years (n = 53)	139 ± 25 (31)	143 ± 30 (22)	0.66
More than 2 years (n = 52)	144 ± 27 (24)	155 ± 20 (28)	0.09

The highest possible score for the total FACT-Hep score is 180.
There are no significant differences when the standard and radical subgroups are compared.

Several previous studies have evaluated health-related QOL in pancreaticoduodenectomy survivors. McLeod et al.[16] published a cross-sectional survey comparing 25 pancreaticoduodenectomy patients to 25 age- and sex-matched cholecystectomy patients using six instruments. All six QOL instruments indicated near-normal well-being, with no significant differences between the two groups. Melvin et al.[18] assessed QOL using the Short Form-36 (SF-36) instrument in 45 patients who had undergone pancreaticoduodenectomy with either pylorus preservation or distal gastrectomy. When the eight domains of the SF-36 were analyzed, there were no differences in the mental health area, but there were significantly lower QOL scores in the group undergoing distal gastrectomy, as compared to the pylorus-preserved group and the age-matched control subjects. Wenger et al.[20] used the European Organization for Research and Treatment of Cancer QLQ-30 instrument to compare 48 patients with pancreatic or periampullary cancer, randomized to pancreaticoduodenectomy with either pylorus preservation (n = 24) or distal gastrectomy (n = 24). Patients were assessed preoperatively and then postoperatively at weeks 2, 6, 12, 24, 36, 48, and 60. Although there were no differences in global QOL, their results revealed a significantly better early (weeks 6 to 36) gastrointestinal QOL in patients undergoing pylorus preservation, with no differences evident beyond 48 weeks postoperatively. Finally, Huang et al.,[21] from our institu-

Table 6. Gastrointestinal outcomes: Weight, enzyme use, and bowel habits

	Standard (n = 17)	Radical (n = 27)	*P* value
Weight (pounds)			
6 mo before PD	178 ± 38	175 ± 35	0.77
Immediately before PD	164 ± 33	162 ± 33	0.85
6 mo after PD	146 ± 24	146 ± 28	0.99
Pancreatic enzyme use			
Immediately before PD	0%	4%	0.43
6 mo after PD	82%	85%	0.81
Number of bowel motions/day			
6 mo before PD	1.1 ± 0.5	1.1 ± 0.3	0.82
Immediately before PD	1.2 ± 0.5	1.3 ± 0.8	0.56
6 mo after PD	1.9 ± 1.0	2.3 ± 1.2	0.21

PD = pancreaticoduodenectomy.
*$P < 0.001$.
†$P < 0.02$.

tion, evaluated QOL in 192 pancreaticoduodenectomy survivors using a minor modification of the City of Hope Medical Center Quality-of-Life Survey.[30,31] Overall QOL scores in all three domains (physical, psychological, and social) were equivalent when pancreaticoduodenectomy survivors were compared to patients undergoing laparoscopic cholecystectomy and healthy control subjects. However, patients undergoing resections for pancreatic adenocarcinoma did have significantly lower scores in the physical and psychological domains (77% and 78%) as compared to laparoscopic cholecystectomy patients (83% and 82%; $P < 0.05$).

The current study used the FACT-Hep questionnaire, a 45-item self-report instrument designed to measure health-related QOL in patients with hepatobiliary cancer. Heffernan et al.[29] have recently shown that the FACT-Hep has excellent test-retest reliability, very high internal consistency, and appropriate convergent and divergent validity. The FACT-Hep instrument was chosen for this study because of its ease of self-administration, focus on assessment of patients with hepatobiliary cancer, prior validation, and availability. Because 55% of the pancreaticoduodenectomy survivors assessed in the current study did not have pancreatic adenocarcinoma, we did not feel compelled to use a pancreas-specific QOL instrument. Interestingly, the QOL scores that were reported by the pancreaticoduodenectomy survivors in this study (scores approximating 145) were comparable to the QOL scores obtained in the study by Heffernan et al.[29] (scores approximating 143). For all these reasons we believe that the choice of the FACT-Hep instrument was appropriate for this group of pancreaticoduodenectomy survivors.

The results of the current study, which involved 105 respondents, reveal no significant differences in QOL when survivors of standard vs. radical pancreaticoduodenal resection are compared. As with any survey that achieves less than a 100% return, we assume that the respondents accurately reflect the entire group. The groups are, however, found to be dissimilar when several parameters are assessed. The group of 50 patients undergoing radical resection had a higher proportion of men (see Table 1), an extended resection to include obligate distal gastrectomy and retroperitoneal lymph node dissection, a longer operative time (see Table 2), a greater number of perioperative complications (e.g., pancreatic fistula, wound infection, and intra-abdominal abscess), and a longer postoperative hospital stay (see Table 3). However, many other parameters were comparable between the two groups in the perioperative period (see Tables 1 to 3), and our QOL assessment occurred, on average, more than 2 years after resection, at a time when recovery was complete. The nearly identical QOL scores for the various FACT-G subscales (physical, social/family, emotional, and functional well-being) and the FACT-Hep subscale yield total FACT-Hep scores that are no different between the standard (143.5) and radical (147.3) groups (see Table 4). Furthermore, the subgroup analyses in Table 5 failed to reveal any differences in FACT-Hep scores when pathologic diagnosis, age at operation, sex, and time since operation were compared. Moreover, the data presented in Table 6 concerning changes in body weight, pancreatic enzyme usage, and number of bowel motions per day indicate no differences between patients undergoing standard and those undergoing radical resection. Undoubtedly, the absence of adverse outcomes in our radical group may be related to the lesser degree of radical (extended) resection performed. Previous reports of disabling postoperative diarrhea and nutritional disturbances[14,15] have followed far more extensive retroperitoneal dissection, often involving circumferential dissection of the celiac axis and the superior mesenteric artery, and more extensive retroperitoneal lymph node dissection. Our technique involved clearing of the lymphatic and neural tissues from the anterior and right lateral aspects of (1) the superior mesenteric vein for approximately a 180- to 270-degree circumference, and (2) the superior mesenteric artery for approximately a 90- to 180-degree circumference. Complete circumferential dissection was not performed.

In all, the data presented herein indicate that, within the context of the outcome parameters assessed in this study, there are no differences in QOL or gastrointestinal functional outcomes in patients undergoing standard vs. radical resection for periampullary adenocarcinoma. Because our previous report[13] revealed a higher complication rate, but has failed to reveal a survival benefit, for patients with periampullary adenocarcinoma who undergo radical (extended) pancreaticoduodenectomy, the available data support the use of a standard, pylorus-preserving pancreaticoduodenectomy as the resectional procedure of choice for such patients.

We thank Donna Adelsberg for her assistance with the manuscript, and Dipayan Chaudhuri and Lydia Bazzano for their help with statistical considerations.

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Discussion

Dr. Warshaw (Boston, MA): This is a wonderful study representing an enormous amount of effort. Quality-of-life concerns are needed in surgical studies. I have several questions and comments. The questionnaire return rate of 70% from those patients who actually were queried is a concern because there may be intrinsic bias in that those who returned the questionnaire might be selected out from those who were doing well. That may contribute to your inability to show a difference. Second, your study closed in mid-2001 and, therefore, it may be somewhat

misleading to use the median time at which the returns were sent as being 31 months. You must have included many with a shorter follow-up for whom the assessment of quality of life would be short term. Your last comment oversteps what your data show. The operation you describe is not the radical resection that is described by the Japanese, which really involves circumferential dissection of the superior mesenteric artery and more extensive lymph node dissection. The poor postoperative status to which you are comparing your outcomes (diarrhea, nutritional deficiencies, long hospitalizations, and rehospitalizations) are from those more extensive operations. Finally, to say that the pylorus-preserving resection should be the standard of care is not shown by this study. You have compared the pylorus-preserving procedure against antrectomy with a more extensive dissection. In fact, one could argue that the quality of life and other outcomes are as good with the clinical Whipple resection, including antrectomy (even with a retroperitoneal lymph node dissection), as with the pylorus-preserving variant.

Dr. T. A. Sohn: We were delighted to obtain a 70% response rate to our quality-of-life surveys, but there is no doubt that there is some intrinsic bias. Certainly some of those patients who were nonresponders were likely dying of their disease, as all of these patients had periampullary adenocarcinoma. When you look at the group of nonresponders, they were statistically identical with regard to demographics, diagnosis, and stage of disease. You commented also on 31 months being our mean time to follow-up in these patients. The 31 months was actually the time to follow-up for our survival data. The average time from operation to completion of this survey was approximately 2 years, but certainly there is a range and, of course, there is some intrinsic bias. This study was not designed to look at different points in time but rather to capture a snapshot in time. That was the way the study was designed. Our radical resection was certainly not the radical resection proposed by Fortner or the Japanese investigators. We do not attempt to extrapolate our data to a more radical resection. We do believe our "radical" resection is more extensive than the standard resection. The average number of lymph nodes that we harvested was 17 in the standard group compared to 28.5 in the radical group, and we do see an increase in the complication rate. I think your last comment regarding our conclusion about pylorus preservation warrants clarification. We believe that pylorus-preservation is favored because it takes less time, has fewer postoperative complications, and has identical survival and postoperative quality of life when compared to our radical resection (which includes antrectomy). It is true that we only compared our radical resection to our standard resection in this report, so we cannot draw a conclusion between pylorus preservation and antrectomy alone without the extended lymph node dissection. Pylorus preservation is favored at our institution.

Dr. G. V. Aranha (Maywood, IL): I have two brief questions: (1) Could you say whether quality of life was different in those who had postoperative complications vs. those that had none? (2) Many of my patients will ask about their chances of needing postoperative enzymes or becoming diabetic? Does your study address these patient concerns?

Dr. Sohn: In terms of postoperative complications, we did not stratify specifically to look at that, so I cannot answer that question directly. Approximately 85% of our patients are given pancreatic enzyme replacement at 6 months after surgery, and 30% to 40% of our patients are diabetic postoperatively, with many of these patients being diabetic preoperatively.

Dr. M. Maggard: (Los Angeles, CA): Some of your outcome points, particularly length of stay, typically do not follow a normal distribution. How did you account for this in your statistical analysis?

Dr. Sohn: The statistical analysis was carried out using standard software. Our length of stay certainly falls within a very clustered range and a fairly normal distribution skewed to one side.

Dr. J.A. Bastidas: (Stanford, CA): I was curious to know what percentage of your patients receive adjuvant chemoradiation and whether you had any subgroup analysis on the impact of radiation on quality of life.

Dr. Sohn: We recommend chemoradiation to all of our patients undergoing Whipple resection for periampullary adenocarcinoma. Approximately 75% of the patients in this series had adjuvant chemoradiation. The distribution between the two groups was similar. We did not do a formal analysis of chemoradiation in terms of quality of life. Most patients had completed their chemotherapy at the time of the survey.

Invited Discussion—Expert Commentator

L. William Traverso, M.D. (Seattle, WA): Extended lymphadenectomy was routinely performed in Japan during the past decade. There were questions about the ability of this radical operation to promote survival, as the number of 5-year survivors was not that different from that achieved with a standard Whipple procedure. Another common question was how the Japanese operation changed the patient's quality of life (QOL). We particularly wanted to know the incidence of diarrhea because the circumferential stripping of the superior mesenteric artery (SMA) and celiac axis was purported to result in severe and disabling diarrhea. No quality-of-life data were available from the Japanese experience, but there is one going on now in Japan. This simultaneously occurs with the trend in Japan away from radical resection and the incorporation of adjuvant therapy. The question of disabling diarrhea

cannot be answered by today's presentation because stripping of the circumferential SMA or celiac axis was not done as the Japanese do it. The Johns Hopkins method stays to the right side of these vessels.

Several papers have examined QOL after a Whipple procedure for malignancy and found significantly lower QOL scores in the group with distal gastrectomy as compared to better QOL scores with the pylorus-preserving variety of the Whipple procedure.

Today's study looks at the QOL in a group of patients who had either a pylorus-preserving Whipple procedure, with the usual lymph nodes removed along with the specimen, vs. a standard Whipple procedure with antrectomy and an extended lymphadenectomy, albeit on the right side of the SMA. Note that 13% of patients in the first group also had a gastrectomy because of localized tumor in the area of the duodenal bulb. There was no difference in QOL at a mean of 2.2 years after surgery. In addition, regardless of what type of operation was performed, the Johns Hopkins investigators observed that each group had a significant weight loss and an increase in bowel motions and the use of exocrine enzymes. It appears that we should be looking at how to improve our results with regard to preserving exocrine function in both groups. This weight loss might have been evaluated in the QOL study if the patients had been entered into the QOL study preoperatively. QOL studies should not be relied on if they are only done in the postoperative period. To overcome this deficiency, specific questions pertaining to gastrointestinal activity should be asked. Table 6 in this report contains these specific questions, and I urge the reader to concentrate on this table.

Mucus Is a Predictor of Better Prognosis and Survival in Patients With Intraductal Papillary Mucinous Tumor of the Pancreas

Yuichi Kitagawa, M.D., Trisha A. Unger, M.D., Shari Taylor, M.D., Richard A. Kozarek, M.D., L. William Traverso, M.D.

The aim of our study was to examine the case histories of patients with intraductal papillary mucinous tumor (IPMT) treated with resection to determine predictors of prognosis. Between 1989 and 2000, all patients treated with pancreatic resection for IPMT (n = 63) were analyzed. The diagnosis of IPMT was made using the surgical specimen and the World Health Organization definition. Predictors were determined using univariate and multivariate analysis. The pathologic findings were benign (n = 30), carcinoma in situ (CIS; n = 5), and invasive carcinoma (n = 28). After univariate analysis, predictors of malignancy (invasive plus CIS) were jaundice (odds ratio = 10.32), elevated serum CA19-9 (odds ratio = 15.0), any abnormal liver function test (odds ratio = 7.69), and p53 overexpression. The only predictor of benign disease was gross mucus observed during endoscopy (odds ratio = 4.35). After multivariate analysis, predictors of malignancy were any abnormal liver function test (odds ratio = 5.09) and p53 overexpression, whereas the only predictor of benign disease was still gross mucus (odds ratio = 5.88). Actuarial 3- and 5-year survival for benign disease was 95% and 83% and for malignant disease 52% and 44%, respectively ($P = 0.0048$). Survival curves also favored p53-negative tumors vs. p53-positive tumors ($P = 0.0055$). In the 33 patients with malignant disease (mean follow-up time = 35 months), the presence of gross mucus was a predictor of prolonged survival after univariate and multivariate analysis (odds ratio = 4.34 and 4.55, respectively), whereas alcohol abuse was a predictor of poor survival (odds ratio = 3.41 and 3.60, respectively). Gross mucus observed during endoscopy is a predictor of benign IPMT and, within the group with malignant IPMT; the presence of gross mucus was associated with better survival. Survival was also strongly associated with either benign IPMT or negative staining for p53 overexpression. (J GASTROINTEST SURG 2003;7:12–19.)

KEY WORDS: Intraductal papillary mucinous tumor, mucus, pancreatic resection, p53 overexpression

An intraductal papillary mucinous tumor (IPMT) is distinctly different from other mucin-producing tumors of the pancreas such as a mucin-producing pancreatic adenocarcinoma or pancreatic mucinous cystic neoplasms such as a mucinous cyst adenoma or mucinous cyst adenocarcinoma. To qualify as an IPMT, these lesions must be shown to connect to the pancreatic ductal system (usually by endoscopic retrograde cholangiopancreatography [ERCP]) and have specific histologic features. The 1996 definition of IPMT by the World Health Organization is useful—that is, an intraductal papillary growth of neoplastic columnar cells and histologic evidence of mucin production in these cells.[1] Most of these tumors emphasize mucin production histologically, but a few show a predominance of papillary growth. Therefore the term intraductal papillary mucinous tumor has been favored. IPMTs are divided into the following three categories: intraductal papillary mucinous adenoma; IPMT with moderate dysplasia; and intraductal papillary mucinous carcinoma. The intraductal papillary mucinous adenoma shows no significant cellular atypia. Mitoses are almost absent. This type of tumor is considered a benign tumor. IPMT with moderate dysplasia

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (oral presentation).

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Published by Elsevier Science Inc.

1091-255X/03/$—see front matter
PII: S1091-255X(02)00152-X

shows foci of moderate dysplasia, and this type of tumor may transform into carcinoma. It is considered a "borderline lesion" and includes carcinoma in situ (CIS). Intraductal papillary mucinous carcinoma shows an intraductal carcinoma composed of papillary proliferations of severe dysplastic epithelium. These tumors are invasive.

The preoperative diagnosis of this disease as malignant or benign is difficult even with new imaging modalities such as new-generation computed tomography (CT),[2–4] magnetic resonance cholangiopancreatography (MRCP),[4–6] and endoscopic ultrasound or intraductal ultrasound.[4,7–9] However, nonhistologic variables have not been established that might help predict benign vs. malignant IPMTs or better survival.

The clinical characteristics of IPMTs were reported from our institution using 31 resected patients with a mean follow-up period of 37 months.[10] The aim of the current study was twofold: first, we wished to analyze twice as many patients with longer follow-up than in our previous study in order to identify preoperative nonhistologic factors that might predict malignant IPMT. The second goal was, with the longer follow-up, to identify the risk factors that affect survival in those patients with malignant IPMT.

PATIENTS AND METHODOLOGY

From June 1989 to July 2000, a total of 63 resected patients were histologically diagnosed in our pancreatic referral center as having IPMT. The World Health Organization histologic definition of IPMT was used for classification. The patients with IPMT were divided into the following three categories: intraductal papillary mucinous adenoma (benign; n = 30), IPMT with moderate dysplasia (CIS; n = 5), and intraductal papillary mucinous carcinoma (carcinoma; n = 28).

For purposes of analysis, the patients were further divided into two groups as follows: benign (n = 30) and malignant (n = 33); the latter included CIS and carcinoma to calculate the odds ratio and relative risk (RR).

Variables analyzed were age at operation, sex, history of pancreatitis, history of diabetes, history of alcohol abuse, duration of symptoms, steatorrhea, diarrhea, weight loss, jaundice, abdominal pain, elevated serum amylase, any abnormal liver function test (including serum alkaline phosphatase, transaminase, or lactic dehydrogenase concentrations), serum CA19-9 elevation, serum carcinoembryonic antigen elevation, cystic mass on CT and/or ultrasound, size of the cystic mass on preoperative imaging, radiographic evidence of calcification in the tumor, gross mucus present during endoscopy, observed defects during ERCP that suggested mucus in the pancreatic ducts, cytologic examination of pancreatic juice performed at ERCP, type of resection (pylorus-preserving Whipple, distal pancreatectomy, total pancreatectomy, or local resection), location of the tumor (head, body/tail, or diffuse), tumor size in the surgical specimen, main pancreatic duct rupture, and according to the tumor location in the pancreatic ductal system—only in the side branches (side branch type; SBT) or main pancreatic duct type (MDT) with or without side branch involvement.

The outcomes after operation were collected by telephone or personal interview in the outpatient clinic. If a patient had died, we recorded the survival time after operation, the date of recurrence, and the cause of death. For surviving patients, we recorded the postoperative survival time and status of recurrence, if applicable.

Histologic Investigations

After resection of the tumor, gross findings and the size of the tumor were recorded. Hematoxylin and eosin (H&E) staining was performed, as well as mucin staining.

Immunohistologic Investigation Using p53

An immunohistologic study for p53 overexpression was performed in 49 patients (28 with benign lesions, 3 with CIS, and 18 with carcinoma). The original H&E-stained tissue sections were reviewed to confirm the diagnosis and to determine the area of the section for p53 investigation. The p53 staining was performed using 2 μm thick sections from paraffin-embedded tissue using an avidin-biotin immunoperoxidase method. The clone used was D07 (DAKO) at a dilution of 1:1000. Briefly, after deparaffinization and rehydration, the slides were incubated in a solution of 3% hydrogen peroxide to block endogenous peroxidase activity. Heat-induced epitope retrieval was performed using a microwave and 10 mmol/L citrate buffer, pH 6.0, for 15 minutes. After microwave treatment, the slides were held in hot buffer for 20 minutes, and then washed in phosphate-buffered saline (PBS) solution. The appropriate dilution of the primary antibody was then applied for 45 minutes at room temperature. After washing with PBS, a biotinylated antimouse antibody (1:200; Vector Laboratories, Burlingame, CA) was applied for 25 minutes. The sections were again washed with PBS, after which the avidin-biotin complex (1:100; Vector Laboratories) was applied for an additional 25-minute incubation. Diaminobenzadine enhanced with a solution of 8% NiCl2 was the chromogen used to develop the black reaction product, followed by a methyl green counterstain. A tumor was considered to have

p53 expression when there was staining of contiguous tumor cell nuclei.[11]

This staining and the evaluation of p53 were performed at the Department of Pathology, University of Washington, and the examiners were blinded to clinical information. A correlation of histologic findings in these patients' IPMTs with p53, K-ras, Her-2/neu, and cyclin D1 is being prepared for publication elsewhere (Taylor SL, Brentnall T, Traverso LW, Kozarek R, Gown AM, Bronner MP. p53 Expression Predicts Invasion in Intraductal Papillary Mucinous Tumors of the Pancreas). The current study compares p53 overexpression with long-term survival after pancreatic resection.

Statistics

For purposes of analysis, the "malignant" group of tumors included both CIS and carcinoma. Statistical comparison was carried out with log-rank analysis for continuous variables (age, duration of symptoms, and length of follow-up) and where more than two variables were being analyzed (tumor location and resection type). All other variables were analyzed with chi-square statistics. Factors found to be predictive on univariate analysis were then subjected to multivariate analysis.

Postoperative survival was calculated using the Kaplan-Meier method, and differences in the survival curves were compared by log-rank test between the benign and malignant IPMT groups. Additionally, within the malignant group, univariate and multivariate analyses were also performed to find the significant predictors for longer or shorter survival.

In each analysis, $P < 0.05$ was considered as significant. All mean data are expressed as ± standard deviation.

RESULTS

Preoperative Findings

The mean age of the 63 patients was 64 ± 11 years; 27 patients (43%) were men and 36 patients (57%) were women. The pathologic findings were benign (n = 30, 48%), invasive carcinoma (n = 28, 44%), and CIS (n = 5, 8%). For purposes of analysis, 33 patients (52%) had "malignant" disease. Preoperative variables are summarized in Table 1 and are listed as benign, total malignant, malignant (CIS), and malignant (invasive). Note that 53 (84%) of 63 patients were symptomatic with abdominal pain and 34 (54%) of 63 had a history of pancreatitis, whereas only 22% were diabetic and 14% had a history of alcohol abuse.

By univariate analysis, significant differences were observed in the malignant group over the benign group, as listed in Table 1 along with the odds ratio. The "malignant" cases were more likely to have a shorter duration of symptoms (an odds ratio of 0.98 means an increase in the duration of symptoms beyond 15 months equals a 2% reduction per month in the odds for malignancy). Malignant cases were more likely to present with jaundice, elevated liver function tests, or elevated serum CA19-9. Benign cases were more likely to have mucus observed during ERCP.

The preceding variables with significant differences by univariate analysis were then subjected to multivariate analysis. The only predictor of malignancy was an elevated liver function test (RR = 5.09), and the only predictor of benign disease was mucus observed during endoscopy (RR = 5.88).

In this series, 20 patients (31.7%) had pancreatic juice collected for cytologic examination during endoscopic pancreatic duct cannulation. Of the 15 patients who were later diagnosed as having malignant disease, only five of them (33.3%) had positive cytologic findings.

Operative and Pathologic Findings

Table 2 summarizes the incidence of operative and postoperative variables divided by benign and malignant disease. No differences were seen between the benign vs. malignant groups by tumor location or type of resection. Note that 35 (56%) of 63 patients had disease localized in the head and an additional 9 (14%) of 63 had diffuse disease. Therefore 73% had a Whipple procedure or total pancreatectomy. Also note that 4 (6%) of 63 patients had noncontiguous multifocal tumors, that is, separate lesions in the head and tail.

Main pancreatic duct rupture was associated with the clinical course of 8 (13%) of 63 patients, with three and five patients in each group, respectively. A significant likelihood for malignancy was noted if the tumor was located in the main pancreatic duct (i.e., the MDT). Note MDT means that the main pancreatic duct was involved with tumor with or without side branch involvement.

As listed in Table 2, 49 patients underwent p53 immunohistochemical studies. A significant likelihood for malignancy was noted in those IPMTs that were in the malignant group ($P < 0.0001$; benign vs. malignant). The odds ratio for p53 staining could not be determined for benign vs. malignant, as all benign patients had negative staining for p53.

Postoperative Survival

The mean follow-up time after operation was 43 months (range 4 to 134 months) for the 63 patients. Actuarial overall 3-, 5-, and 10-year survival was 95.2%,

Table 1. Preoperative variables in 63 IPMT patients

	Benign (n = 30)	Total malignant (n = 33)	CIS (n = 5)	Malignant invasive (n = 28)	Odds ratio	*P* value
Age (yr)	65 ± 11	63 ± 11	58 ± 10	64 ± 11	0.98	NS
Male	11 (37%)	16 (48%)	2 (40%)	14 (50%)	1.63	NS
History of pancreatitis	18 (60%)	16 (77%)	4 (80%)	12 (43%)	0.63	NS
Diabetes	7 (23%)	7 (21%)	1 (20%)	6 (21%)	0.89	NS
Ethanol abuse	2 (7%)	7 (21%)	1 (20%)	6 (21%)	3.77	NS
Symptom duration months (range)	35.2 (1–180)	15.1 (1–96)	14.8 (3–41)	15.1 (1–96)	0.98	0.032
Steatorrhea	7 (23%)	2 (6%)	0 (0%)	2 (7%)	0.21	NS
Diarrhea	9 (30%)	4 (12%)	0 (0%)	2 (7%)	0.32	NS
Weight loss	13 (43%)	12 (36%)	2 (40%)	10 (36%)	0.75	NS
Jaundice	2 (7%)	14 (42%)	2 (40%)	12 (43%)	10.32	0.001
Abdominal pain	24 (80%)	29 (88%)	5 (100%)	24 (86%)	1.81	NS
Documented amylase elevation	13 (43%)	15 (45%)	3 (60%)	12 (43%)	1.03	NS
LFT elevation	5 (17%)	20 (61%)	4 (80%)	16 (57%)	7.69	0.0004
CA 19-9	3/21 (14%)	17/21 (81%)	3 (60%)	12/16 (75%)	15.0	0.0002
CEA	1/8 (12%)	4/8 (50%)	0/1 (0%)	4/7 (57%)	4.67	NS
Mass on CT and/or US	25 (83%)	25 (78%)	5 (100%)	20 (71%)	0.63	NS
Cystic mass size (cm) in specimen	3.4 ± 2.6	3.2 ± 1.7	2.0 ± 0.7	3.4 ± 1.7	0.97	NS
Calcifications by x-ray	1/25 (4%)	3/25 (12%)	1 (20%)	2/20 (10%)	2.90	NS
Gross mucus by endoscopy	19/29 (66%)	9/26 (35%)	2 (40%)	7/21 (33%)	0.23	0.006
Filling defects on ERP	15/29 (52%)	11/26 (42%)	3 (60%)	8/21 (38%)	0.68	NS

When mean is used the data ± standard deviation, LFT elevation = AST, ALT, LDH, or alkaline phosphatase; statistics are benign (n = 30) vs. total malignant (n = 33).

82.9%, and 66.8% for patients with benign tumors and 52.4%, 43.7%, and 32.8%, respectively for the total malignant group (including CIS). Mean follow-up for the subgroups was as follows: benign, 53 months (range 4 to 113 months); total malignant, 35 months (range 7 to 73 months); CIS, 32 months (range 13 to 73 months); and invasive malignant, 35 months (range 7 to 34 months).

In Fig. 1 patient survival with benign IPMTs was statistically better than with malignant IPMTs ($P = 0.0048$). The survival of the same patients was compared using p53 staining in 49 patients (see bottom of Table 2) rather than benign vs. malignant. In Fig. 2 patient survival in the p53 negative–staining group (n = 38) was statistically better ($P = 0.0055$) than the patients with p53-positive tumors (n = 11). We further looked at just the patients in the invasive IPMT group who had p53 staining (n = 18), where 11 of 18 were positive for p53. No difference in survival was observed for p53-negative malignant tumors (n = 7) vs. p53-positive malignant tumors (n = 11).

Predictors of Survival in Patients With Malignant Disease

Among the 33 patients with malignant disease, there was one significant predictor of good survival after univariate and multivariate analysis—mucus observed during endoscopy (RR = 4.34 and 4.55, respectively). There was one predictor of poor survival—a history of alcohol abuse (RR = 3.41 and 3.60, respectively).

DISCUSSION

Any cystic lesion connected to the pancreatic ductal system is an IPMT until proved otherwise. Resection of the IPMT is required if the patient is a candidate for surgery. As can be seen in our study, resection is required for two reasons: amelioration of symptoms and prevention or treatment of a malignant process. In the current study of 63 patients, 84% were symptomatic with abdominal pain and 54% had a documented history of pancreatitis. The high incidence of malignancy (52%) further supports removal of all of these lesions in patients who are candidates for surgery. In our earlier study of 31 resected patients,[10] and in the current group of 63 resected patients, malignancy was observed in 28% and 43%, respectively, of SBT lesions, whereas malignancy was found in 60% and 73%, respectively, of main pancreatic duct lesions. Lesions in the main pancreatic duct are most likely to be malignant but even the SBT lesions have such a high incidence that, regardless of the location, these tumors need to be removed. Also, benign lesions are considered premalignant and should be removed.

Table 2. Operative and postoperative variables of IPMT patients

	Benign (30)	Malignant total (33)	Malignant CIS (5)	Malignant carcinoma (28)	Odds ratio	*P* value
Head	15	19	5	14	1.00*	
Body and/or tail	9	7	0	7	0.61	
Diffuse	3	6	0	6	1.58	
Head and tail	3	1	0	1	0.26	NS
Gross tumor size (cm ± SD)	3.6 ± 2.6	3.6 ± 1.9	2.4 ± 1.1	3.8 ± 1.9	0.99	NS
MDT	13	24	4	20	1.00*	
SBT	17	9	1	8	0.29	0.02
PPW	16	20	5	15	1.00*	
Distal	8	7	0	7	0.70	
Total	5	5	0	5	0.80	
Local	1	1	0	1	0.80	NS
P53 staining	0/28 (0%)	11/21 (52%)	0/3 (0%)	11/18 (61%)	†	<0.0001

MDT = Main pancreatic duct type, SBT = side branch type, PPW = pylorus preserving Whipple procedure; statistics are benign (n = 30) vs. malignant (n = 33).
*Reference at 1.00 for which the others in their group are compared.
†Odds ratio could not be calculated as all of the benign group were negative for p53 staining.

The preceding discussion suggests that the clinician should decide to resect by focusing on establishing the diagnosis of IPMT rather than on preoperative predictors of malignancy. However, preoperative predictors of malignancy would be helpful to discuss prognosis with patients or their referring physicians. They would also be helpful to decide whether resection is indicated in a poor surgical candidate, and diligent efforts should be employed to improve a comorbid condition that might enable surgical resection. Unfortunately, there are few preoperative predictors of malignancy to consider. Preoperative nonhistologic predictors of malignancy are only reliable when they are considered as part of the clinical findings. Some items that have been associated with malignancy are from preoperative imaging studies such as the variables of main pancreatic duct type,[10,12] size of the mass,[12–14] tumor location,[13] and CA19-9 elevations.[14,15] In our study we found the following to be significantly associated with malignancy: a tumor that resulted in jaundice; abnormal liver function tests; short duration of symptoms; and serum CA19-9 elevation.

Gross mucus observed during endoscopy was associated with benign IPMTs after both univariate and multivariate analysis. This may be a useful predictor of benign disease as we found mucus in 65% of the

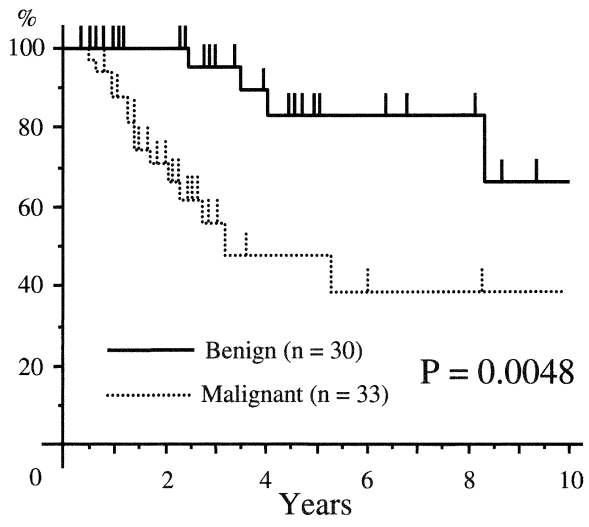

Fig. 1. Kaplan Meier survival curves were significantly different as the benign IPMT group had greater than 80% 5-year survival, but the malignant group showed less than 50% 5-year survival after surgery ($P = 0.0048$).

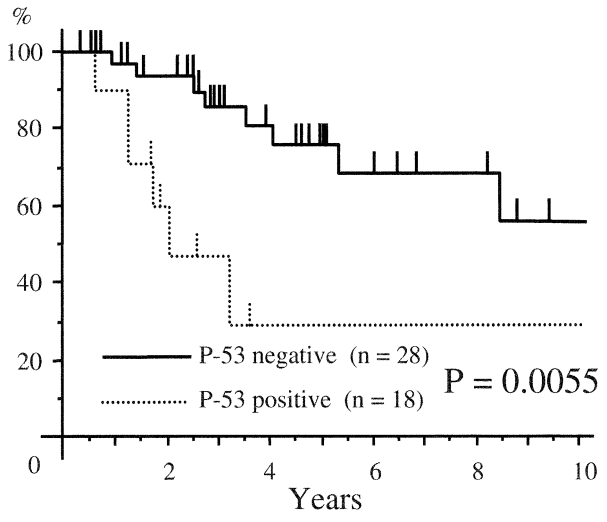

Fig. 2. Kaplan Meier survival curves were significantly different when groups with and without p53 staining were compared. The p53-negative group had a 75% 5-year survival, whereas the p53-positive group (which was associated with invasive tumors) showed a 30% 5-year survival ($P = 0.0055$).

benign tumors and only 35% of malignant cases. The presence of mucus may predispose to symptoms through pancreatic duct obstruction and bring the patients to medical attention sooner. For whatever reasons, the presence of mucus means an earlier stage of IPMT that also translates into improved survival. In the 33 patients with malignant IPMTs, if mucus was observed the survival was better. The presence of mucus means a good prognosis, but surgical resection is still required—that is, one third are malignant and mucus causes symptoms that are relieved by resection.[10]

Most predictors of malignancy for IPMT are histologic (and therefore obtained after resection). Preoperatively, histologic studies of the pancreas are risky. In addition, cytologic examination of pancreatic juice is not helpful. In our study, only one third of the malignant cases had positive cytologic findings. Mucus is a welcome factor to search for in the workup.

Examples of factors that have been associated with malignancy in cases of IPMT after surgery (i.e., postoperative factors) are the histologic type of mucin (sulphomucin), K-ras, p53, Ki-67, proliferating cell nuclear antigen, p27 protein, and cyclin E.[14,16–19] The tumor suppressor gene, p53, is interesting. Our study suggests that p53 overexpression is associated with invasive IPMTs. Among our 63 patients, we found no overexpression in the benign or CIS groups but in 52% of the patients with invasive malignancy. In addition, p53 by itself was at least as good as histologic findings (benign or malignant) at determining prognosis and survival (see Fig. 2). The tumor suppressor gene, p53, is located on chromosome 17p. The gene is related to cell growth, and an overexpression is associated with human neoplasms such as pancreatic cancer. Overexpression of p53 was found in 0% to 57% of patients with mucin-hypersecreting tumors.[20–23] Our finding of p53 overexpression isolated to invasive tumors is new, as none of these other studies concluded that p53 overexpression was associated with invasion.

These lesions are being diagnosed with increasing frequency at our referral center. Over the last decade 75% of the lesions have been resected within the last 5 years. A suspicion is raised for the presence of an IPMT when a symptomatic patient has a cystic structure seen on CT scan or ERCP, or the cystic structure is discovered incidentally on ultrasound or CT scan. Others have also noted these findings using imaging studies that include primarily ERCP[7,24,25] and CT.[2,4] Some investigators reported on the usefulness of selective MRCP.[4–6] Others have reported endoscopic or intraductal ultrasound to be helpful.[4,7–9] The use of pancreatoscopy has been described for observing and obtaining biopsy specimens in the intraductal tumors of the major pancreatic ducts.[26] We have found that CT and ERCP are sufficient because any cystic mass in the pancreas that connects to the pancreatic ductal system needs to be removed. These lesions are not subtle and are not usually clinically or anatomically similar to a pseudocyst. IPMT should be considered high on the differential list in a patient with idiopathic pancreatitis and a suggestion of a cystic lesion or lesions in the pancreas. It is interesting to note that survival of those with malignant IPMTs and alcohol abuse was poorer, although we observed alcohol abuse in only 7 (21%) of 33 of our patients with malignant disease. The pancreatitis caused by ductal obstruction with mucus can be associated with ductal disruption and pseudocyst. Although ductal disruption was seen in only 13% of the 63 patients, these were severe cases of pancreatitis. In the absence of alcohol abuse or gallstones, the presence of an occult IPMT should be considered in patients with recurrent idiopathic pancreatitis.

We thank Matthew Barnett for statistical support.

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Discussion

Dr. H.A. Pitt (Milwaukee, WI): I noticed that you had more of the side-branch type in your benign patients, and I would like to know what criteria you used for differentiating IPMT side branch vs. mucinous cystic neoplasms or mucinous cystic tumors?

Dr. Y. Kitagawa: To be classified as an IPMT, all cystic lesions had to have a direct connection to the pancreatic duct proven by ERCP. If the tumor was then histologically proven to involve just the major side branches, it was called a side branch type and if it was involving the main pancreatic duct, whether it was in the side branches or not, it was termed a main duct type. Mucinous cystic tumors, like the mucinous cystadenoma of the pancreas, do not connect to the pancreatic ductal system and were excluded from this study. Of course, all of these mucinous lesions, IPMTs or mucinous cystic lesions, are either malignant or premalignant and should be treated the same, with resection, as you implied.

Dr. A.L. Warshaw (Boston, MA): This is a very interesting study. I would like to ask you a question based on the mucus production, in particular. To my knowledge, there is no histologic difference between IPMTs that hyperproduce and those that do not. Why would that be? In our series, which comprises about 80 resected patients, 30 of whom were clearly malignant, we have made the observation that it is more likely that there is hyperproduction of mucus presenting at the ampulla when the tumor is located in the head of the pancreas, rather than the tail. Your series apparently does not show that difference. We have hypothesized that this is a local obstructive phenomenon in part, that mucus is more likely to accumulate when the tumor is in the proximal duct. Such patients may be more likely to present with symptoms of pancreatitis because of the obstructive nature of the location within the head. Could that be part of the explanation for why those are more likely to be benign, that they are presenting earlier because they are symptomatic?

Dr. Kitagawa: As you point out, our study shows tumors more common in the head of the pancreas, and half of them are in the side branches and not in the main pancreatic duct. Your speculation could be correct that the reason mucus is associated with benign disease is that the mucus causes symptoms, and therefore the diagnosis is made earlier.

Dr. B. Wait (Springfield, MA): You have classified your in situ cancers with the other malignancies. Although I realize that these are small numbers, could you tell us if you have separated these out to determine whether there is a difference in survival between patients with in situ disease and those with invasive disease. Second, what are your recommendations for follow-up of patients with either in situ or invasive disease?

Dr. L.W. Traverso (Seattle, WA): Maybe I can answer that. There are too few cases of CIS to provide meaningful survival curves. There are two additional questions to answer

here—how to follow-up these patients plus when and how much to resect. We do not know the answer to the first question, but we are following them every 6 months for recurrent symptoms and with imaging studies. Because the majority of patients are symptomatic, a few of them come back with symptoms, and we have to decide what to do. In regard to how much to resect, our goal is to get rid of dysplasia, but we are not chasing mucinous hyperplasia and continuing to resect. These cases demand pioneering evaluations where only long-term follow-up can answer the questions.

Dr. J.P. Hoffman (Philadelphia, PA): I found it curious that in your analysis of the malignant tumors you did not find a significant prognostic implication for positive lymph nodes. I assume you looked at that. Also, we heard a few days ago that the colloid variety of cancer does better than the ductal variety. Did you look at that?

R.A. Prinz (Chicago, IL): I have two short questions. First, why did your patients with the benign disease die? Secondly, you did not share with us any recurrence data. Did you find recurrences in either your malignant or your benign group?

Dr. Kitagawa: All of the patients with benign disease died of other causes rather than IPMT. In patients with malignant disease, we made a big effort to resect to negative margins (as far as to have no dysplasia). Only three patients had recurrence after malignant tumor resection.

Regulation of Kupffer Cell TNF Gene Expression During Experimental Acute Pancreatitis: The Role of p38-MAPK, ERK1/2, SAPK/JNK, and NF-κB

Michel M. Murr, M.D., Jun Yang, M.D., Adam Fier, B.A., Scott F. Gallagher, M.D., Gay Carter, B.A., William R. Gower, Jr., Ph.D., James G. Norman, M.D.

We have demonstrated that Kupffer cell–derived tumor necrosis factor (TNF) mediates pancreatitis-associated liver injury. The aim of this study was to determine the role of p38 mitogen-activated protein kinase (MAPK), extracellular stress-related kinase 1/2 (ERK1/2), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and nuclear factor–κB (NF-κB) in TNF gene expression within Kupffer cells. TNF and TNF-mRNA were measured in rat livers perfused with elastase. TNF, TNF-mRNA, NF-κB activation, and phosphorylated p38-MAPK, SAPK/JNK, and ERK1/2 were determined in Kupffer cells treated with elastase. Elastase increased TNF and upregulated TNF-mRNA in livers ($P<0.03$) and Kupffer cells ($P<0.001$). Phosphorylated p38-MAPK, SAPK/JNK, and ERK1/2 and activated NF-κB were detected in Kupffer cells at 7 minutes; at 60 minutes, TNF-mRNA peaked and NF-κB returned to baseline, whereas all three kinases remained activated. Gadolinium inhibited elastase-induced upregulation of TNF-mRNA ($P < 0.001$), TNF production ($P<0.001$), and attenuated SAPK/JNK, as well as ERK1/2, but not p38-MAPK. Both UO126 and SB203580 significantly inhibited elastase-induced upregulation of TNF-mRNA and TNF production ($P<0.001$), but only UO126 inhibited activation of NF-κB. It was concluded that pretranscriptional regulation of TNF gene expression in Kupffer cells follows an orderly activation of p38-MAPK, ERK1/2, and SAPK/JNK that may not converge on NF-κB. The seemingly limited duration of NF-κB activation may be important in "switching off" the cytokine cascade during acute pancreatitis. (J GASTROINTEST SURG 2003;7:20–25.)

KEY WORDS: Acute pancreatitis, Kupffer cells, TNF, signal transduction, liver injury

The morbidity and mortality associated with severe acute pancreatitis is largely attributable to the associated distant organ dysfunction and exacerbation of the systemic inflammatory response. Liver injury is a marker of the systemic inflammatory response during acute pancreatitis and is an important clinical prognostic indicator. We have demonstrated that pancreatitis-associated liver injury is mediated by noxious inflammatory cytokines that are produced within Kupffer cells.[1,2] In that regard, the liver is a unique organ because Kupffer cells are the largest population of fixed tissue macrophages and have been shown to have a distinct role in sepsis and hemorrhage.[3–5] Pancreatic elastase induces production of tumor necrosis factor (TNF) in Kupffer cells via activation of nuclear factor–kappa B (NF-κB).[1] However, the role of upstream regulators of NF-κB and TNF gene expression in Kupffer cells is not well characterized.

The current study was undertaken to determine the role of the following upstream regulators: p38 mitogen-activated protein kinase (MAPK), extracellular stress-regulated kinase 1/2 (ERK1/2), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and NF-κB in TNF gene expression within Kupffer cells.

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (poster presentation), and published as an abstract in *Gastroenterology*, July 2002.
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Supported by SSAT Career Development Award 2001-2003 (M.M.) and by a VA Merit Award (J.N.).
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Published by Elsevier Science Inc.
1091-255X/03/$—see front matter
PII: S1091-255X(02)00053-7

METHODS

Animal care was in accordance with the guidelines of the Department of Laboratory Animal Medicine at the University of South Florida, a facility accredited by the American Association for Accreditation in Laboratory Animal Care. Studies were conducted after approval was secured from the Institutional Animal Care and Use Committee of the University of South Florida.

In Situ Liver Perfusion

Male Sprague-Dawely rats (250–350 g) were anesthetized (Nembutal, 50 mg/kg intraperitoneally). The portal vein and the suprahepatic inferior vena cava were cannulated, and the inferior vena cava was ligated to close the circuit, as described by Hems et al.[6] The liver was left in situ and perfused with oxygenated (95% O_2–5%CO_2) Krebs-Henseleit bicarbonate (KHB) buffer at 3 to 4 ml/g until the effluent was clear. Perfusion pressure (<20 cm H_2O) and perfusate temperature (37° C) were kept constant by a heater pump. The entire perfusion system consisted of a reservoir, pump (Brinkmann Instruments, Inc., Roxbury, NY), bubble trap, filter, and oxygenator. The O_2 content of the perfusate was maintained at 19 to 20 mg/ml to ensure tissue viability. Subsequently the livers were perfused for 3 hours as follows: (1) control: KHB buffer, n = 5; and (2) elastase (1 U/ml), n = 5. The dose of elastase was based on dose-response experiments performed on human monocytes (unpublished data) and rat macrophage cell lines that had been validated previously in this model.[1] The optimal dose was determined to be the lowest dose that resulted in the maximal amount of TNF response that did not affect cell viability.

The effluent from the liver was collected every 15 minutes and stored at −80° C. TNF protein in the effluent was measured by means of a commercially available rat enzyme-linked immunosorbent assay (ELISA) kit (BioSource International, Camarillo, CA). At the conclusion of the experiment, the liver parenchyma was harvested to measure TNF-mRNA.

Kupffer Cell Tissue Cultures

Rat Kupffer cells were isolated from male Sprague-Dawley rats by in situ sequential digestion of the liver with pronase and collagenase, low-speed centrifugation to remove parenchymal cells, and subsequent separation of a Kupffer cell–enriched fraction by discontinuous arabinogalactin gradient centrifugation.[7] Cells were incubated in Dulbecco's minimum essential medium–5% fetal calf serum for 24 hours before any treatment was begun, and nonadherent cells were removed. Kupffer cell viability was assessed by exclusion of trypan blue.

Tissue cultures of pure Kupffer cells (>98%) were plated in 24-well plates (2.5×10^6 cells/well), and pretreated with gadolinium chloride (Gd; 0.5 mg/ml to inhibit TNF production from Kupffer cells via NF-κB) for 24 hours,[1] SB203580 (1 μmol/ml to inhibit p38-MAPK) for 1 hour, or UO126 (1 mmol/ml to inhibit ERK1/2) for 2 hours. TNF protein was determined in the supernate 2 hours after treatment with elastase (1 U/ml).

In separate experiments, 10^7 cells/well were seeded in 100 ml dishes and treated with elastase (1 U/ml); TNF-mRNA and NF-κB activation were determined. To determine the role of p38-MAPK, ERK1/2, and SAPK/JNK, 10^7 cells/well were seeded in 100 ml dishes and pretreated with SB203580 or UO126 for 2 hours prior to elastase. Phosphorylated forms of these kinases were determined at 0, 7, 15, 30, 60, and 120 minutes.

Kupffer Cell TNF-mRNA

TNF-mRNA was measured by semiquantitative differential reverse transcription–polymerase chain reaction (RT-PCR), as previously outlined.[1] Briefly, total Kupffer cell RNA was isolated by guanidium thiocyanate/acid phenol extraction and primed using oligo (dT) (Gibco, Gaithersburg, MD) and subsequently reversed transcribed with reverse transcriptase (Superscript II; Gibco). The cDNA products were coamplified in the presence of murine-specific TNF and beta-2 microglobulin (BMG) primers for 20 to 25 cycles of polymerase chain reaction in a UNO-Thermoblock (Biometra, Tampa, FL). The sequence for the TNF primer was sense 5′ATGAGCACAGAAAGCATGATC3′ and antisense 5′TACAGGCTTGTCACTCGAATT3′. The BMG primer sequence was sense 5′CTCCCCAAATTCAAGTGTACTCTCG3′ and antisense 5′GAGTGACGTGTTTAACTCTGCAAGC3′ (Ransom Hill Biosciences, Ramona, CA). All primers are known to span at least one intron. The reaction products were separated with electrophoresis in 2.5% metaphor agarose gel containing ethidium bromide and photographed digitally under ultraviolet light using the ultraviolet gel documentation system (UVP, Upland, CA). Band intensity of each sample was determined by means of gel documentation system (GDS) image analysis software (UVP), and TNF/BMG cDNA ratios were calculated for analysis.

Determination of NF-κB Activation by Electrophoretic Mobility Shift Assay

NF-κB–specific consensus oligonucleotide (5′AGTTGAGGGTTTCCCAGGC3′, Promega Corp.,

Madison, WI) was 5′ end-labeled with γ^{32}P adenosine triphosphate (ICN, Costa Mesa, CA) using polynucleotide kinase (Gibco). Samples containing 10 μg of nuclear protein extract were incubated in binding buffer (10 mmol/L Tris [pH 7.5], 100 mmol/L NaCl, 1 mmol/L EDTA, 4% glycerol, and 80 μg/ml sonicated sperm DNA) with or without excess unlabeled NF-κB–specific oligonucleotide for 15 minutes on ice. End-labeled NF-κB (1.5×103 cpm) was added, and samples were incubated for an additional 45 minutes at room temperature. Free oligonucleotide and oligonucleotide-bound protein were separated by electrophoresis on a native 6% polyacrylamide gel. Gels were dried under vacuum on Whatman paper and exposed to Kodak BioMax MS film for 3 to 6 hours at −80° C. Absence of binding in the presence of excess unlabeled NF-κB–specific oligonucleotide confirmed NF-κB binding specificity.

Determination of p38-MAPK, ERK1/2, and SAPK/JNK by Immunoblotting

The phosphorylated forms of p38-MAPK, ERK1/2, and SAPK/JNK were determined by Western blotting. Protein extracts from Kupffer cell cultures were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electrotransferred to a nitrocellulose membrane. Nonspecific binding was blocked with 5% bovine serum albumin, then immunoblotted overnight at 4° C with 1:1000 dilution of rabbit polyclonal phosphospecific p38-MAPK, ERK1/2, or SAPK/JNK antibodies. Subsequently the membrane was washed and incubated with horseradish peroxidase–conjugated antirabbit antibody for 2 hours at room temperature. The immunoblot was washed and the bands were detected with an enhanced chemiluminesence kit (LumiGlo; New England Biolabs, Beverly, MA), and then quantified by densitometry.

Gadolinium chloride ($GdCl_3.6H_2O$), pancreatic elastase, SB203580, and UO126 were all purchased from Sigma (St. Louis, MO).

Statistical Analysis

Experiments were repeated in triplicate (not gels) and averaged. Data are presented as mean ± SEM. Student's *t*-test was used. Significance was set at $P < 0.05$.

RESULTS

In Situ Liver Perfusion (TNF and TNF-mRNA)

Elastase induced a dramatic increase in TNF protein content in the effluent (1448 ± 71 pg/ml vs. 20 ± 2 pg/ml; $P < 0.001$ vs. sham). In addition, elastase upregulated TNF-mRNA. TNF/BMG was 0.22 ± 0.03 vs. 0.06 ± 0.03 ($P = 0.03$ elastase vs. sham) signifying de novo synthesis of TNF in the liver.

TNF Production and Upregulation of TNF-mRNA in Kupffer Cell Cultures

Elastase induced production of TNF in primary cultures of Kupffer cells in a time-dependent manner (baseline = 30 ± 2 pg/ml; 30 minutes = 47 ± 8 pg/ml; 60 minutes = 200 ± 17 pg/ml) that peaked at 120 minutes (1448 ± 71 pg/ml; $P < 0.0001$ elastase vs. control). Similarly, elastase upregulated TNF-mRNA within Kupffer cells; TNF/BMG = 0.45 ± 0.02, 0.52 ± 0.09, 0.90 ± 0.01, and 0.79 ± 0.02 at 15, 30, 60, and 120 minutes, respectively, as compared to a control value of 0.02 ± 0.001 ($P < 0.0001$ at all time points).

Activation of NF-κB in Kupffer Cell Cultures

Treatment of Kupffer cells with elastase induced a marked activation of NF-κB that was detected in nuclear extracts at 7 minutes, peaked at 15 minutes, and returned to baseline at 60 minutes (Fig. 1)

Activation of p38-MAPK, ERK1/2, and SAPK/JNK in Kupffer Cells

Elastase induced a time-dependent phosphorylation of p38-MAPK, ERK1/2, and SAPK/JNK. Phosphorylated p38-MAPK and SAPK/JNK were detected as early as 7 minutes and remained activated at 60 minutes. ERK1/2 activation peaked abruptly at 15 minutes, then progressively decreased but remained activated at 60 minutes (Fig. 2).

Inhibition of TNF Production and Downregulation of TNF-mRNA in Kupffer Cells

Gd, SB203580, or UO126 significantly inhibited elastase-induced TNF production from Kupffer cells (Fig. 3, *A*) and the elastase-induced upregulation of TNF-mRNA (TNF/BMG: 0.1 ± 0.06, 0.4 ± 0.01, 0.3 ± 0.04 vs. 1.1 ± 0.1; all $P < 0.005$ vs. elastase, respectively (Fig. 3, *B*).

Inhibition of P38-MAPK, ERK1/2, SAPK/JNK, and NF-κB in Kupffer Cells

To characterize the relative roles of the upstream regulators of TNF gene expression, the activity of each of the three kinases was investigated while inhibiting the remaining two, respectively.

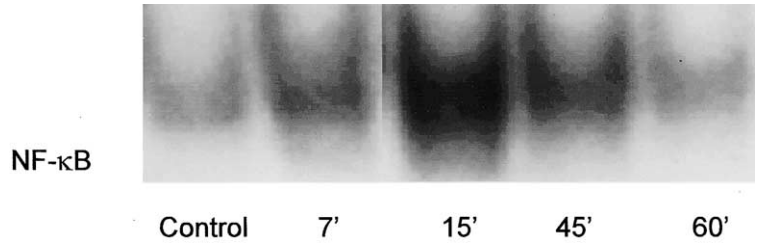

Fig. 1. Activation and nuclear translocation of NF-κB (electrophoretic mobility shift assay) was induced in primary Kupffer cell cultures as early as 7 minutes but returned to baseline by 60 minutes.

Gd attenuated elastase-induced phosphorylation of ERK1/2 and SAPK/JNK but not p38-MAPK (Fig. 4) SB203580 and UO126 did not inhibit elastase-induced phosphorylation of SAPK/JNK. Moreover, UO126 did not inhibit elastase-induced phosphorylation of p38-MAPK.

On the other hand, activation of NF-κB was inhibited by Gd and UO126 but not by SB203580. Gd attenuated elastase-induced activation of NF-κB at 7 and 15 minutes and to greater extent at 30 minutes after treatment with elastase (Fig. 5). Similarly, UO126 significantly attenuated activation of NF-κB between 15 and 30 minutes after treatment with elastase (data not shown).

DISCUSSION

Both Ranson's criteria and the Acute Physiology and Chronic Health Evaulation II (APACHE II) incorporate liver injury into their scoring systems for predicting the severity of acute pancreatitis. By the same token, the role of the liver in amplifying the systemic inflammatory response during acute pancreatitis is unique because it houses the largest population of fixed tissue macrophages and warrants further investigation.

Although the production of many inflammatory cytokines in large quantities is induced during acute pancreatitis, TNF appears to be pivotal in the systemic progression of pancreatitis and a determinant of mortality in laboratory animals.[8,9] In various models, pancreatic enzymes—namely, elastase—mimicked the effects of pancreatitis by inducing production of proinflammatory cytokines from macrophages in specific organs such as the liver and lungs.[1,2,10] In a model of pancreatitis-induced liver injury, we have demonstrated that elastase-induced and Kupffer cell–derived TNF induce hepatocyte injury both in vivo and in vitro.[1]

In that particular model, elastase induced TNF production by activating NF-κB within Kupffer cells.[1] NF-κB is a transcription factor that regulates not only TNF gene expression but also the expression of many inflammatory cytokines.[11] NF-κB is activated by stress-regulated protein kinases that belong to the mitogen-activated protein kinase superfamily.[12] These upstream regulators of NF-κB (namely, p38-MAPK, ERK1/2, and SAPK/JNK) are phoshorylated in response to diverse extracellular stimuli and trigger a cascade that regulates multiple transcription factors in addition to NF-κB.[13] These kinases are ubiquitously present in all cell types, and their role in the cell's response to injury continues to receive much attention because the ability to manipulate them before the full cytokine cascade is activated may have important therapeutic implications.

We investigated TNF gene expression in an established in situ liver perfusion model. Elastase upregulated TNF-mRNA and induced TNF pro-

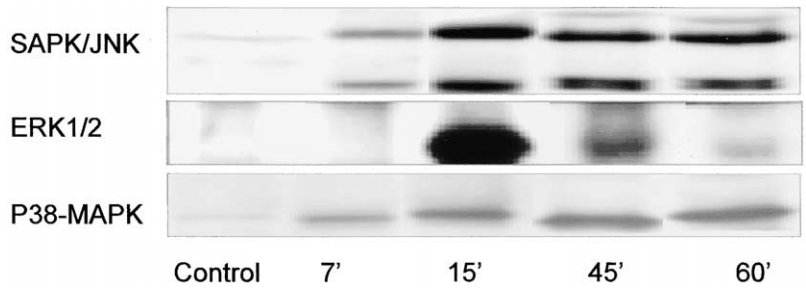

Fig. 2. Time course of activation of p38-MAPK, ERK, and SAPK/JNK in Kupffer cells treated with elastase. These second messenger systems remained activated at 60 minutes after NF-κB returned to baseline activity.

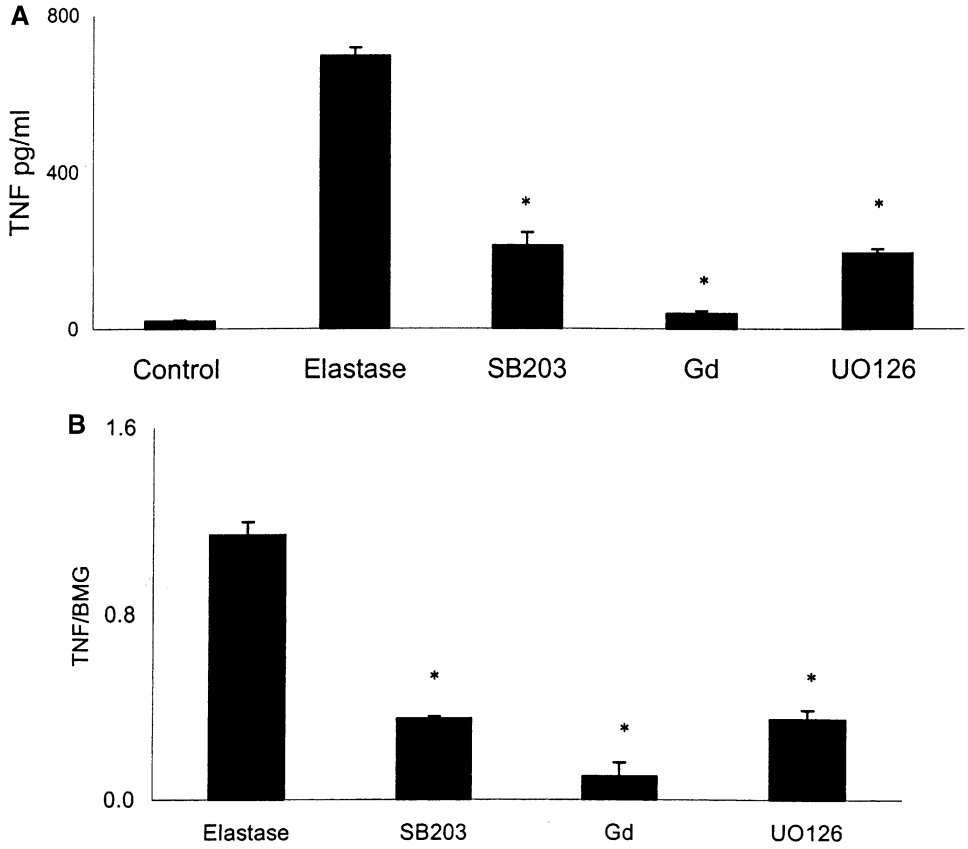

Fig. 3. A, Elastase-induced production of TNF from Kupffer cells in large quantities as compared to control values. Gd, SB203580, or UO126 significantly attenuated the elastase-induced TNF production from Kupffer cells (*$P<0.001$ vs. elastase). **B**, Similarly, Gd, SB203580, or UO126 inhibited elastase-induced upregulation of TNF-mRNA in Kupffer cells (*$P<0.001$ vs. elastase). These experiments were carried out after 60 minutes of treatment with elastase when TNF-mRNA upregulation was at a peak.

duction; in this model, TNF is primarily hepatic in origin because the liver is devoid of circulating blood cells. These findings are in line with previous reports from our laboratory demonstrating that elastase mimics acute pancreatitis by inducing liver inflammation and upregulating TNF-mRNA in an identical manner to that seen in diet-induced pancreatitis.[1,2]

Furthermore, signal transduction pathways in Kupffer cells were studied by mapping the time course of activation of p38-MAPK, ERK1/2, SAPK/JNK, and NF-κB in response to treatment with pancreatic elastase, and by employing known inhibitors of these systems (Gd:NF-κB; SB203580:p38-MAPK; and UO126:ERK1/2). Elastase induced a prompt and dramatic increase in phosphorylated (activated) p38-MAPK, ERK1/2, and SAPK/JNK and activated NF-κB. Interestingly, although p38-MAPK and SAPK/JNK and to a lesser extent ERK1/2 remain activated at 60 minutes, NF-κB activation returned to baseline. As expected, the peak of TNF-mRNA followed that of the three kinases and that of NF-κB, but started to decrease by 120 minutes. These data suggest that NF-κB plays a central role in TNF gene expression and may be pivotal in "switching on/switching off" the cytokine cascade.

On the other hand, TNF production and TNF-mRNA were significantly attenuated by Gd, SB203580, and UO126 (see Fig. 3). To further characterize the cellular pathways that govern TNF gene expression, we examined whether activation of NF-κB and the MAP kinases is interdependent. Gadolinium and UO126, but not SB203580, inhibited activation of NF-κB. Moreover, Gd inhibited activation of ERK1/2 and SAPK/JNK but had no effect on p38-MAPK.

Inhibition of p38-MAPK by SB203580 had no effect on activation of SAPK/JNK. Similarly, inhibition of ERK1/2 by UO126 had no effect on activation of SAPK/JNK or p38-MAPK. These findings may be due, in part, to the structural differences between mem-

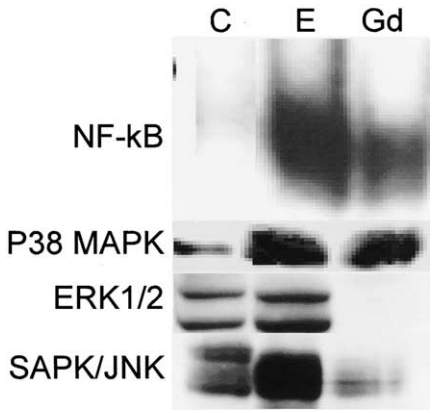

Fig. 4. Elastase (*E*)-induced activation of p38-MAPK, ERK1/2, and SAPK/JNK within Kupffer cells as compared to control (*C*) values. Pretreatment with gadolinium (*Gd*) inhibited elastase-induced activation of ERK1/2 and SAPK/JNK in addition to inhibiting NF-κB but not p38-MAPK.

bers of the same mitogen-activated protein kinase superfamily (i.e., p38-MAPK, SAPK/JNK, and ERK1/2) that explain the selective inhibition of p38-MAPK by the class of SB203580 compounds.[14]

Gadolinium had a global inhibitory effect on upstream regulators of TNF gene expression, whereas the effect of UO126 was limited to inhibiting activation of NF-κB. The mechanism by which SB203580 inhibited TNF-mRNA without attenuating activation of NF-κB cannot be ascertained and warrants further investigation. These data suggest that p38-MAPK, ERK1/2, and SAPK/JNK are independent and may not converge on NF-κB. One plausible explanation is that ERK1/2 and SAPK/JNK are known to translocate to the nucleus after being activated, whereas the exact subcellular location of activated p38-MAPK is less defined.[13]

We conclude that TNF gene expression within Kupffer cells follows an orderly activation of signal transduction pathways. Pretranscriptional regulation of TNF gene expression by p38-MAPK, ERK1/2, and SAPK/JNK exhibits no interdependence among these various kinases and may not converge on NF-κB. Nevertheless, the seemingly limited duration of NF-κB activation may be an important step in "switching on/switching off" the cytokine cascade during acute pancreatitis.

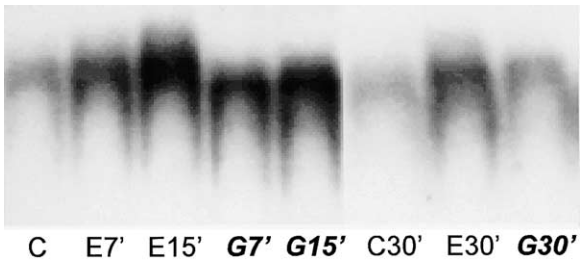

Fig. 5. Elastase-induced activation of NF-κB within Kupffer cells at 7, 15, and 30 minutes (E7′, E15′, E30′) as compared to control values. Gadolinium attenuated elastase-induced activation of NF-κB at 7 and 15 minutes and to a greater extent at 30 minutes (G7′, G15′, G30′); the control values for 7 and 15 minutes are different from that for 30 minutes.

We thank Martha Entel for her administrative help in the preparation of this manuscript.

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Intestinal Hypoperfusion Contributes to Gut Barrier Failure in Severe Acute Pancreatitis

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Intestinal barrier failure and subsequent bacterial translocation have been implicated in the development of organ dysfunction and septic complications associated with severe acute pancreatitis. Splanchnic hypoperfusion and ischemia/reperfusion injury have been postulated as a cause of increased intestinal permeability. The urinary concentration of intestinal fatty acid binding protein (IFABP) has been shown to be a sensitive marker of intestinal ischemia, with increased levels being associated with ischemia/reperfusion. The aim of the current study was to assess the relationship between excretion of IFABP in urine, gut mucosal barrier failure (intestinal hyperpermeability and systemic exposure to endotoxemia), and clinical severity. Patients with a clinical and biochemical diagnosis of acute pancreatitis were studied within 72 hours of onset of pain. Polyethylene glycol probes of 3350 kDa and 400 kDa were administered enterally, and the ratio of the percentage of retrieval of each probe after renal excretion was used as a measure of intestinal macromolecular permeability. Collected urine was also used to determine the IFABP concentration (IFABP-c) and total IFABP (IFABP-t) excreted over the 24-hour period, using an enzyme-linked immunosorbent assay technique. The systemic inflammatory response was estimated from peak 0 to 72-hour plasma C-reactive protein levels, and systemic exposure to endotoxins was measured using serum IgM endotoxin cytoplasmic antibody (EndoCAb) levels. The severity of the attack was assessed on the basis of the Atlanta criteria. Sixty-one patients with acute pancreatitis (severe in 19) and 12 healthy control subjects were studied. Compared to mild attacks, severe attacks were associated with significantly higher urinary IFABP-c (median 1092 pg/ml vs. 84 pg/ml; $P < 0.001$) and IFABP-t (median 1.14 μg vs. 0.21 μg; $P = 0.003$). Furthermore, the control group had significantly lower IFABP-c (median 37 pg/ml; $P = 0.029$) and IFABP-t (median 0.06 μg; $P = 0.005$) than patients with mild attacks. IFABP correlated positively with the polyethylene glycol 3350 percentage retrieval ($r = 0.50$; $P < 0.001$), CRP ($r = 0.51$; $P < 0.001$), and inversely with serum IgM EndoCAb levels ($r = -0.32$; $P = 0.02$). The results of this study support the hypothesis that splanchnic hypoperfusion contributes to the loss of intestinal mucosal integrity associated with a severe attack of pancreatitis. (J GASTROINTEST SURG 2003;7:26–36.)

KEY WORDS: Ischemia/reperfusion, acute pancreatitis, permeability, IFABP

Acute pancreatitis is an enigmatic disease, the pathophysiology of which remains poorly understood. It continues to claim an overall mortality rate of approximately 10%, despite improvements in intensive therapy and radiologic and surgical interventions.[1–3] In patients with severe acute pancreatitis, multiorgan system failure (MOSF) is responsible for early deaths from the disease, whereas sepsis supervenes later and remains the major cause of late deaths.[4–6] Secondary infection supervenes in 30% to 70% of patients with pancreatic necrosis,[7–10] and is associated with significantly increased mortality.[7] Enteric gram-negative organisms are responsible for the majority of these infections,[7,11–15]suggesting that the gut itself acts as a source of infection. Abnormally increased intestinal permeability to large molecules, such as endotoxin, and possibly even to bacteria, are central to the hypothesis implicating the gut in the development of MOSF and sepsis.[16] Endotoxin is shed from (dying) gram-negative bacteria

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (oral presentation).

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1091-255X/03/$—see front matter
PII: S1091-255X(02)00090-2

and has been proved to cause a wide variety of detrimental physiologic responses.[17,18] It is detectable in peripheral blood from most patients with severe and fatal acute pancreatitis.[19–21] Experimental and clinical studies have demonstrated increased intestinal permeability to macromolecules[22–24] and the gut has been identified as an important source of infection during acute pancreatitis.[25,26] Exley et al.[20] detected endotoxemia at presentation more commonly in nonsurvivors of acute pancreatitis (91% vs. 35%), and levels were significantly higher in severe and fatal attacks.[20] Similar findings were reported by Ammori et al.,[24] who demonstrated a sevenfold increase in intestinal macromolecular permeability during severe attacks, which was strongly associated with increased anti-endotoxin antibody levels indicating greater endotoxin entry into the systemic circulation. Further evidence exists to implicate the gut in the development of sepsis and multiple organ failure.[16,27–30] Circulating endotoxins are bound by anti-endotoxin antibodies, the levels of which fall inversely to the amount of endotoxin challenge,[31] and the level of decline is a reflection of the clinical severity.[32] More compelling evidence implicating the gut as a source of bacterial and endotoxin translocation comes from experimental studies. Bacteria were observed to translocate from the intestinal lumen to the mesenteric lymph nodes and subsequently to other extraintestinal sites after the induction of acute pancreatitis.[26,25,33] Although the route of migration of microorganisms from the intestinal lumen remains obscure,[34–37] bacterial translocation seems to be the most important route of bacterial infection[34] and suggests an underlying failure in intestinal barrier function. Several mechanisms have been suggested whereby increased intestinal permeability may lead to translocation of endotoxin and enteric bacteria, including mucosal ischemia,[38–44] ischemia/reperfusion injury,[45,46] impaired immune defenses,[47–51] and changes in indigenous intestinal microbial ecology leading to bacterial overgrowth.[52–55]

Microcirculatory impairment caused by ischemia/reperfusion injury has been postulated as a causative factor of acute pancreatitis, and has been associated with increased severity in experimental models. Visceral hypoperfusion has been demonstrated to aggravate pancreatic injury in experimental and clinical situations, and visceral arteriovenous shunting has been observed in several experimental pancreatitis models.[22] The gut capacity of oxygen extraction is compromised by insufficient intestinal microcirculation at the subserosal level, even with near-normal flow in the arteries,[56] that may lead to gut mucosal damage.

The aim of the present study was to investigate whether small bowel mucosal injury resulting from hypoperfusion contributes to intestinal barrier failure in patients with acute pancreatitis. This was to be achieved by measuring the urine concentrations of intestinal fatty acid binding protein (IFABP) and correlating these levels with changes in intestinal macromolecular permeability and systemic exposure to endotoxins. IFABP is a 15 kDa protein that constitutes 2% of intestinal mucosal protein by mass and is believed to be important in fatty acid transport and storage. IFABP is located mainly within the mucosa of the tips of the small intestinal villi,[57] the earliest site of ischemic injury.[58] IFABP has been demonstrated to be an accurate marker for reversible, intestinal mucosal injury resulting from ischemia. Previous studies in humans have established that IFABP is not detected in significant concentrations in the blood or urine of healthy individuals, but becomes measurable following acute intestinal ischemia.[59] It is thought that increased epithelial cell membrane permeability permits IFABP to leak through from cytosol, thus entering the systemic circulation and then urine. Excretion, therefore, represents a useful biochemical marker for epithelial injury in the small bowel.[60]

PATIENTS AND METHODS

A consecutive series of adults with a clinical diagnosis of acute pancreatitis, supported by hyperamylasemia exceeding three times the upper normal range, were included in the study. Patients were studied within 48 hours of onset of severe abdominal pain. Written informed consent was obtained from all of them. Patients with inflammatory bowel disease, previous bowel resection, renal disease (oliguric or anuric [<400 ml/day]), and those with already established organ failure at the time of admission were excluded.

Clinical progress was prospectively recorded, and attacks were classified as "mild" (uncomplicated) or "severe" (life-threatening organ system dysfunction or pancreatic collection) according to the Atlanta clinical criteria.[10] Control subjects were healthy (departmental staff) age- and sex-matched volunteers. The Leeds Teaching Hospitals NHS Trust ethics committee approved the study.

METHODS

Acute Physiology and Chronic Health Enquiry (APACHE) II scores were determined from data collected within 24 hours of admission.[3,61] The acute-

phase reactant, C-reactive protein (CRP), was measured daily for the first 5 days after the attack, and the peak value during that phase was determined. All measurements of intestinal permeability were completed within 72 hours of onset of severe abdominal pain.

Measurement of Urine IFABP

Intestinal FABP concentrations (IFABP-c) were determined from 24-hour total urine collections using the HyCult Biotechnology bv (Uden, The Netherlands) human IFABP enzyme-linked immunosorbent assay (ELISA) kit. Blinded duplicate assays of the specimens were performed in batches, using an internal standard curve with each assay. Briefly, the IFABP test kit is a solid-phase ELISA based on the sandwich principle. All IFABP standards were reconstituted in accordance with the manufacturer's instructions (standard range 20 to 5000 pg/ml), and 100 ml of each standard urine test sample and control sample were used. After incubation with anti-IFABP antibody, excess antibody was removed by washing, and a second biotinylated tracer antibody to human IFABP was added. The bound antibody was subsequently conjugated with streptavidin-peroxidase and, after an additional washing, tetramethylbenzidine was added to the wells and incubated in the dark at 25° C. The reaction was stopped by the addition of citric acid and the absorbance at 450 nm was measured with a spectrophotometer. The total quantity of IFABP excreted over 24 hours (total IFABP-t) was calculated with reference to the urine volume.

Measurement of Intestinal Permeability

Intestinal permeability was determined by measuring the differential urinary excretion of enterally (oral or nasogastric tube) administered 100 ml solution of two polyethylene glycol (PEG) molecules (PEG 3350/400 ratio) over 24 hours, using high-flow liquid chromatography as previously described.[24,62]

Measurement of IgM Anti-Endotoxin Core Antibody

Anti-endotoxin core IgM antibodies to core glycolipid antigens were measured by a direct enzyme-linked immunosorbent assay (ELISA), as previously described by Barclay et al.[63,64] The normal ranges of these antibodies were determined from 1024 healthy adult blood donors, and the results were expressed as a percentage of the median (MU = median units) of the normal adult range: median IgM endotoxin cytoplasmic core antibody (EndoCAb) was 100.5 MU (tenth to ninetieth percentiles 38.84 to 258.81 MU, lower and upper quartiles 63.80 and 156.61 MU, respectively).

C-reactive Protein Assay

CRP was measured with the use of a standard ELISA kit (Dako, High Wycombe, UK). The normal CRP concentration in serum is less than 10 mg/L.

Statistical Analysis

Results were summarized using medians and interquartile ranges, and intergroup comparisons were performed using nonparametric tests—the Mann-Whitney or Pearson (rank) correlation test, as appropriate. An α probability of $P = 0.05$ was used to determine statistical significance.

RESULTS

A total of 61 patients with acute pancreatitis and 12 healthy control subjects were studied (Table 1). Groups were similar with regard to age and sex distribution. The etiology of acute pancreatitis was identified in 59 of 61 patients as follows: gallstones in 36, alcoholism in 13, endoscopic retrograde cholangiopancreatography in six, hyperlipidemia in three, and drug-related in one. No adverse effects of PEG were identified in this study. Clinical outcomes in patients with severe acute pancreatitis are shown in Table 2. Of the 19 severe attacks, 15 were complicated by single (n= 6) or multiple (n = 9) organ system failure; eight patients had significant pancreatic necrosis (≥50% of the gland on contrast-enhanced CT) and four patients subsequently developed pseudocysts. Among the nine patients with MOSF, two had necrosis affecting 50% or more of the gland (all died), and seven had no demonstrable necrosis (three died). Only one patient had CT-guided biopsy confirmation of infected pancreatic necrosis and subsequently underwent surgical debridement. No other patients underwent surgical intervention in the acute setting. Of the patients who had a severe attack, 11 (58%) were on total parenteral nutrition, and 15 (79%) were receiving prophylactic antibiotics (cephalosporins) intravenously.

The median APACHE II score within the initial 24 hours of admission was significantly higher in patients with severe attacks (11.5; range 5 to 27) compared to those with mild attacks (8; range 0 to 16) ($P = 0.002$; Mann-Whitney U test).

Table 1. Details of patients and control subjects

	Mild pancreatitis (n = 42)	Severe pancreatitis (n = 19)	Control (n = 12)	*P* value
Median age (yr)	60 (range 43–74)	63 (range 49–73)	56 (range 23–82)	NS†
Sex ratio (M/F)	23/19	12/7	7/5	NS*
Etiology				
Gallstones	27	9		
Alcohol	9	4		
ERCP	3	3		NS*
Hyperlipidemia	2	1		
Drug-related	0	1		
Idiopathic	1	1		
APACHE II score on admission				
Median	8 (range 0–16)	11.5 (range 5–27)		0.002†

ERCP = endoscopic retrograde cholangiopancreatography; NS = not significant.
*Chi-square test (± Yates correction as appropriate).
†Mann-Whitney U test.

Urine IFABP Levels

Intestinal FABP concentrations in 24-hour urine collections were significantly increased in patients with severe acute pancreatitis (median 1091.7 pg/ml [range 39.0 to 2890 pg/ml]) compared to patients with mild pancreatitis (median 84.5 pg/ml [range 0 to 2022 pg/ml; $P < 0.001$) and healthy control subjects (median 37.3 pg/ml; [range 1 to 83.6 pg/ml]; $P < 0.0001$) (Fig. 1). Patients with a mild attack had significantly higher urine IFABP concentrations compared to healthy control subjects ($P = 0.029$).

In view of significantly greater 24-hour urine volumes among patients with mild (median 2200 ml [range 1000 to 5100 ml]) as compared to severe (median 1350 ml [range 600 to 3800 ml; $P < 0.001$]) acute pancreatitis, total IFABP excretion was determined (IFABP concentration × 24-hour urine volume). Total IFABP was significantly greater among patients with a severe attack (median 1.14 μg [range 0.09 to 3.67 μg]) compared to those with a mild attack (median 0.21 μg [range 0 to 3.28 μg]; $P = 0.003$), and the latter was significantly greater than in healthy control subjects (median 0.06 μg [range 0 to 0.14 μg]; $P = 0.005$). A strong positive correlation was observed between the urine IFABP concentration and the total IFABP ($P < 0.001$, $r = 0.89$), as shown in Fig. 2.

Table 2. Clinical outcome of 19 patients with severe acute pancreatitis

Pseudocyst	4
Pancreatic necrosis	8
Single organ failure	6
MOSF	9
Death	5

Urine IFABP and Clinical Outcome

Patients who subsequently developed MOSF had higher urinary concentrations of IFABP (median 1097 pg/ml [range 39 to 2891 pg/ml]) compared to those in whom MOSF was absent (median 120 pg/ml

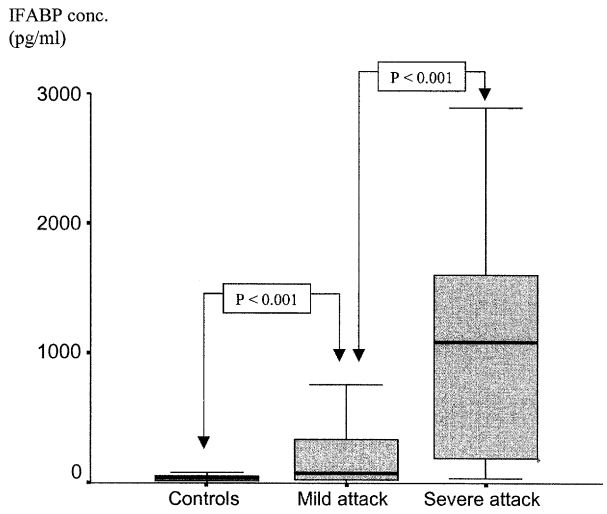

Fig. 1. Urine IFABP concentrations in patients with acute pancreatitis and healthy control subjects. There was a significant correlation between severity of disease and urine IFABP concentration; a severe attack was associated with a higher IFABP concentration compared to a mild attack ($P < 0.001$) and the latter with values from healthy control subjects ($P < 0.001$).

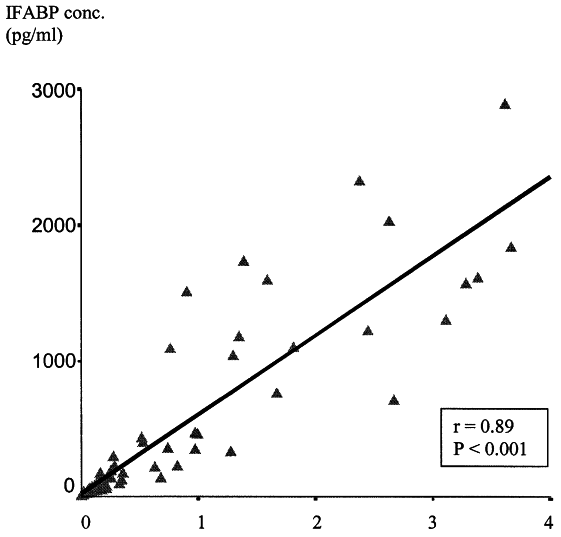

Fig. 2. Urine IFABP concentration vs. total IFABP excreted over 24 hours in patients with acute pancreatitis. A very strong positive correlation was observed ($r = 0.89$, $P < 0.001$).

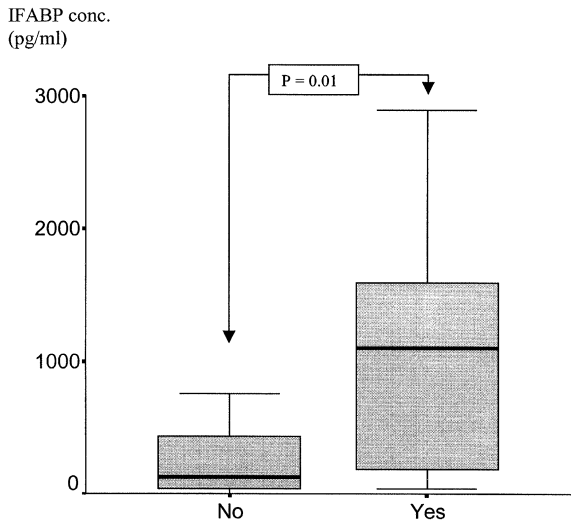

Fig. 3. Urine IFABP concentrations in patients with acute pancreatitis with and without MOSF. Patients who developed MOSF had a significantly higher IFABP concentration in urine compared to patients in whom MOSF was absent ($P = 0.01$).

[range 0 to 2022 pg/ml]; $P = 0.01$, Mann-Whitney U test) (Fig. 3). No correlation was observed with respect to the presence or absence of pancreatic necrosis. Of interest, the highest IFABP level was seen in a patient who developed infected pancreatic necrosis (>50%) and MOSF.

IgM Endotoxin Cytoplasmic Antibody and C-reactive Protein

IgM EndoCAb levels were significantly reduced in patients with severe attacks (median 43 MMU/ml [range 10.5 to 225.5 MMU/ml]) compared to those with mild attacks (median 65.6 MMU/ml [range 15.9 to 344.5 MMU/ml]; $P = 0.02$). Peak serum CRP was significantly increased in patients with severe attacks (median 272.5 mg/L [range 108 to 383 mg/L]) compared to those with mild attacks (median 115 mg/L [range 1 to 410 mg/L]; $P < 0.0001$, Mann-Whitney U test).

Intestinal Permeability

Intestinal permeability to macromolecules, as measured by the percentage of urinary excretion of PEG 3350, was significantly increased in patients with severe attacks of pancreatitis (median 1.24, range 0.03 to 6.78, SD 2.07) compared to patients with mild attacks (median 0.08, range 0.026 to 0.41, SD 0.087) and healthy control subjects (median 0.12, range 0.08 to 0.16, SD 0.07) ($P < 0.0001$) (Fig. 3). There was no difference in the excretion of the micromolecule PEG 400 between patients with severe or mild attacks and healthy control subjects (P = NS.).

The ratio of the urinary excretion of the two PEG molecules (PEG 3350/400) corrects for possible changes in intestinal motility and absorption between groups. It was significantly increased in patients with severe disease (median 0.072 [range 0.02 to 0.36]), compared to those with mild disease (median 0.007 [range 0.001 to 0.026]) and control subjects (median 0.009 [range 0.005 to 0.012]); ($P < 0.00001$). No significant differences in the excretion of either PEG molecule were observed between the group with mild disease and the control subjects, suggesting minimal changes in intestinal permeability, motility, and absorption.

Correlation Between Urine IFABP and Intestinal Macromolecular Permeability

A positive correlation was observed between the percentage of urinary excretion of the macromolecule PEG 3350 and both the urine IFABP concentration ($r = 0.5$; $P < 0.001$) (Fig. 4) and the total IFABP excreted ($r = 0.32$; $P = 0.02$, Pearsons' correlation test).

Correlation Between Urine IFABP, C-reactive Protein, and IgM EndoCAb

A significant positive correlation was observed between IFABP and plasma CRP levels (IFABP-c:r =

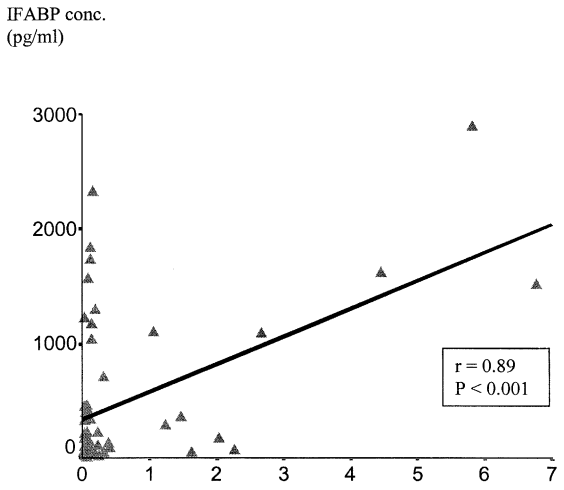

Fig. 4. Urine IFABP concentration vs. PEG 3350% retrieval in patients with acute pancreatitis. A strong positive correlation was observed ($r = 0.56$; $P < 0.001$).

0.54, $P < 0.001$ and IFABP-t excreted: r = 0.46, $P < 0.001$). Furthermore, serum IgM EndoCAb demonstrated a significant inverse correlation with the IFABP concentration ($r = -0.32$; $P = 0.02$) (Fig. 5).

DISCUSSION

Under normal physiologic conditions, the gastrointestinal tract receives a fifth of the cardiac output and is regulated by the nervous system and circulating vasoactive peptides. Relative hypovolemia, either through hemorrhagic, septic, or cardiogenic shock blood, is preferentially shunted to preserve the vital organs at the expense of the splanchnic circulation. The mature epithelium depends on a terminal arteriole for its blood supply, and the positioning of the venules around a central arteriole in the villus, and thus a countercurrent mechanism exists. A decrease in splanchnic perfusion results in a concomitant decrease in oxygen delivery to the intestinal mucosa; this coupled with the consequences of reperfusion leads to histologic evidence of mucosal ischemia. Loss of cell membrane integrity and cytoskeletal alterations during hypoperfusion results in the leakage of cytoplasmic proteins such as IFABP, which are subsequently delivered to the peripheral circulation and excreted in urine. We have demonstrated that increased IFABP levels in urine correlate with the severity of acute pancreatitis, as significantly higher levels were observed in patients with MOSF and necrosis compared to patients with mild attacks ($P < 0.001$). In addition, a strong positive correlation was also demonstrated with peak 0 to 72-hour plasma CRP levels ($r = 0.53$; $P < 0.001$). The observed levels of IFABP concentration in patients with a severe attack were almost 14-fold higher than in patients with mild attacks, and 30-fold greater than in the healthy population. Furthermore, urine concentrations of IFABP strongly correlated with total IFABP excreted in the urine over 24 hours ($r = 0.8$; $P < 0.001$), suggesting that levels were not simply a reflection of low excretion volumes. The study supports the hypothesis that a loss of functional integrity of the intestinal epithelium is related to a severe attack of acute pancreatitis, by showing a strong correlation between IFABP levels and gut macromolecular permeability, as determined by both PEG 3350 retrieval and the PEG 3350/400 index. Gollin et al.[59] have shown experimentally that ischemia, even in a relatively short segment, produced an elevation in the IFABP level similar to that observed when the entire small bowel was affected. Furthermore, it appears that these levels only increased after the restoration of blood flow after a period of ischemia, unless the ischemic episode was of a significantly prolonged duration. A number of previous studies have demonstrated the specificity of IFABP to ischemic bowel injury in the clinical setting. Kanda et al.[65] demonstrated similar serum levels of IFABP in healthy control subjects and patients with acute abdominal pain (peptic ulcers, appendicitis, gallstones, ischemic colitis, diverticulitis, peritonitis, and ureteric calculi). Levels appeared to be significantly elevated in patients with mesenteric ischemia and infarction. In a prospective study of 100 consecutive patients in a surgical intensive care unit, Lieberman et al.[66] re-

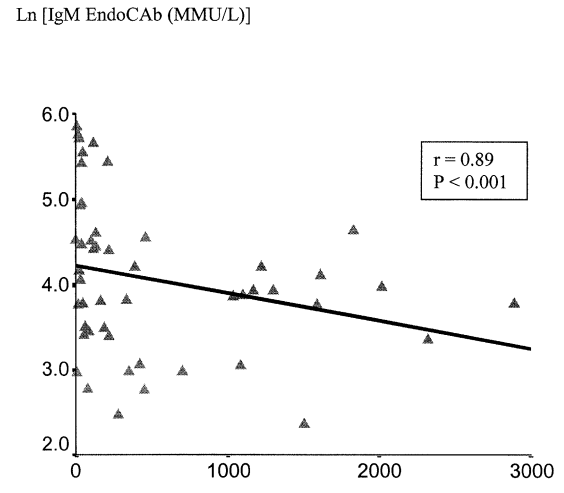

Fig. 5. Urine IFABP concentration and serum IgM EndoCAb concentration (natural logarithm). A significant inverse correlation was observed ($r = -0.32$; $P = 0.02$).

ported that elevated levels of IFABP correlated with the clinical development of systemic inflammatory responses. However, the median levels were only three- to fourfold different between patients with a systemic inflammatory resonse and both those without a systemic inflammatory response (with infections and/or organ failure) and a healthy control group. Furthermore, SIRS was only present in five patients, in whom the underlying pathology was not stated. It is therefore likely that splanchnic hypoperfusion coupled with reperfusion injury contributed to the significantly higher levels of IFABP observed in our patients with severe attacks of acute pancreatitis. It was interesting to observe that the highest IFABP level was found in the one patient with infected pancreatic necrosis, who subsequently underwent surgical debridement and a right hemicolectomy for what was observed to be an ischemic bowel. Mucosal injury in such circumstances may be mediated by oxygen-derived free radicals,[67–70] proinflammatory cytokines,[71] arachidonic acid metabolites, and lipopolysaccharides. A number of experimental studies have demonstrated this phenomenon in acute pancreatitis. In a retrograde taurocholate-induced model of acute pancreatitis, alterations in the pancreatitis-associated intestinal microcirculation were evaluated by measuring carbon dioxide metabolism.[22] An increase in production and transport of CO_2 from tissue to blood was detected in response to hypovolemia. A compromise in gut capacity to extract oxygen was additionally observed, principally in the subserosal regions.[56] Hotz et al.[72] showed that an impairment of colonic capillary perfusion correlates with the severity of pancreatitis and precedes the development of increased gut permeability. Microcirculatory disturbances in colonic blood flow have also been reported by Klar,[73] who demonstrated increased survival and pancreatic perfusion in an experimental model of isovolemic hemodilution. Hunt and Mildenhall[74] and Russell et al.[75] suggested that pancreatic inflammation might involve the mesentery and cause ischemic changes through a circulatory disturbance, according to their findings of hemosiderin-laden macrophages in the mesentery and evidence of submucosal vascular thrombosis in their examination of resected specimens. Jensen and Bradley[76] reported four cases of segmental intestinal infarctions associated with acute mesenteric vein thrombosis after acute pancreatitis.

In patients with severe acute pancreatitis, MOSF is responsible for early disease mortality, whereas sepsis supervenes later and remains the principal cause of death.[4] An increase in intestinal permeability to large toxic molecules, such as endotoxin, and possibly bacteria is a derangement in gut barrier function that is central to the hypothesis implicating the gut in the development of sepsis and MOSF.[16] Previous experimental and clinical studies demonstrated an increase in intestinal permeability to macromolecules[22–24] and identified the gut as an important source of infection during acute pancreatitis.[25,26] Wang et al.[22] demonstrated an increase in the mucosal permeability of the distal ileum and colon in vitro to the macromolecule ovalbumin. A number of studies have also demonstrated increased exposure to endotoxins in patients with severe acute pancreatitis.[20,24,32,77] Further evidence of endotoxin exposure is drawn from observing the serum concentrations of IgM anti-endotoxin core antibodies, which bind to circulating endotoxin before their subsequent removal by the reticuloendothelial system.[64] IgM anti-endotoxin antibodies are almost exclusively found in the circulation and have been shown to fall in the presence of endotoxin within the circulation.[24,32,64,77] The present study not only confirms previous reports of a decrease in IgM anti-endotoxin antibodies associated with severe acute pancreatitis,[24,32] but also has demonstrated an inverse correlation with IFABP levels. The results of our study therefore suggest that loss of small bowel mucosal integrity because of splanchnic hypoperfusion significantly contributes to the loss of gut barrier function that is observed in severe acute pancreatitis. The development of systemic endotoxemia may, in turn, act through a positive feedback mechanism, either directly or through the release of cytokines,[78] to further increase intestinal permeability,[79,80] impair host immunity,[81] and promote bacterial translocation from the gut,[80,82] thus resulting in a vicious circle. Abnormalities in immune function, such as a reduction in circulating levels of CD4-positive (T-helper) lymphocytes,[83] a decrease in delayed-type skin hypersensitivity,[84] impaired cell-mediated immunity (depressed production of interleukin-2),[85] and systemic phagocytic function[86] have all been described in experimental pancreatitis. Disruption of intestinal microflora was found in the small intestine and cecum of animals after the induction of acute pancreatitis, with a significant increase in gram-negative bacterial counts.[25] Such overgrowth of bacteria may be the product of decreased intestinal motility.[25,87] In acute pancreatitis, it has been shown that intravenous antibiotic prophylaxis,[88,89] selective decontamination of pathologic bacteria from the gut,[89–92] therapy with immunomodulators,[86] and stabilization of increased gut permeability[93] reduce pancreatic infections in experimental and human settings. Of greater interest, and perhaps greater relevance to our findings of splanchnic ischemia/reperfusion injury contributing to severe disease, hyperoxia,[94] nitric oxide,[46] inducible nitric oxide synthase inhibitors,[95] allopurinol (a free radical scavenger),[96,97] and

platelet-activating factor antagonists[97–99] have all been shown to enhance intestinal barrier function and restore intestinal permeability in experimental models of intestinal reperfusion injury and acute pancreatitis. It must be emphasized that although clinical trials that employed platelet-activating factor antagonists,[100] or allopurinol,[101] have failed to show a significant benefit in infectious complications or overall mortality in acute pancreatitis, secondary end points have not included the integrity of the gut mucosal barrier.

Although it remains unclear from this study as to whether ischemia/reperfusion injury or mucosal hypoxia is responsible for the loss of mucosal integrity, the significantly elevated levels of IFABP strongly support the former. Our findings suggest that splanchnic hypoperfusion and loss of small bowel mucosal integrity are associated with a severe attack of acute pancreatitis. The clinical implications of our findings are that goal-directed therapy with the use of gastric tonometry or IFABP, together with agents that improve mucosal blood flow and protect against ischemia/reperfusion injury, and avoidance of agents that redistribute blood away from the mucosa, may preserve intestinal permeability. The benefit of such an approach will need to be tested in well-conducted prospective clinical trials.

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Discussion

Dr. M. G. Sarr (Rochester, MN): You have used the term intestinal ischemia. How do you know that the mucosa is really ischemic? I understand your implications, but couldn't this protein be released from some other factor other than ischemia?

Mr. S. Rahman: From what we gather from animal studies, ischemia appears to be the cause of the release, but I think we should perhaps use the term perfusion abnormalities. There are no experimental data to suggest whether hypoxia, per se, rather than ischemia can, in fact, result in a similar type of injury. So your point is well taken, but we are basing our report on what is already known. Certainly hypoxic injury may induce similar types of problems.

Dr. Sarr: I was also hoping you were going to be able to correlate this with pancreatic necrosis that becomes infected, because the next implication is that you have affected permeability; that is, you have increased, theoretically, bacterial reflux into some type of a system, and supernecrosis and/or superinfection. Have you looked at infection?

Mr. Rahman: We have two studies that are in progress. One is a prospective follow-up study and, to answer your question regarding necrosis, I think the numbers here are far too small, but this is something that we will be looking at in the future.

Dr. M.D. Duncan (Baltimore, MD): You have demonstrated a relationship between markers of pancreatitis and the effect of PEG absorption or permeability. I am wondering if you have looked at, or others have looked at,

mediators such as platelet-activating factor (PAF) and the use of the PEG permeability test as a way to test the efficacy of such mediators, or blockade of the mediators such as the PAF antagonist?

Mr. Rahman: Not that I am aware. My predecessor, Basil Ammori, in the unit showed a direct correlation between PEG absorption and pre-procalcitonin, and also endotoxin exposure, but we have not looked at whether the PEG molecule itself is injurious to the mucosa, as well.

Dr. J.B. Matthews (Cincinnati, OH): I am confused as to how FABP appears in the bloodstream. You drew diagrams showing how serum FABP reflects epithelial permeability, but I do not understand how a villous cell marker, which is where FABP is expressed, leaks backward through the endothelium to gain access to the bloodstream. When the epithelium is ischemic, the villous tips slough into the lumen. Do you know the mechanism of how FABP would get absorbed? Do you have any evidence to help us understand whether there are region-specific changes in mucosal permeability? You have ascribed it to small intestine, but there actually are methods to distinguish gastric versus small intestinal versus colonic permeability using multiple markers with different permeability properties. I wonder whether you have had a chance to look at that?

Mr. Rahman: In answer to your first question, I think if you refer back to the original work done on intestinal FABP, the mechanism has not been described on why intestinal FABP gets into the circulation, but certainly the levels have been shown to rise in animal studies. In answer to your second question, permeability changes may occur throughout the colon. I think that most of the experiments that have been done, both in animals and humans, suggest that permeability changes may occur toward the distal ileum and the proximal colon, but I think the purpose of this study was to show that the small bowel is indeed a player in permeability changes, and perhaps enteral nutrition may play a key role in trying to limit the damage. But certainly we are looking at markers specifically toward the large bowel now.

Invited Discussion—Expert Commentator

Richard A. Hodin, M.D.: This paper relates to the issue of the gut mucosal barrier, which appears to be impaired in a variety of severe illnesses including trauma and sepsis. Most of our present knowledge with regard to gut barrier dysfunction comes from animal models, but the true incidence and importance of this issue in human illnesses has not been clarified. This study is, therefore, extremely interesting because the investigators looked at a population of patients (i.e., humans) with either mild or severe acute pancreatitis. Gut barrier function was determined by examining the urinary excretion of macromolecular probes delivered into the gastrointestinal tract. In addition, urinary levels of the intestinal FABP were measured, because this has been shown in animals to be a marker for gut barrier dysfunction.

The results demonstrate a strong correlation between illness severity and gut barrier dysfunction. As such, this is one of the few studies in humans that provides convincing evidence that this gut mucosal dysfunction is not just an experimental phenomenon but may be important in the clinical arena. The assumption that the gut was ischemic must be questioned, however, because we do not know that was the case. It will be of interest in future studies to determine the actual mechanism underlying the gut barrier dysfunction in patients with severe acute pancreatitis. In addition, the true clinical value, if any, of measuring urinary intestinal FABP levels in severely ill patients should be determined in proper controlled studies.

Restoring Apoptosis in Pancreatic Cancer Cells by Targeting the Nuclear Factor-κB Signaling Pathway With the Anti-Epidermal Growth Factor Antibody IMC-C225

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We have previously demonstrated that RelA is constitutively activated in the majority of human pancreatic cancers and plays an important role in tumorigenesis and metastasis. The antiapoptotic gene *bcl-xl* is a downstream target of RelA, and regulation of *bcl-xl* transcription is mediated directly by the nuclear factor κB (NF-κB) binding sites present in the upstream promoter element of the *bcl-xl* gene. In this study we investigated the effects of inhibition of epidermal growth factor receptor (EGFR) signaling pathway with the anti-EGFR monoclonal antibody IMC-C225 on constitutive NF-κB activation and regulation of apoptosis-related genes in human pancreatic cancer cells. We found that activation of EGFR can be blocked with the anti-EGFR antibody IMC-C225 in the human pancreatic cancer cell line MDA Panc-28, leading to a marked decrease in constitutive NF-κB DNA binding activity. Our data also suggest that downregulation of NF-κB DNA binding activity by IMC-C225 leads to a decrease in *bcl-xl* and *bfl-1* expression. Therefore, targeting the NF-κB signaling pathway with an anti-EGFR antibody may be one strategy to restore apoptosis in human pancreatic cancer cells, thereby enhancing the effect of chemotherapy and radiation therapy. (J GASTROINTEST SURG 2003;7:37–43.)

KEY WORDS: Pancreatic cancer, epidermal growth factor receptor, NF-κB, bcl-xl, apoptosis

Pancreatic cancer exhibits a highly malignant phenotype characterized by perineural invasion and early metastasis to distant organs.[1] Conventional therapies including chemotherapy and irradiation have had a limited impact on the disease. The resistance of pancreatic cancer cells to apoptosis is thought to render most chemotherapeutic agents and radiation therapy ineffective.[2,3]

RelA/nuclear factor–kappa B (NF-κB) is a family of pleiotropic transcription factors that orchestrate the expression of a plethora of genes essential in growth, oncogenesis, differentiation, apoptosis, tumorigenesis, and immune and inflammatory responses.[4–7] These factors are activated by many stimuli, including proinflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1).[4,5] The transcriptional activity of NF-κB proteins is regulated by their interaction with the family of NF-κB inhibitors, known as IκB. This interaction results in the formation of inactive NF-κB:IκB complexes in the cytoplasm. The inducible phosphorylation by IκB kinase and subsequent proteasome-mediated degradation of IκB inhibitors release NF-κB for translocation into the nucleus to regulate transcription of specific genes.[4,8] Several reports have suggested that members of the RelA/NF-κB and IκB families are involved in the development of cancer[9–13] and that RelA plays an important role in protecting

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002, (oral presentation).

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Supported by The Lockton Fund for Pancreatic Research at the University of Texas M.D. Anderson Cancer Center and The Cancer League of Bern, Switzerland (G.M.S.).

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Published by Elsevier Science Inc.
1091-255X/03/$—see front matter
PII: S1091-255X(02)00088-4

cells from proapoptotic stimuli.[14–16] Recently we showed that RelA and the RelA target gene urokinase-type plasminogen activator (*uPA*) were constitutively activated in approximately 70% of human pancreatic cancers and in 9 of 11 human pancreatic cancer cell lines but not in normal pancreatic tissues and nontumorigenic pancreatic epithelial cells.[17,18] We also showed that the antiapoptotic gene *bcl-xl* is a downstream target of RelA with two putative NF-κB binding sites on its promoter and that transcription of *bcl-xl* is induced by constitutive RelA/NF-κB activity in human pancreatic cancer cells.[19]

Human tumors express high levels of growth factors and their receptors, and many malignant tumors exhibit autocrine- or paracrine-stimulated growth.[20] Among the best-studied growth factor receptor systems is the epidermal growth factor receptor (EGFR) family (also known as type I receptor tyrosine kinases or ErbB tyrosine kinase receptors), comprising four homologous receptors: EGFR (ErbB1 or HER1), ErbB2 (HER2/neu), ErbB3 (HER3), and ErbB4 (HER4).[21,22] Approximately one third of all human epithelial cancers express high levels of the EGFR.[23] EGFR is overexpressed in 30% to 50% of human pancreatic tumors,[24,25] and simultaneous overexpression of EGFR and its ligands is associated with enhanced tumor growth and metastasis.[26] Several studies have shown that EGFR can activate NF-κB in various human cancer cell lines.[27] There is also evidence that transactivation of EGFR by TNF-α leads to NF-κB activation[28] and that a high level of EGFR expression is optimal for epidermal growth factor–induced NF-κB activation.[27] However, the signals generated by EGFR that lead to NF-κB activation remain unknown.[29–32]

The cumulative data suggest that the overexpression of EGFR in human pancreatic adenocarcinoma cell lines may be critical to the mediation of constitutive NF-κB activity and resistance to apoptosis. Therefore we investigated the effects of inhibition of EGFR with the anti-EGFR monoclonal antibody (mAb), IMC-C225, on NF-κB DNA binding activity and regulation of apoptosis-related genes.

MATERIAL AND METHODS

Cell Line and Tissue Culture

The human pancreatic tumor cell line MDA Panc-28 was maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin (Gibco BRL), and 10 mg/ml streptomycin (Gibco BRL).[33] Adherent monolayer cultures were maintained on plastic and incubated at 37° C in a humidified 5% CO_2/95% air atmosphere for use in the experiments that follow.

Cell Proliferation Assays

Cell proliferation assays were performed in six-well culture plates in triplicate. Cells were seeded onto plates and allowed to attach for 24 hours prior to treatment. To examine the inhibitory effects of the anti-EGFR mAb IMC-C225 (kindly provided by ImClone Systems, New York, NY), the cells were cultured in DMEM supplemented with 10% FBS in the presence or absence of 20 nmol/L IMC-C225 for 24 to 96 hours. Cell numbers were determined with a Coulter Z1 particle counter (Beckman Coulter, Miami, FL) after the cells were harvested by trypsinization.

Western Blot Analysis for Epidermal Growth Factor Receptors

For Western blot analysis, cells were lysed in NP-40 lysis buffer (50 mmol/L Tris-HCl [pH 7.4], 150 mmol/L NaCl, 0.5% NP-40, 50 mmol/L NaF, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonyl fluoride, 25 g/ml leupeptin, and 25 g/ml aprotinin) at 4° C after 6, 12, 24, 36, and 48 hours of incubation with IMC-C225. The supernatants were then cleared by centrifugation, and total protein concentrations were measured using the Coomassie plus protein assay reagent (Pierce Chemical, Rockford, IL). Twenty micrograms of protein sample (per lane) were mixed with an equal volume of Laemmli sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 minutes at 100° C. The samples were separated using 10% SDS-polyacrylamide gel electrophoresis and then transferred onto a polyvinyldenedifluoride (PVDF) membrane (Osmonics, Westborough, MA). The membrane was blocked with 5% nonfat milk in phosphate-buffered saline (PBS) containing 0.2% Tween-20 and incubated with polyclonal rabbit antibodies against EGFR (Santa Cruz Biotechnology, Santa Cruz, CA) or bcl-xl (Santa Cruz Biotechnology), or with monoclonal mouse antibodies against tyrosine-phosphorylated EGFR (BD Transduction Laboratories, San Diego, CA) or β-actin (Sigma-Aldrich, St. Louis, MO). Membranes were washed in PBS containing 0.2% Tween-20 and probed with horseradish peroxidase-coupled secondary goat antirabbit or antimouse antibodies (Amersham, Arlington Heights, IL). Antibodies were detected using the Lumi-Light Western blotting substrate (Roche, Indianapolis, IN) according to the manufacturer's instructions.

Electrophoretic Mobility Shift Assay

An electrophoretic mobility shift assay (EMSA) was performed using nuclear extracts prepared as described previously.[34] After preparation, 10 μg of nuclear extract was incubated with 1 μg of poly (dI-dC) (Pharmacia, Piscataway, NJ) in 10 μl of binding buffer (75 mmol/L NaCl, 15 mmol/L Tris-HCl [pH 7.5], 1.5 mmol/L EDTA, 1.5 mmol/L dithiotreitol (DTT), 25% glycerol, and 20 μg/ml bovine serum albumin) for 30 minutes at 4° C. The ^{32}P-labeled probes used contained the following double-stranded oligonucleotides: wild-type κB (5′-AGTTGAGGG GACTTTCCCAGGC-3′) and Oct-1 (5′-TGTCG AATGCAAATCACTAGAA-3′) consensus sequences. Equal loading of nuclear extracts was monitored by assessment of Oct-1 binding. The probes were added to the extracts and allowed to bind for 20 minutes, at room temperature. Reaction mixtures were analyzed on 4% polyacrylamide gels containing 0.25× tris-borate-EDTA (TBE) buffer (22.5 mmol/L Tris, 22.5 mmol/L borate, and 0.5 mmol/L EDTA [pH 8.0]). Each gel was then dried for 1 hour at 80° C and exposed to Kodak film (Eastman Kodak, Rochester, NY) at −80° C.

Ribonuclease Protection Assay

After treatment with IMC-C225 for 6 to 36 hours, cells were harvested, and total RNA was prepared using Trizol reagent (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. A custom-made Riboquant Multiprobe RNase Protection Assay System (Pharmingen, San Diego, CA) and multiprobe hAPO-2 template set (Pharmingen) were used for the RNase protection assay. The ^{32}P-labeled riboprobes were hybridized for 18 hours with 20 μg of total RNA. The hybridized RNA was digested with RNase, and the remaining RNase-protected probes were purified and resolved on denaturing polyacrylamide gels. Equal loading of mRNA samples was monitored by assessment of the two housekeeping genes, *L32* and *GAPDH*.

Determination of DNA Fragmentation

DNA fragmentation in apoptotic cells was measured by DNA gel electrophoresis. The cells were treated for 48 hours with 20 nmol/L IMC-C225, 50 μmol/L gemcitabine, or both. The cells were then collected and washed with PBS, followed by incubation with extraction buffer (10 mmol/L Tris [pH 8.0], 0.1 mmol/L EDTA, 0.5% SDS, and 20 μg/ml RNase) at 37° C for 1 hour. The sample was incubated at 50° C for 3 hours with 4 μmol/L/ml proteinase K. DNA was extracted with phenol:chloroform and chloroform. The aqueous phase was precipitated with two volumes of 100% ethanol and 1/10 volume of 3 mol/L sodium acetate on ice for 30 minutes. The DNA pellet was then washed with 70% ethanol and resuspended in 50 μl Tris-EDTA buffer. The absorbance of the DNA solution at 260 nm and 280 nm was determined by spectrophotometry. The extracted DNA (40 μg/lane) was electrophoresed on a 2% agarose gel, and the gel was stained with 50 μg/ml ethidium bromide for 30 minutes, and then photographed.

RESULTS

We observed inhibition of MDA Panc-28 cell growth after 1 day of treatment with 20 nmol/L IMC-C225 (Fig. 1). Growth inhibition increased over time and reached nearly 30% after 96 hours, compared with nontreated MDA Panc-28 cells. In accordance with previous reports,[20,23,36,37] there was no significant difference in cell viability between treated and control cells, as determined by exclusion staining with Trypan blue (data not shown).

To determine whether IMC-C225 blocks EGFR activation, we incubated MDA Panc-28 cells with IMC-C225 for up to 48 hours and performed Western blot analysis using antibody specific against tyrosine-phosphorylated (activated) EGFR. Phosphorylation of EGFR was markedly decreased after 12 hours of incubation and no longer detectable after 24 hours, whereas no changes in the level of total EGFR were detected (Fig. 2). These data strongly suggest that activation of EGFR in pancreatic cancer cells can be blocked by IMC-C225.

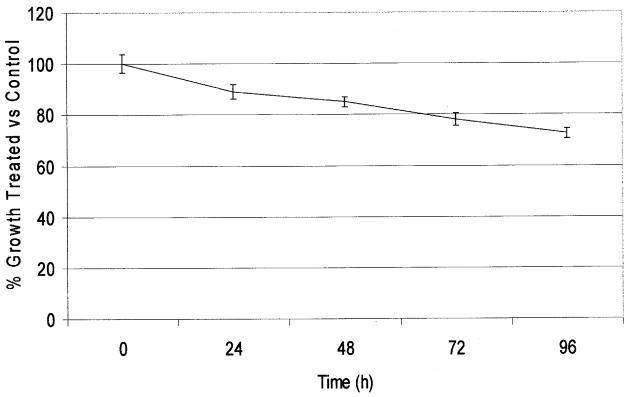

Fig. 1. Monoclonal antibody IMC-C225 inhibits cell proliferation in human pancreatic cancer cell line MDA Panc-28. Incubation of MDA Panc-28 cells with 20 nmol/L IMC-C225 increases growth inhibition over time. Inhibition is nearly 30% after 96 hours of incubation.

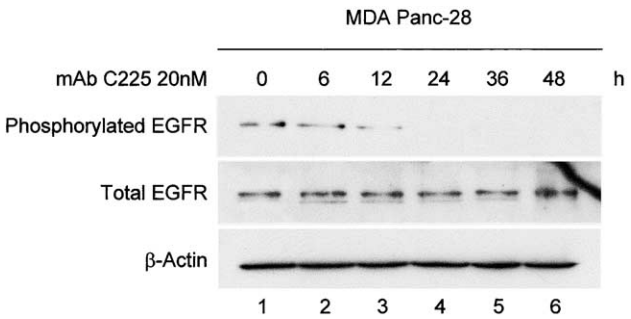

Fig. 2. Monoclonal antibody (*mAb*) IMC-C225 blocks activation of EGFR in human pancreatic cancer cell line MDA Panc-28. Western blot analysis shows that phosphorylation and subsequent activation of EGFR are markedly decreased after 12 hours of incubation with 20 nmol/L IMC-C225 and no longer detectable after 24 hours. No changes in the level of total EGFR are detected. β-Actin serves as the loading control.

To determine if the DNA binding activity of NF-κB can be reduced by blocking EGFR, we assessed DNA binding activity after incubating MDA Panc-28 cells with 20 nmol/L IMC-C225. After 48 hours of treatment, the DNA binding activity of NF-κB was significantly decreased (Fig. 3). This effect was also present after 12 hours of treatment with IMC-C225.

Recently it has been suggested that *bcl-xl* is a downstream target of NF-κB.[19,35] Therefore we treated MDA Panc-28 cells with 20 nmol/L IMC-C225 and determined NF-κB DNA binding activity to the two NF-κB binding sites in the bcl-xl promoter. After 12 hours of treatment, NF-κB DNA binding activity and the level of bcl-xl protein were already decreased; 48 hours of exposure to IMC-C225 resulted in a significant decrease in NF-κB DNA binding activity (by electrophoretic mobility shift assay [EMSA]) as well as in bcl-xl protein level (by Western blot analysis) (Fig. 4, *A* and *B*). IMC-C225 had a similar effect on the expression of *bcl-xl*- and *bfl-1* mRNA, another antiapoptotic member of the bcl-2 family (Fig. 4, *C*). Our data suggest that NF-κB DNA binding activity and expression of *bcl-xl* and *bfl-1* are downregulated in response to treatment with IMC-C225.

To determine whether inhibition of constitutive NF-κB activation by IMC-C225 sensitizes pancreatic

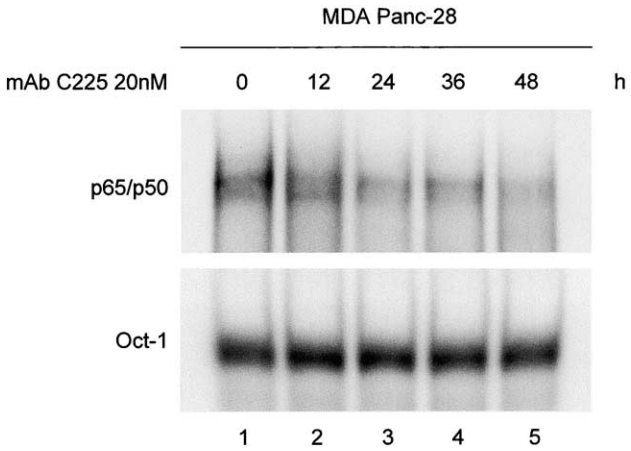

Fig. 3. Blocking of EGFR by IMC-C225 leads to a decrease in NF-κB DNA binding activity in human pancreatic cancer cell line MDA Panc-28. After 48 hours of treatment with 20 nmol/liter IMC-C225, the DNA binding activity of NF-κB is significantly reduced. The observed effect starts after 12 hours of treatment with IMC-C225. Oct-1 serves as the loading control.

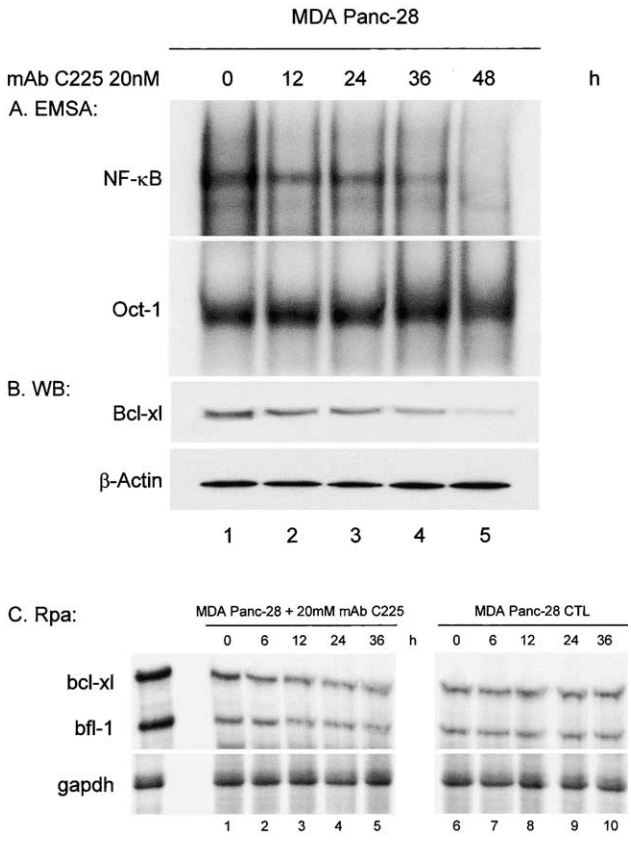

Fig. 4. Downregulation of *bcl-2* family members in MDA Panc-28 cells after treatment with anti-EGFR IMC-C225. After 48 hours of treatment with 20 nmol/L IMC-C225, a drastic decrease in NF-κB DNA binding activity as well as the amount of bcl-xl protein is observed (**A** and **B**). β-Actin and Oct-1 serve as the loading controls. Besides *bcl-xl*, a remarkable effect on bfl-1 mRNA (**C**) is detectable after 12 hours of treatment with 20 nmol/L IMC-C225. GAPDH serves as the loading control.

cancer cells to chemotherapy-induced apoptosis, MDA Panc-28 cells were incubated with IMC-C225, gemcitabine, or both for 48 hours, and then apoptosis was determined by assessment of DNA fragmentation (Fig. 5). IMC-C225 alone and gemcitabine alone induced weak apoptosis (see Fig. 5, lanes 2 and 3), whereas treatment with both IMC-C225 and gemcitabine induced strong apoptosis (see Fig. 5, lane 4).

DISCUSSION

Activation of the *K-ras* oncogene by point mutation and EGFR by overexpression, and functional inactivation of the tumor suppressor genes *p16*, *p53*, and *DPC4* are the frequent genetic alterations in human pancreatic cancer.[3] However, the interplay of these genetic alterations and specific signaling cascades that mediate chemotherapy-resistant pancreatic cancer phenotypes is poorly understood. To develop more effective therapeutic strategies for pancreatic cancer, it is important to identify targets that play key roles in cellular sensitivity to chemotherapy-induced apoptosis. A mechanism by which tumor cells may gain resistance to apoptosis is the activation of NF-κB,[36] a genetic alteration recently reported by our group.[17] Several reports have shown that RelA/NF-κB plays an important role in protecting cells from proapoptotic stimuli.[14]

In the present study we sought to restore pancreatic cancer cell apoptosis by targeting the NF-κB signaling pathway with an anti-EGFR monoclonal antibody. We found that treatment of MDA Panc-28 cells with the anti-EGFR monoclonal antibody IMC-C225 increased growth inhibition over time (see Fig. 1). The observed growth inhibitory effect of IMC-C225 was probably due to cell cycle modulation. This is in accord with previous studies showing that treatment with IMC-C225 slows cell proliferation and arrests the cell cycle in the G1 phase.[37,38] Activation of EGFR was no longer detectable after 24 hours of treatment with IMC-C225, whereas no changes in the level of total EGFR could be detected (see Fig. 2). This strongly suggests that activation of EGFR in pancreatic cancer cells can be blocked by IMC-C225. Finally, the constitutive DNA binding activity of NF-κB in MDA Panc-28 cells was significantly reduced after 48 hours of treatment with IMC-C225 (see Fig. 3), suggesting that constitutive activation of NF-κB may be triggered by the EGFR signaling pathway.

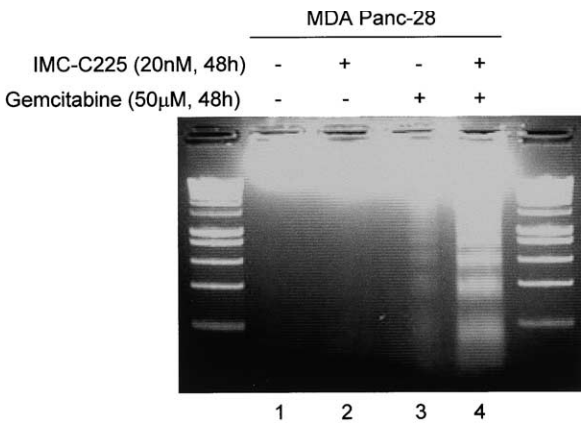

Fig. 5. DNA fragmentation of apoptotic cells. Low levels of apoptosis are detectable after 48 hours of treatment with either IMC-C225 or gemcitabine alone (*lanes 2 and 3*). Strong apoptosis is observed after combined treatment with IMC-C225 and gemcitabine for 48 hours (*lane 4*). No apoptosis is detectable in control cells (*lane 1*).

In previous studies we demonstrated that the antiapoptotic *bcl-xl* gene is a downstream target of RelA and that its transcription is induced by constitutive RelA/NF-κB activity.[35] Further, the regulation of *bcl-xl* transcription may be directly mediated by the NF-κB binding sites present in the upstream promoter element of the *bcl-xl* gene.[19] Thus the dysregulation of RelA/NF-κB activity in tumor cells is critical to the inhibition of proapoptotic stimuli. We therefore hypothesized that apoptosis may be restored in tumor cells by targeting the NF-κB signaling pathway with the anti-EGFR IMC-C225. After treatment of MDA Panc-28 cells with IMC-C225 for 48 hours, NF-κB DNA binding activity as well as expression of bcl-xl (see Fig. 4, *A* and *B*) were dramatically decreased. Further, *bcl-xl* transcription was markedly suppressed after 36 hours of treatment with IMC-C225 (Fig. 4, *C*). The same effect was detected on *bfl-1* mRNA, another antiapoptotic *bcl-2* family member. These results suggest that IMC-C225 is an effective agent in the suppression of NF-κB DNA binding activity and of *bcl-xl* expression in MDA Panc-28 cells.

We also studied whether pancreatic cancer cell apoptosis could be enhanced by the combination of gemcitabine and anti-EGFR–induced downregulation of constitutive NF-κB activity. After 48 hours of treatment with IMC-C225 or gemcitabine alone, low levels of apoptosis were observed; however, treatment of the cells with both IMC-C225 and gemcitabine significantly increased apoptosis. These data suggest that IMC-C225 may sensitize pancreatic cancer cells to chemotherapy-induced apoptosis by downregulation of antiapoptotic genes.

In summary, we have demonstrated that activation of EGFR can be blocked with the monoclonal

antibody IMC-C225 in the human pancreatic cancer cell line MDA Panc-28. Inhibition of EGFR leads to a marked decrease in the constitutive NF-κB DNA binding activity in these cells, suggesting an important role of EGFR in NF-κB activation. Two members of the *bcl-2* family, *bcl-xl* and *bfl-1*, are regulated by NF-κB, and downregulation of NF-κB DNA binding activity by IMC-C225 in pancreatic cancer cells leads to a decrease in *bcl-xl* and *bfl-1* transcription. These results suggest that the blockade of EGFR by IMC-C225 may be effective in the downregulation of antiapoptotic genes. Therefore targeting the NF-κB signaling pathway with an anti-EGFR monoclonal antibody may be a valuable tool in restoring apoptosis to pancreatic cancer cells, thereby potentiating the anticancer activity of chemotherapy and/or radiation.

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Discussion

Dr. K.S. Kirkwood (San Francisco, CA): First of all, this is an extraordinary amount of data. Congratulations. You show complete blockade of activated EGFR and downregulation of downstream mediators as well, but only a modest reduction in the cell proliferation assay, 30% or so. I was wondering if you are attributing that to other dominant factors that contribute there, or what your hypothesis is?

Dr. G. Sclabas: C225 is not a very powerful cytotoxic agent; it mainly is cytostatic. One sees growth inhibition of approximately 20% to 30% in different cell lines, in addition to the pancreatic cancer in vitro model as shown (Ye D et al. Clin Cancer Res 1999;5:2171–2177). The therapeutic goal is more to sensitize the cells to apoptosis induced by chemotherapeutic agents.

Dr. M.P. Callery (Boston, MA): I have a follow-up to Dr. Kirkwood's question. Do you know if any of the cell cycle checkpoint regulatory proteins may have accumulated that are otherwise dependent for their turnover with NK-κB metabolism?

Dr. Sclabas: We did not further study this because previous publications have shown that C225 leads to GI cell cycle arrest (Wu X et al. Oncogene 1996;12:1397–1403; and Peng D et al. Cancer Res 1996;56:3666–3669).

Dr. Callery: You may find some of these proteins will accumulate and that will help with respect to the cell proliferation data. Also, as apoptosis is restored, can other effectors of apoptosis be identified, for example, the caspases increasing and such?

Dr. Sclabas: No. In the RNase protection assay we did not see any impact on transcription of the initiator caspases 8 and 9. But I have to say that we did not investigate the activation of these caspases or the transcription or the activation of the effector caspases (Shi Y et al. Mol Cell 2002;9:459–470).

Dr. J.P. Hoffman (Philadelphia, PA): I am wondering if your hypothesis about the sensitization toward apoptosis has been confirmed? If the gemcitabine is given first, does that abrogate the response before the C225?

Dr. Sclabas: Yes, the cells must be pretreated to block the EGFR and then gemcitabine must be given. Otherwise the effect of C225 will not be seen (Bruns CJ et al. Clin Cancer Res 2000;6:1936–1948).

Invited Discussion—Expert Commentator

L. William Traverso, M.D. (Seattle, WA): Lack of apoptosis is one of the reasons why chemotherapy does not work in pancreatic cancer. The authors are trying to restore apoptosis by treating a cancer cell line with an antibody to EGFR (the receptor for epithelial growth factor). After antibody treatment, they were able to block these receptors, whereas overall levels of the EGF remained the same, and therefore EGF is presumably inactive. At the same time there was a decrease in NF-κB, a signal protein in the nucleus that prevents apoptosis. Cell proliferations were also noted to decrease by 30%. These investigators have yet to prove that they can reverse chemoresistance. They will have to show increased apoptosis in an animal model—the two groups would be chemotherapy with and without antibody. Apoptosis would have to be measurably higher in the chemotherapy plus antibody group. Note the paradox of not enough apoptosis in cancer and too much apoptosis in severe pancreatitis—a paper that will be reviewed next.

Clinical Outcomes After Laparoscopic Antireflux Surgery in Patients With and Without Preoperative Endoscopic Esophagitis

Ketan M. Desai, M.D., Margaret M. Frisella, R.N., Nathaniel J. Soper, M.D.

A wide spectrum of endoscopic findings exists in patients with gastroesophageal reflux disease (GERD). This study compared clinical outcomes after laparoscopic antireflux surgery (LARS) in patients who had GERD with and without preoperative endoscopic esophagitis. From 1992 to 2001, a total of 414 patients who underwent LARS with 6 months or more of follow-up were prospectively entered into a database. Among these patients, 84 (20%) had no endoscopic evidence of esophagitis on preoperative endoscopy (group 1), whereas 330 (80%) did have esophagitis (group 2). Perioperative outcomes, GERD symptom relief, and the use of acid-reducing medication were assessed. Preoperative DeMeester scores in groups 1 and 2 were 44 ± 29 and 61 ± 62 ($P < 0.05$) and mean esophageal peristaltic amplitude was 86 ± 32 mm Hg vs. 60 ± 42 mm Hg, respectively ($P < 0.05$). Although procedure time was significantly shorter in group 1, other perioperative outcomes were similar. At postoperative follow-up, the use of proton pump inhibitors was reduced in both groups (86% to ≤14%). With the exception of postoperative dysphagia, there was no difference in GERD symptom relief between groups 1 and 2. The presence or absence of preoperative esophagitis has minimal effect on favorable symptomatic outcomes after LARS. Thus LARS is an effective treatment option for patients, irrespective of endoscopic evidence of esophagitis, leading to excellent symptom relief and a marked reduction in the use of proton pump inhibitors. (J GASTROINTEST SURG 2003;7:44–52.)

KEY WORDS: Gastroesophageal reflux disease, esophagitis, laparoscopy, surgery

Gastroesophageal reflux disease (GERD) is a common disorder of the foregut with multifactorial etiology. Reflux of gastric acid may cause esophagitis with resulting impairment of esophageal peristalsis.[1] Deterioration of esophageal clearance function prolongs contact of the refluxate with the esophagus and enhances mucosal damage. As a result, patients with GERD exhibit a wide range of endoscopic findings (normal to severe esophagitis) with the extent of mucosal injury influenced by prior use of acid suppressive medication.

Reflux esophagitis was first recognized in the 1930s.[2] Since then, the major focus of treatment has been on erosive or ulcerative esophagitis and its complications (stricture, Barrett's esophagus). Until recently, endoscopy-negative reflux disease was thought to be a diagnostic challenge of limited clinical importance and a milder form of GERD that did not warrant aggressive therapy.[3]

Over the past few years, evidence has emerged that patients with endoscopy-negative GERD are similar to those who exhibit esophagitis with respect to patterns of reflux, severity of symptoms, and levels of medical treatment.[4–6] Furthermore, outcomes analysis suggests that in endoscopy-negative patients low-level therapy (cisapride, H_2 receptor antagonist) is often unsuccessful, and these patients require high-dose proton pump inhibitors, exhibit high rates of relapse, and have inferior response rates to omeprazole in comparison to those with esophagitis.[4,5,7,8] Such evidence suggests that endoscopy-negative reflux disease is a morbid disease and has a substantial impact on quality of life. For these reasons, management strategies for endoscopy-negative GERD

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (oral presentation).
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Published by Elsevier Science Inc.
1091-255X/03/$—see front matter
PII: S1091-255X(02)00135-X

should be based on the same principles as those for reflux esophagitis.

Different treatment strategies for GERD are available depending on the needs of the patient. Acid-reducing medication and lifestyle modifications are usually recommended for patients with endoscopy-negative reflux disease. Consideration of surgical treatment in symptomatic patients without esophagitis requires further physiologic testing and a careful selection process. Patients with endoscopy-negative reflux disease may be advised to avoid surgical treatment because of a lack of evidence of reflux and the thought that it is a milder form of GERD. In addition, symptomatic patients without evidence of esophagitis may have multiple causes for their symptoms[9] and therefore may exhibit less favorable outcomes after surgical treatment.

Antisecretory medications do not completely inhibit reflux of gastroduodenal contents. Through the creation of an effective antireflux valve, laparoscopic antireflux surgery (LARS) provides durable symptomatic relief and a reduction in the use of medication in most patients.[10,11] However, data on clinical outcomes of endoscopy-negative GERD patients who undergo LARS are limited.[11] Patients who have GERD with erosive disease and its complications (stricture, Barrett's esophagus) also may pose a technical challenge during the laparoscopic approach because of the presence of periesophageal inflammation or a foreshortened esophagus.

The aim of the current study was to compare clinical outcomes after LARS in GERD patients with and without endoscopic evidence of esophagitis. We hypothesized that patients with endoscopy-negative reflux disease would have better perioperative results than patients with esophagitis. However, given the outcome data of medical therapy for endoscopy-negative GERD, we hypothesized that these patients would have less favorable long-term postoperative outcomes when compared to the esophagitis-positive population.

MATERIAL AND METHODS

Patient Population

From May 1992 to October 2001, a total of 597 consecutive patients undergoing LARS by the senior author (N.J.S.) were entered prospectively into a computerized database, and their records were analyzed retrospectively. Of these patients, 414 (69%) had both preoperative endoscopy and 6 months or more of postoperative follow-up, and they constitute the study population. Results of endoscopy were negative for esophagitis on preoperative endoscopic examination in 84 patients (20%; group 1). The remaining 330 patients (80%; group 2) exhibited varying degrees of esophagitis on endoscopy. The study was approved by the Institutional Review Board of Washington University School of Medicine.

Preoperative Evaluation

All patients were evaluated on the basis of a detailed history that included global health status; patients were also questioned as to the use of antacids or acid-reducing medication and the presence or absence of typical and atypical symptoms of GERD. Responses were elicited on a yes/no basis, with any degree of the given symptom indicated as a "yes" response. Midway through the accrual of data, typical symptoms were also scored quantitatively. The severity and frequency of typical esophageal symptoms (heartburn, regurgitation) were scored on a scale of 0 to 4 (0 = none; 4 = very severe, or occurring at least once daily). Upper endoscopy was performed in all patients to determine the presence or absence of esophagitis, hiatal hernia, stricture, or Barrett's esophagus. Endoscopic grading of reflux esophagitis and classification of patient groups was based on the Savary-Miller scores;[12] however, many patients undergoing endoscopy at other institutions were not assigned specific grades when esophagitis was noted (Table 1). The vast majority (≥97%) of patients in each group underwent preoperative esophageal manometry. Preoperative 24-hour pH testing with calculation of a modified DeMeester score[13] was performed in 74 patients (88%) in group 1 and 197 (60%) in group 2. Patients in group 1 who did not undergo preoperative 24-hour pH testing were unable to tolerate the test and exhibited both typical

Table 1. Patient groups*

	Endoscopic findings	No. of patients (%)
Group 1		
Grade 0	Normal mucosa without lesions	84 (20)
Group 2		
Grade 1	≥1 erosions in one mucosal fold	58 (14)
Grade 2	Several erosions in several mucosal folds	32 (8)
Grade 3	Circumferential erosions	35 (8)
Grade 4	Ulcer(s), strictures, shortening of esophagus	48 (12)
Grade 5	Barrett's esophagus	77 (19)
UTD	Esophagitis-positive: grade unavailable	80 (19)

UTD = unable to determine grade.
*Based on Savary-Miller classification.[12]

GERD symptoms and evidence of GERD as determined by reflux on barium swallow.

Operative Technique

In all patients the fundoplication was constructed around a 50 to 60 F Maloney dilator after posterior crural closure with interrupted nonabsorbable sutures.[14] The short gastric vessels were divided routinely to allow for full fundic mobilization. For Nissen fundoplications, a 2 cm wrap was created using three nonabsorbable sutures, and the wrap was secured to the esophageal wall. Patients with severe esophageal dysmotility, defined by a mean contraction amplitude of 35 mm Hg or less and/or greater than 30% nonpropagating peristaltic waves, underwent posterior partial (270-degree) fundoplication.

Postoperative Care and Evaluation

For the first 24 hours after surgery, patients were given intravenous ondansetron and ketorolac. Nasogastric tubes were not used. Clear liquids were offered the morning after surgery, and patients were allowed to advance to a soft diet later that day, followed by discharge from the hospital. Full, unrestricted activity was resumed as tolerated.

Routine postoperative clinical assessments were performed at 1 month, 6 to 12 months, and annually thereafter. Patients completed questionnaires that were identical to those used preoperatively with regard to the use of medication and GERD-related symptomatology at each postoperative evaluation. In addition, satisfaction and overall improvement in esophageal symptoms were determined by means of a visual analogue scale. Patients who were unable to return for outpatient visits were contacted by telephone by a research nurse. Early in our experience, 96% of a cohort of unselected patients were demonstrated to have normalization of acid exposure time by 24-hour pH study performed after LARS. Subsequently, postoperative endoscopic, radiologic, and/or physiologic evaluations of the upper intestinal tract were not performed routinely. However, patients who reported unusual abdominal or chest discomfort, GERD-related symptoms, or dysphagia underwent testing. The percentage of patients reporting symptoms and their use of acid-reducing medication were calculated and compared to preoperative data and between groups. $P < 0.05$ was considered significant as determined by chi-square analysis and Student's t test. Summary data are presented as a mean ± standard deviation or percentage.

RESULTS

Preoperative Findings

Among the patients who underwent LARS, 84 patients (group 1) had no preoperative evidence of esophagitis on endoscopy. However, in 77 patients (23% of group 2), preoperative endoscopy revealed varying degrees of esophagitis along with the presence of Barrett's esophagus (see Table 1). The 84 patients in group 1 were similar to the 330 patients in group 2 with respect to age, body mass index, and duration of preoperative symptoms (Table 2). However, the sex distribution of the groups was markedly different, with 65% of group 1 being female as opposed to only 38% of patients in group 2. Results of preoperative physiologic tests are presented in Table 3. Mean amplitude of esophageal body contractions was significantly greater in group 1 than in group 2, but lower esophageal sphincter pressure was similar in the two groups. Patients in group 2 had higher DeMeester scores ($P < 0.05$) than those in group 1, but overall esophageal acid exposure time did not differ. Preoperatively the presence of typical and atypical symptoms of GERD, use of antireflux medications, and severity of typical symptoms were similar in the two groups (Tables 4 to 6).

Perioperative Outcomes

In both groups, 91% or more of patients underwent complete fundoplication; however, a partial fundoplication was more commonly required in group 2 (Table 7). The incidence of intraoperative complications (pleural injury, bleeding, splenic injury, and liver injury) was low and similar in the two groups. Only 3% of patients in each group had postoperative complications that were grade 2b or higher.[15] Postoperative complications included myocardial infarction, cardiac arrhythmias, respiratory failure, pneumonia, deep venous thrombosis, and urinary tract retention/infections. Although procedure times were longer in group 2, perhaps reflecting the greater frequency of partial fundoplications,

Table 2. Patient characteristics

	Group 1 (N = 84)	Group 2 (N = 330)	*P* value
Age (yr)	47 ± 13	47 ± 12	NS
% Female	65	38	<0.05
Body mass index	30 ± 5	29 ± 5	NS
Duration of symptoms (mo)	76 ± 49	79 ± 43	NS

Mean ± standard deviation; NS = not significant.

Table 3. Results of preoperative endoscopic and physiologic studies

	Group 1	Group 2	*P* value
Endoscopic esophagitis (%)	0	100	—
Barrett's esophagus (%)	0	23	—
Modified DeMeester score	44 ± 29	61 ± 62	<0.05
Esophageal acid exposure time (%)	12 ± 6	13 ± 8	NS
Amplitude of contraction (mm Hg)	86 ± 32	60 ± 42	<0.05
LES pressure (mm Hg)	10 ± 7	8 ± 6	NS
LES length (cm)	2.7 ± 1.2	3.1 ± 1.1	<0.05

LES = lower esophageal sphincter; NS = not significant; Mean ± standard deviation.

the intervals for advancement of diet, hospital stay, and return to work were similar in both groups (see Table 7).

Postoperative Clinical Outcomes

Mean follow-up for group 1 was 20 ± 14 months (range 6 to 58 months) and for group 2 was 35 ± 24 months (range 6 to 109 months) ($P < 0.05$; Table 8). LARS markedly decreased the use of acid-reducing medications and GERD symptoms in both groups (see Tables 4 and 5). Preoperatively, 98% of patients in group 1 and 99% in group 2 were using various acid-reducing medications, as compared to 18% of patients in group 1 and 16% of patients in group 2 postoperatively. Use of proton pump inhibitors markedly declined from 86% to 14% or less in each group ($P < 0.05$; Fig. 1). Significant and durable symptomatic improvement was noted in comparison with the preoperative evaluation. The presence of typical (heartburn, regurgitation, dysphagia) and atypical (chest pain, water brash, nocturnal aspiration) symptoms of GERD was significantly reduced after LARS in each group (Fig. 2; $P < 0.05$). Postoperatively, with the exception of dysphagia, there was no statistical difference in the use of acid-reducing medications or GERD symptoms between groups. In addition, subgroup analysis after LARS revealed significant improvement in heartburn severity, heartburn frequency, regurgitation severity, and regurgitation frequency to extremely low levels (see Table 6). After surgery, predominantly mild dysphagia was evident in 21 patients (25%) in group 1 vs. 46 patients (14%) in group 2 ($P < 0.05$), significantly less than the preoperative values in both groups. Although the incidence of preoperative abdominal bloating was present in 12% in group 1 and 14% in group 2, this symptom was present postoperatively in 29 patients (35%) in group 1 and 106 patients (32%) in group 2. Postoperative satisfaction after LARS was more than 90% in both groups (see Table 8).

Improvement in esophageal symptoms, as assessed by a 10 cm visual analogue scale, was 8.7 ± 2.4 in group 1 vs. 8.9 ± 2.1 in group 2 (P = NS). Patients with recurrent or atypical symptoms were studied endoscopically and/or with an upper gastrointestinal contrast study. Subsequent physiologic testing was performed as deemed necessary. Surgical failures (wrap disruption, intrathoracic migration, and slippage aborally) required reoperation in 3% of each group.[16]

DISCUSSION

Endoscopic evaluation of patients with symptomatic GERD can reveal varying grades of erosive esophagitis. However, a considerable number of patients with symptomatic GERD have no mucosal abnormalities on endoscopy.[4,8,17] A recent multicenter trial of patients with symptoms of GERD, excluding those who had previously used acid-reducing medications, determined the prevalence of endoscopy-

Table 4. Medication use before and after LARS

	Group 1			Group 2		
	Preop	Postop	*P* value*	Preop	Postop	*P* value*
Medication use	98%	18%	<0.05	99%	16%	<0.05
Antacids	23%	2%	<0.05	24%	4%	<0.05
H_2 receptor antagonist	13%	0%	<0.05	23%	3%	<0.05
Proton pump inhibitors	86%	14%	<0.05	86%	11%	<0.05

*Preop (preoperative) vs. postop (postoperative) values; no statistical differences in preop or postop values between groups.

Table 5. Response of typical and atypical symptoms to LARS

	Group 1			Group 2		
	Preop	Postop	*P* value*	Preop	Postop	*P* value*
Heartburn	98%	15%	<0.05	93%	15%	<0.05
Regurgitation	85%	7%	<0.05	85%	7%	<0.05
Dysphagia	45%	25%†	<0.05	48%	14%	<0.05
Water brash	83%	11%	<0.05	77%	7%	<0.05
Nocturnal aspiration	54%	1%	<0.05	55%	2%	<0.05
Chest pain	65%	23%	<0.05	54%	14%	<0.05
Abdominal bloating	12%	35%	<0.05	14%	32%	<0.05
Able to belch	—	90%		—	88%	

*Preop (preoperative) vs. postop (postoperative) values.
†No statistical differences in preop or postop values between groups, except for dysphagia.

negative GERD to range from 38% to 84%.[17] Although the degree of esophageal acid exposure generally increases with the severity of esophagitis, as shown by esophageal pH monitoring,[18] there is a wide variation among patients with GERD such that the amount of acid exposure is a poor predictor of the presence of esophagitis. In a study of 451 patients, 63% of patients with endoscopy-negative reflux disease had elevated esophageal acid exposure.[19] Symptoms of GERD are also similar in those with and without endoscopic evidence of esophagitis such that symptom severity and frequency are poor predictors of the presence of erosive esophagitis.[4,6,20]

Recently, treatment recommendations for patients with endoscopy-negative GERD have become similar to those for patients with erosive disease. In the past, endoscopy-negative reflux disease was considered a milder form of the disease to be treated medically.[3] However, trials evaluating the short-term relief of symptoms in endoscopy-negative patients showed adequate control of heartburn in only 23% to 45% of patients on cisapride,[8,21] and in 24% to 40% of those on an H_2 receptor antagonist.[7,20] Even high-dose proton pump inhibitors provided only 60% to 70% of endoscopy-negative patients with adequate symptomatic relief.[3,7,21,22,23] In addition, comparisons between those with and without esophagitis suggest that omeprazole may provide less symptomatic relief in endoscopy-negative patients.[4,5] Within 6 months after cessation of medical therapy, relapse of symptoms in endoscopy-negative patients has been reported in as many as 75% of patients.[4,24] For this reason, some form of long-term therapy is usually warranted.

In the current study, 20% of the patients referred specifically for surgical treatment of documented GERD had no evidence of esophagitis on endoscopy. Most of these patients had been treated with proton pump inhibitors. However, this therapy did not eliminate reflux, as evidenced by the presence of ongoing preoperative heartburn and regurgitation. The two groups undergoing LARS in the current study differed with respect to endoscopic esophagitis, as well as gender and esophageal physiology. The percentage of women was much higher in the endoscopy-negative population, confirming the greater incidence of erosive disease in men.[4,20]

Preoperative physiologic testing revealed higher DeMeester scores in those patients with esophagitis,

Table 6. Scoring of typical esophageal symptoms before and after LARS

	Group 1		Group 2	
Symptom measures	Preoperative*	Postoperative	Preoperative*	Postoperative
Heartburn frequency	2.4 ± 1.4	0.3 ± 1.0	2.3 ± 1.6	0.2 ± 0.7
Heartburn severity	2.3 ± 1.4	0.2 ± 0.6	2.0 ± 1.5	0.2 ± 0.7
Regurgitation frequency	2.1 ± 1.4	0.2 ± 0.8	2.0 ± 1.5	0.2 ± 0.7
Regurgitation severity	1.8 ± 1.4	0.2 ± 0.5	1.9 ± 1.5	0.2 ± 0.8

*$P < 0.05$ (vs. postoperative values); mean ± standard deviation; no statistical differences in preoperative or postoperative values between groups.
0–4 scale: 0 = none; 4 = very severe or symptoms occurring once or more daily.

Table 7. Perioperative outcomes

	Group 1	Group 2	*P* value
% Partial fundoplication	1 (N = 1)	9 (N = 29)	<0.05
Procedure time (min)	109 ± 36	129 ± 47	<0.05
Postoperative stay (days)	1.4 ± 1.4	1.4 ± 0.8	NS
Return to work (days)	14 ± 8	13 ± 7	NS
Postoperative complications* (%)	3	3	NS

Mean ± standard deviation; NS = not significant.
*Grade 2b or higher.[15]

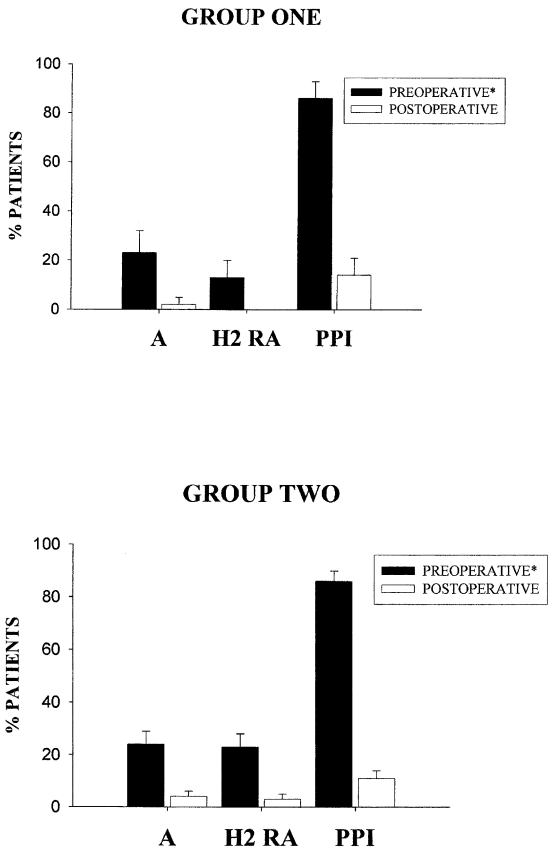

Fig. 1. Medication use before and after LARS. *$P < 0.05$ (preoperative vs. postoperative). Data are expressed with 95% confidence interval. A = antacids; H_2 RA = histamine-2 receptor antagonist; PPI = proton pump inhibitors.

despite similar acid exposure times between groups. Reflux of gastric contents may lead to esophageal injury and esophageal body dysfunction.[25] In the current study those with endoscopic esophagitis had lower mean amplitudes of esophageal body contraction, which suggests a higher incidence of esophageal dysmotility in patients with increased mucosal inflammation. This difference in motility characteristics was reflected in the greater application of partial fundoplications in group 2. This fact, along with the possibility of increased difficulty of hiatal dissection in patients with significant inflammatory disease of the esophagus, likely led to the observed increased operative times in this group. However, despite differences in procedure times, overall hospital stay, return to work, and complication rates were equivalent between groups. Thus major end points of perioperative outcomes were similar in patients with and without esophagitis.

Over a duration of follow-up ranging from 6 months to 9 years, LARS provided the vast majority of patients in both groups with relief from typical and atypical symptoms and a reduction in the use of proton pump inhibitors from 86% (preoperatively) to 14% or less (postoperatively). Absolute relief of postoperative heartburn and regurgitation was present in 85% and 93% of each group according to simple "yes/no" responses. Low esophageal symptom scores further confirm the overall efficacy of LARS in patients with and without esophagitis. These results compare favorably with those of another trial evaluating the impact of esophagitis on postoperative outcomes after LARS.[11] The patients with endoscopy-negative GERD, therefore, had significant symptomatic relief and a marked reduction in the use of medication after LARS that was nearly identical to that in patients with preoperative esophagitis.

Our study is limited by the fact that patients did not undergo routine postoperative esophageal function tests. However, that these operations effectively cure GERD is supported, in part, by the results of postoperative pH testing in a subset of patients, 96% of whom had normalization of esophageal acid exposure. In general, compliance with postoperative pH studies is low given that most patients are asymptomatic or have mild symptoms of GERD, as is evident from the objective and quantitative symptom scoring. An accepted alternative measure of success

Table 8. Long-term outcomes

	Group 1	Group 2	*P* value
Months follow-up	20 ± 14	35 ± 24	<0.05
% Satisfaction	97	92	NS
Improvement in esophageal symptoms (VAS)	8.7 ± 2.4	8.9 ± 2.1	NS
% Reoperation	3 (N = 3)	3 (N = 11)	NS

Mean ± standard deviation; NS = not significant; VAS = visual analogue scale.

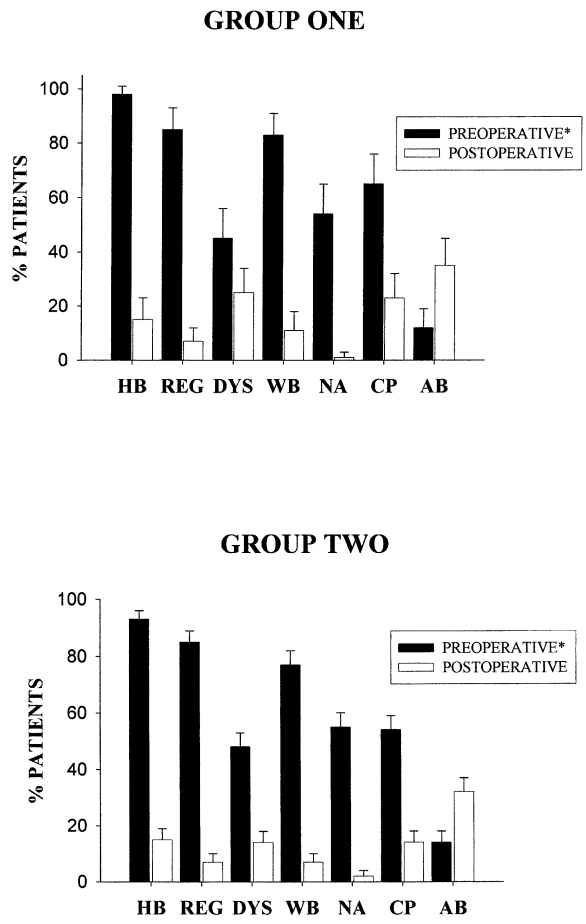

Fig. 2. Symptom response following LARS. *$P < 0.05$ (preoperative vs. postoperative). Data are expressed with 95% confidence interval. HB = heartburn; Reg = regurgitation; Dys = dysphagia; WB = water brash; NA = nocturnal aspiration; CP = chest pain; AB = abdominal bloating.

after antireflux surgery is improvement in GERD symptoms.[26] Responses to medical treatment and symptom scores are useful in the evaluation of GERD treatment and can be predictive of improvement after antireflux surgery.[27,28]

Our results indicate that patient outcomes and complication rates compare favorably not only between the two groups in the present study, but also with other series evaluating the effectiveness of LARS.[29,30] After LARS, symptomatic relief (heartburn and dysphagia) in the endoscopy-negative patient at short-term follow-up (median 12 months) has been reported.[11] However, little is known about differences in perioperative and long-term clinical outcomes between these two distinct study populations. The effectiveness and durability of long-term proton pump inhibitor therapy in those with endoscopy-negative reflux disease is debatable. It is unclear from current outcome studies of medical therapy whether patients with endoscopy-negative GERD have adequate symptomatic relief and improvement in quality of life. We have shown that after LARS, patient satisfaction and symptomatic outcome are equally good, irrespective of the presence or absence of endoscopic esophagitis.

CONCLUSION

Our hypotheses that endoscopy-negative reflux disease patients would have better perioperative results than patients with esophagitis and that these patients would have less favorable long-term postoperative outcomes when compared to the esophagitis-positive population proved to be false. LARS is an effective treatment strategy for GERD, with no significant clinical differences between patients with and without preoperative esophagitis. Although endoscopy is a valuable diagnostic tool in the evaluation of GERD, the presence or absence of preoperative esophagitis does not influence perioperative or long-term postoperative outcomes. For patients with endoscopy-negative reflux disease, LARS provides significant improvement of symptoms and a marked reduction in the use of proton pump inhibitors.

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Discussion

Dr. D. Shibata (Baltimore, MD): There are some clinicians who hesitate to refer patients for antireflux surgery in the setting of no visible esophagitis on endoscopy. What should be the minimal workup that would be required to provide the objective evidence necessary to convince such clinicians to refer their patients for surgical intervention?

Dr. K. Desai: To justify referral of patient with endoscopy-negative reflux disease, the clinician should perform a 24-hour pH test and esophageal manometry to document objective evidence of GERD.

Dr. S.R. DeMeester (Los Angeles, CA): Did you look at the subgroup specifically that had a partial antireflux operation in the endoscopy-positive group? There have been reports that esophagitis, and more severe reflux disease, tends to do less well with a partial fundoplication. Did that fall out in your analysis? My second question is, did it make any difference, or did you analyze the data based on any history of esophagitis rather than esophagitis at the most recent endoscopy? Gastroenterologists would tell patients that if they have esophagitis at their most recent endoscopy, they are simply not taking enough proton pump inhibitors. Did it make a difference whether you looked at groups based on any history ever of esophagitis?

Dr. Desai: I will answer your second question first. All patients had an extensive preoperative history and physical examination. Patients were questioned as to the presence or absence of signs and symptoms of reflux esophagitis. Results of preoperative endoscopy were reviewed. Only a small percentage of our patients underwent preoperative endoscopy on more than one occasion. All endoscopy-positive patients in our study had grade 1 through 4 esophagitis according to the Savary-Miller classification, and all of them did equally well. We did not perform a subgroup analysis of our endoscopy-positive patients to determine the effects of partial vs. total fundoplication in this patient population.

Dr. W. Rattner (Boston, MA): I noticed that there was a significant difference in DeMeester scores between the two groups, and I wonder if you could give us information on the amount of upright vs. supine reflux. Did the magnitude of supine reflux alone account for the difference in the DeMeester scores between patients with and without esophagitis?

Dr. Desai: Those with esophagitis had higher DeMeester scores in our study as opposed to the endoscopy-negative patients, who had lower DeMeester scores. However, when we conducted a subgroup analysis of those with supine and upright reflux, there was no major difference in those findings.

Dr. T. M. Quinn (New York, NY): I noticed that 10% of your patients were still on proton pump inhibitors.

Why do you think that is, and is it the group that had the partial fundoplication that accounts for this, or have they actually been evaluated with pH and motility studies?

Dr. Desai: After LARS, a 10% rate of postoperative use of proton pump inhibitors compares very favorably with rates in the existing literature. A few patients required postoperative proton pump inhibitors for symptomatic relief. However, a number of them were on proton pump inhibitors for other medical conditions such as gastritis. Our patients, who underwent partial fundoplication, did not have a higher rate of proton pump inhibitor use in this study. A number of patients are placed on proton pump inhibitors and are not taken off of these medications by their internal medicine physicians despite relief of symptoms and esophagitis. Did we routinely perform postoperative pH testing and manometry? No. However, early on in our experience, 96% of a subgroup of our patients had normalization of acid exposure time after LARS.

Invited Discussion—Expert Commentator

Jeffrey H. Peters, M.D. (Los Angeles, CA): The first paper, a report from Washington University, compares outcomes of laparoscopic Nissen fundoplication in patients with nonerosive reflux disease to outcomes in those with erosive esophagitis. The question of the appropriateness of antireflux surgery in the setting of nonerosive reflux disease is not new and predates the introduction of laparoscopic Nissen fundoplication. It is only recently, however, that large cohorts of patients have accumulated allowing the question to be well studied. As this and other studies have shown, it is clear that outcomes in carefully selected patients with nonerosive disease do indeed justify antireflux surgery; that is, most patients will be relieved of their reflux symptoms and the vast majority are satisfied with the results. The data provide another important lesson, however. It is also clear that outcome is affected by the severity of the disease. Patients who need the operation the most tend to benefit the most. This fact underscores the need for careful patient selection.

The Effect of Chronic Pain Syndromes and Psychoemotional Disorders on Symptomatic and Quality-of-Life Outcomes of Antireflux Surgery

Vic Velanovich, M.D.

Psychoemotional disorders (PED) and chronic pain syndromes (CPS) are common problems. Many patients with these disorders also suffer from gastroesophageal reflux disease (GERD). It is unclear how PED/CPS affect outcomes of antireflux surgery; therefore, the purpose of this study was to determine if PED/CPS adversely affects the results of surgical therapy for GERD. All patients referred for surgical therapy for GERD completed both the GERD-HRQL symptom severity instrument and the SF-36 generic quality-of-life instrument prior to surgery. To be candidates for surgery, patients must have symptomatic GERD and objective evidence of pathologic reflux by upper endoscopy, esophageal manometry and 24-hour pH monitoring. Patients underwent either laparoscopic or open Nissen or Toupet fundoplication. Six to 24 months postoperatively, patients were evaluated for satisfaction and quality-of-life. Ninety-three percent of control patients compared to 25% of PED/CPS patients were satisfied with surgery ($P < 0.001$). Dissatisfaction in PED/CPS patients was generally due to persistent or new somatic complaints. Median total GERD-HRQL scores improved for both groups, although postoperative scores were worse in the PED/CPS group. PED/CPS patients had significantly worse SF-36 scores both preoperatively and postoperatively compared to control patients. SF-36 scores improved in four of eight domains in control patients and none in the PED/CPS patients. In conclusion, PED/CPS patients are generally dissatisfied with antireflux surgery. Although some patients do benefit from surgery, careful patient selection is required. (J GASTROINTEST SURG 2003;7:53–58.) © 2003 The Society for Surgery of the Alimentary Tract, Inc.

KEY WORDS: Gastroesophageal reflux disease, antireflux surgery, fibromyalgia, anxiety disorder, depression

Gastroesophageal reflux disease (GERD) is a relatively common disorder affecting 7% of the adult United States population on a daily basis.[1] GERD can cause significant impairments in quality-of-life,[2,3] and antireflux surgery has been shown to not only improve symptoms, but also improve quality-of-life.[4,5] Therefore, surgery can be an effective treatment for GERD in appropriately selected patients.

Psychoemotional disorders (PED), such as depression and anxiety, are also very common. An episode of major depression occurs in about 15% of the population, and 6 to 8% of all outpatient primary care clinic visits are for depression.[6] Similarly, 10 to 15% of clinic visits are related to anxiety and other stress disorders.[6] Chronic pain syndromes (CPS) are also common. For the purpose of this study, chronic pain syndromes are those named disorders which cause persistent pain requiring indefinite treatment. The most common of these is fibromyalgia. The prevalence of fibromyalgia in the United States using the American College of Rheumatology's diagnostic criteria was 3.4% in women and 0.5% in men.[7] It is also common that patients with fibromyalgia also suffer from depression and anxiety.[7] Therefore, it can be expected that many patients will suffer from both GERD and PED and/or CPS.

This is not inconsequential as PED can affect patients with GERD. Sir William Osler recognized the

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (poster presentation).

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1091-255X/03/$—see front matter
PII: S1091-255X(02)00136-1

association between GERD-like symptoms and PED, "oesophagismus is met with in hysterical patients and hypochondriacs . . . the idiopathic form is found in females of a marked neurotic habit."[8] There have been other studies since then documenting the association of GERD and PED.[9,10] Specifically, with respect to antireflux surgery, both personality factors[11] and psychiatric illness[12] have been reported to affect outcomes. I have not found a study evaluating the effect of CPS, specifically fibromyalgia, on GERD. Therefore, the purpose of this study was to better characterize the effect of PED and CPS on the surgical treatment of GERD.

PATIENTS AND METHODS

Patients

All patients referred for surgical management of GERD were evaluated with history and physical examination. All patients were on some type of acid suppression medication with most, but not all, having at least some symptomatic relief. Patients with symptoms consistent with GERD were then evaluated with upper endoscopy, esophageal manometry, 24-hour esophageal pH monitoring, and, selectively, with contrast upper gastrointestinal series and/or gastric emptying scintigraphy. If these studies confirmed pathologic reflux, patients were then counseled as to the risks and benefits of antireflux surgery. Prior to surgery, patients completed the GERD-HRQL symptom severity questionnaire (Table 1)[13] and the generic quality-of-life instrument, the SF-36.[14] The GERD-HRQL is a Likert-type questionnaire of 10 items. Each item is scored from 0 to 5, the total score is the sum of the scores of the 10 questions. The worst possible score is 50 (incapacitated in all items), and the best possible score is 0 (asymptomatic in all items). The SF-36 assesses eight domains: physical functioning (PF), role-physical (RP), role-emotional (RE), bodily pain (BP), vitality (VT), mental health (MH), social functioning (SF), and general health (GH). The scores are normalized so the best possible score is 100, and the worst possible score is 0. The patients who make up this report underwent antireflux surgery for symptomatic GERD with at least 6 months follow-up. Patients with paraesophageal hernias were excluded. After surgery, patients were contacted either by telephone or clinic visit 6 to 24 months and were simply asked whether or not they were satisfied with their surgery and, if dissatisfied, why; and to complete both the GERD-HRQL and the SF-36.

During the initial history and physical examination, patients, in addition to other past medical history, were assessed for the presence of PED or CPS. Patients were considered to have a PED if they carried a diagnosis which was recognized by the DSM-IV as a psychiatric disease *and* were being treated by a psychiatrist or primary care physician with medications or other psychological treatments. Patients were considered to have a CPS if they carried a diagnosis of one of the arthritidies recognized by the

Table 1. The gastroesophageal health related quality-of-life instrument

Scale							
0 = No symptoms							
1 = Symptoms noticeable, but not bothersome							
2 = Symptoms noticeable and bothersome, but not every day							
3 = Symptoms bothersome every day							
4 = Symptoms affect daily activities							
5 = Symptoms are incapacitating—unable to do daily activities							
Questions about symptoms (circle one for each question)							
1. How bad is your heartburn?	0	1	2	3	4	5	
2. Heartburn when lying down?	0	1	2	3	4	5	
3. Heartburn when standing up?	0	1	2	3	4	5	
4. Heartburn after meals?	0	1	2	3	4	5	
5. Does heartburn change your diet?	0	1	2	3	4	5	
6. Does heartburn wake you from sleep?	0	1	2	3	4	5	
7. Do you have difficulty swallowing?	0	1	2	3	4	5	
8. Do you have pain with swallowing?	0	1	2	3	4	5	
9. Do you have gassy or bloating feelings?	0	1	2	3	4	5	
10. If you take medication, does this affect your daily life?	0	1	2	3	4	5	
11. How satisfied are you with your present condition?	Satisfied			Neutral			Dissatisfied

American College of Rheumatology[7] and were receiving treatment specifically for this disease. I believe these definitions represent "real world" working diagnostic criteria.

Operations

This study assessed patients over a 4-year time span. During that time, my practice was to tailor surgery based on prior abdominal surgeries, esophageal physiology and anatomy, and patient preference. In patients with adequate esophageal motility (i.e., primary esophageal peristaltic propulsive contractions with amplitudes of greater than 30 mm Hg in our laboratory) without esophageal foreshortening, the laparoscopic Nissen fundoplication was my procedure of choice. If there was significant dysmotility (i.e., aperistalsis or primary amplitudes of less than 30 mm Hg), then the laparoscopic Toupet fundoplication was my procedure of choice. Recently, with reports of high recurrence rates after laparoscopic Toupet fundoplication,[15] I now perform laparoscopic Nissen fundoplication exclusively. Although most procedures were planned as laparoscopic cases, some were planned as open procedures. All redo fundoplications were done through an upper, midline laparotomy. Patients requiring an additional procedure(s) which were not generally done laparoscopically were also done using the open approach. A few patients requested open operations. Patients who had foreshortened esophagi underwent Collis-Nissen fundoplications, which was done through an open approach. An esophagus was considered foreshortened if, after the hiatus and esophagus were completely mobilized, the gastroesophageal junction could not be brought into the abdomen without tension.

Statistical Analysis

All data was analyzed using the True Epistat statistical computer program.[16] Nominal data was analyzed using the chi-squared test. Data derived from the GERD-HRQL was ordinal in nature, while data derived from the SF-36 was not Gaussian in nature. Therefore, these data were analyzed nonparametrically using the Mann-Whitney U-test. A P value of ≤ 0.05 was considered significant.

RESULTS

Demographics

Table 2 presents the demographic data of the patients studied. The median follow-up was 12 months (range 6 to 24) even though this study was done over a 4-year period. There were a total of 134 control patients and 28 PED/CPS patients. This group included patients previously reported.[12] The PED/CPS patients were predominantly female, while the majority of control patients were male. Average age, as well as the distribution of the type of operation were similar between the groups. There were a total of 51 PED/CPS diagnoses among the 28 PED/CPS patients. Of the patients with more than one diagnosis, the combination of fibromyalgia with depression, anxiety, or both were most common. Control patients were all patients who did not have either a PED or a CPS.

Satisfaction and Symptom Outcomes

Ninety-three percent of control patients were satisfied with their surgical result, compared to 25% of PED/CPS patients ($P < 0.0001$). Causes for dissatis-

Table 2. Patient demographics

	PED/CPS (n = 28)	Controls (n = 134)
Male	6 (21%)	84 (63%)
Female	22 (79%)	50 (37%)
Average age (yr ± S.D.)	42.4 ± 10.3	46.8 ± 13.8
Operations performed		
Laparoscopic Nissen	22 (79%)	100 (75%)
Laparoscopic Toupet	0 (0%)	11 (8%)
Converted from laparoscopic	4 (14%)	10 (7%)
Planned open	2 (7%)	13 (10%)
Psychoemotional and chronic pain diagnoses*		
Depression	18	
Anxiety disorder	14	
Schizophrenia	2	
Fibromyalgia	14	
Chronic fatigue syndrome	1	

*Number of diagnoses add up to more than 28 due to some patients carrying more than one diagnosis.

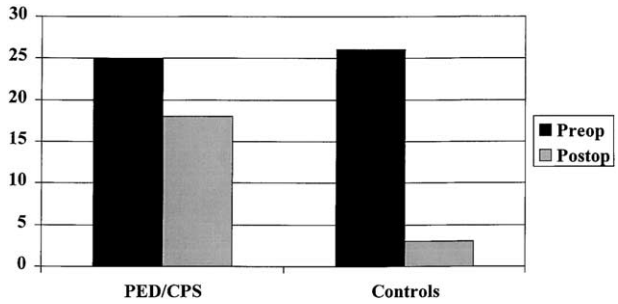

Fig. 1. Median preoperative and postoperative GERD-HRQL scores for patients with psychoemotional disorders or chronic pain syndromes (PED/CPS) and control patients. PED preoperative vs. PED postoperative, $P = 0.006$; control preoperative vs. control postoperative, $P < 0.0001$; PED preoperative vs. control preoperative, P = NS; PED postoperative vs. control postoperative, $P < 0.0001$.

faction among the 10 dissatisfied control patients were symptomatic recurrence in 6 and surgical complications in 4. Six of these patients underwent postoperative 24-hour pH monitoring confirming recurrent reflux. Causes for dissatisfaction in the 21 dissatisfied PED/CPS patients were vague somatic complaints in all 21; in addition, three patients complained of dysphagia, and 1 recurrent heartburn. Ten of the PED/CPS patients agreed to repeat physiologic testing with contrast upper gastrointestinal series, esophageal manometry, and 24-hour pH monitoring. None had objective pathologic evidence of GERD.

Figure 1 presents the preoperative and postoperative median total GERD-HRQL scores for both groups. Although PED/CPS patients and control patients had similar preoperative scores, PED/CPS patients' median total GERD-HRQL scores improved only 7 points (25 to 18; $P = 0.006$), while control patients' median total score improved by 23 points (26 to 3; $P < 0.0001$). PED/CPS patients had a significantly worse postoperative median score compared to control patients ($P < 0.0001$).

Quality-of-Life Outcomes

Table 3 presents the median preoperative and postoperative scores of all domains of the SF-36 for PED/CPS and control patients. There were no statistically significant changes in the PED/CPS patients' median preoperative and postoperative scores in any of the SF-36 domains. On the other hand, control patients showed statistically significant improvements in four of eight domains. In all eight domains both preoperatively and postoperatively, control patients had better scores compared to PED/CPS patients.

DISCUSSION

A symptom is a patient perceived phenomenon. It is the patient's conscious perception of the chain of events which begins with a peripheral noxious stimulus that activates primary nociceptors; this neural activation travels through the central pain pathways via the spinal cord to the thalamus. The message is then relayed to the frontal cortex and somatosensory cortex of the brain, where it becomes a conscious perception for the patient. From here, the signal enters the pain modulation network, where the perception of pain can be both dampened and increased.[17] Hence, pain, or any symptom, is both a sensation and an emotion. Therefore, factors involved in the manifes-

Table 3. Median scores of the SF-36 preoperatively and postoperatively for control and PED/CPS patients (range)

	PF	RP	RE	BP	VT	MH	SF	GH
PED/CPS preop	72.5 (95)	50 (100)	67 (100)	42 (74)	45 (55)	44 (64)	62.5 (100)	52 (65)
PED/CPS postop	75 (100)	0 (100)	0 (100)	31 (100)	30 (70)	44 (92)	37.5 (100)	55 (87)
Control preop	90 (85)	100 (100)	100 (100)	74 (100)	60 (85)	84 (84)	100 (100)	72 (85)
Control postop	95 (90)	100 (100)	100 (100)	84 (100)	65 (80)	84 (76)	100 (75)	77 (68)
P value PED/CPS preop vs. PED/CPS postop	NS	NS	NS	NS	NS	NS	NS	NS
P value control preop vs. control postop	0.05	NS	0.04	0.004	NS	NS	0.007	NS
P value PED/CPS preop vs. control preop	0.008	0.01	0.006	<0.001	<0.001	<0.001	0.003	0.002
P value PED/CPS postop vs. control postop	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Abbreviations: PF = physical functioning; RP = role-physical; RE = role-emotional; BP = bodily pain; VT = vitality; MH = mental health; SF = social functioning; GH = general health; PED/CPS preop = psychoemotional disorder/chronic pain syndrome patients' median preoperative SF-36 score, score range in parentheses; PED/CPS postop = median postoperative score; Control preop = median control patients preoperative score; Control postop = median control patients' postoperative score.

tation of pain include the peripheral stimulus, the neural signaling caused by this stimulus, and the patient's conscious interpretation and reaction to this signaling. Surgeons generally address and focus on the peripheral stimulus (e.g., the inflamed appendix in appendicitis, the pathologic reflux of GERD). Occasionally, they will address the neural signaling (e.g., a celiac plexus block for pancreatic cancer). However, there is no surgical intervention for the emotional response to pain, and this aspect may be overlooked. Patients with PED/CPS diagnoses have a heightened awareness and response to pain, and this pain requires a multi-modality approach.[6,7]

PED/CPS affect how patients perceive their symptoms of GERD and how they response to treatment. Bradley et al.[18] found that patients with high levels of "gastrointestinal susceptibility" on the Millon Behavioral Health Inventory reported more episodes of "heartburn" even though these episodes were not associated with actual pathologic esophageal acid exposure. Compared to healthy controls, GERD patients display a higher level of anxiety, obsessionality, pessimism, and gastrointestinal susceptibility.[19] It has been suggested that the focused attention on symptoms that medical management provides may induce or aggravate these psychological differences.[20]

From the patient's viewpoint, there is no greater level of "focused attention" than surgery. Therefore, it should come as no surprise that surgery can aggravate symptoms related to fibromyalgia,[7] depression, and anxiety.[6] Previous publications have documented the adverse affects of PED and personality traits on the outcomes of antireflux procedures.[10–12,21] Although no study has examined CPS specifically in its relation to GERD, CPS patients have many of the same characteristics as PED patients. What is interesting is that PED/CPS patients' dissatisfaction with surgery stem not from GERD recurrence, but rather other "non-specific" complaints. This phenomenon of non-specific side effects related to medication use has been termed the *nocebo phenomenon*.[22] The nocebo phenomenon is when a patient experiences adverse side effects that are *not* a direct result of the specific pharmacological action of the drug. The several patient factors that have been associated with the nocebo phenomenon include the patient's expectations of adverse effects at the time of treatment, a process of conditioning in which the patient learns from prior experiences to associate medication-taking with somatic symptoms, certain psychological characteristics such as anxiety, depression, and the tendency to somatize, and situational and contextual factors.[22] Clearly, many PED/CPS patients have these characteristics. A factor that is unique to surgery is preoperative counseling—that is, patients are told of the complications that may be the result of surgery; therefore, they know which adverse events to expect. Many of these patients have been in pain for a long time; the preoperative median bodily pain (BP) score of 42 (see Table 3) is worse than the mean score of 50 for a group of patients with psychiatric and serious medical conditions.[23] In fact, both preoperatively and postoperatively, PED/CPS patients have worse scores compared to control patients in all domains of the SF-36. They also respond less well symptomatically to antireflux surgery compared to controls as measured by the GERD-HRQL instrument, despite normalization of 24-hour pH monitoring scores in 10 of the dissatisfied patients tested. This implies that this is a unique group of patients who may not be ideal candidates for antireflux surgery.

I propose that PED/CPS patients referred for antireflux surgery be approached more cautiously than patients without these disorders. Most of these patients should be treated medically for GERD and aggressively for their underlying PED/CPS. Perhaps consideration should be given to a diagnosis of a functional esophageal disorder, with a trial of tricyclic antidepressants.[24] Kamolz et al.[25] have demonstrated that psychological intervention influences the outcome of laparoscopic antireflux surgery in patients with stress-related symptoms. Therefore, eliciting the help of a psychologist or psychiatrist may be beneficial. The quandary for the surgeon is that many of these patients clearly have GERD-related problems such as strictures or Barrett's esophagus and warrant surgical intervention; yet, detailed counseling as to the side effects may in fact promote the appearance of these side effects. There is no clear answer for this. Therefore, if antireflux surgery is contemplated, careful patient selection is required.

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Laparoscopic Paraesophageal Hernia Repair, a Challenging Operation: Medium-Term Outcome of 116 Patients

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Laparoscopic paraesophageal hernia repairs performed in 116 patients between 1992 and 2001 were prospectively analyzed. Perioperative outcomes were assessed and follow-up was performed under protocol. There were 85 female and 31 male patients who had a mean (± SD) age of 65 ± 13 years and an American Society of Anesthesiology score of 2.3 ± 0.6. All but two patients underwent an antireflux procedure. Gastropexy was performed in 48 patients, an esophageal lengthening procedure in six patients, and prosthetic closure of the hiatus in six patients. Major complications occurred in five patients (4.3%) with two postoperative deaths (1.7%). Mean follow-up was 30 ± 25 months; 96 patients (83%) have been followed for more than 6 months. Among these patients, 73 (76%) are asymptomatic, 11 (11%) have mild symptoms, and 12 (13%) take antacid medications. Protocol barium esophagograms were obtained in 69% of patients at 6 to 12 months' follow-up. Recurrence of hiatal hernia was documented in 21 patients (22% overall and in 32% of those undergoing contrast studies). Reoperation has been performed in three patients (3%). When only the patients with recurrent hiatal hernias are considered, 13 (62%) are symptomatic but only six (28%) require medication for symptoms. Laparoscopic paraesophageal hernia repair is generally safe, even in this high-risk group. This study confirms a relatively high incidence of recurrent hiatal abnormalities after paraesophageal hernia repair; however, most recurrent hiatal hernias are small and only 3% have required reoperation. Protocol esophagograms detect recurrences that are minimally symptomatic. Improved techniques must be devised to improve the long-term outcomes of laparoscopic paraesophageal hernia repair. (J GASTROINTEST SURG 2003;7:59–67)

KEY WORDS: Hernia, hiatal, laparoscopy, fundoplication

The classic definition of paraesophageal hernia (PEH) is a protrusion of the gastric fundus through the diaphragmatic hiatus while the lower esophageal sphincter remains in its normal anatomic position (type II hiatal hernia). More frequently, both the fundus and the lower esophageal sphincter are herniated into the thorax (type III hiatal hernia).[1] As the name indicates, in both types of PEH (types II and III), the herniated stomach lies alongside the thoracic esophagus. Even though they account for only 5% of all hiatal hernias, PEHs are important because they represent a potentially serious disease.[2] Unlike sliding (type I) hiatal hernias, PEHs imply a greater risk for the patient because, when left untreated, life-threatening complications may occur, including hemorrhage, strangulation, volvulus, and perforation.[3,4] For these reasons, surgical repair of PEH is generally recommended. However, the operative strategy is a matter of debate, and there is no single technique that guarantees uniform long-term success. Indeed, the recurrence rates for PEH repair, performed either as an open or a laparoscopic procedure, have been disappointingly high. In patients followed closely, the reported recurrence rates have ranged from 10% to 42%,[5–8] especially when routine contrast x-ray studies are performed.

At present, laparoscopy is accepted as the standard approach for the surgical treatment of gastroesophageal reflux disease,[9] and it is also widely used for repair of PEH. Although technically demanding, this

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (oral presentation).
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1091-255X/03/$—see front matter
PII: S1091-255X(02)00151-8

approach provides better exposure of the surgical field than open transabdominal procedures and adds the known general advantages of laparoscopy in terms of reduced morbidity, shorter hospital stay, rapid recuperation, and decreased pain medication requirements.[10] These advantages may be especially valuable in the PEH population because most patients are elderly and have multiple comorbid conditions. Because a high percentage of recurrent PEHs are initially asymptomatic,[7] contrast imaging is necessary to accurately detect recurrences. The purpose of this study was to assess our experience in 116 patients who had undergone laparoscopic PEH repair since 1992 with close follow-up including protocol barium swallow.

MATERIAL AND METHODS

Patients

The study population consisted of 119 patients who underwent attempted laparoscopic PEH repair at the Washington University Medical Center/Barnes-Jewish Hospital from September 1992 to October 2001. There were 87 female and 32 male patients who had a mean age (± SD) of 65 ± 13 years. Patient body mass index (BMI) ranged from 18 to 41 (29 ± 6). The mean American Society of Anesthesiology (ASA) score was 2.3 ± 0.6. Preoperative evaluation included upper endoscopy in all patients, barium esophagograms in 103 patients (89%), and esophageal manometry in 111 patients (96%). Manometry was not performed in five patients because of inability to tolerate the study. Preoperative 24-hour pH tests were not performed routinely, because fundoplications are generally performed during the operative procedure. Despite the preoperative workup, in 15 patients the diagnosis of PEH was made only at the time of surgery. Table 1 summarizes the patient characteristics and results of preoperative evaluation. Hernia size was defined as the distance measured from the diaphragmatic hiatus to the intrathoracic fundus (PEH) or to the gastroesophageal junction (sliding). The lower esophageal sphincter was considered hypotensive on manometry if the resting pressure was 6 mm Hg or less.

Surgical Technique

Our technique has been previously described[7] and has evolved with increasing experience; here we describe the procedure as it is currently performed. After careful reduction of the herniated contents into the peritoneal cavity, an incision at the crural ring is made to develop a plane between the peritoneum and the pleura so that the entire sac can be removed from the mediastinum. Systematic division of the short gastric vessels using ultrasonic shears is done routinely, and the distal esophagus is circumferentially mobilized taking care to identify and protect the vagal trunks. The hiatal defect is closed posterior to the esophagus with 0-gauge interrupted braided polyester sutures, which pushes the esophagus anteriorly in relation to the diaphragm, thereby obtaining additional effective intra-abdominal esophageal length. In the event that primary repair is not possible because of undue tension, prosthetic repair of the hiatal defect is undertaken. After closure of the hernia defect, a fundoplication is performed. A 360-degree Nissen fundoplication is done unless manometry

Table 1. Paraesophageal hernia: Presenting features in 116 patients

Age (yr)*	65 ± 13	(range 24–91)
Symptom duration (mo)*	53 ± 43	(range 1–120)
Presenting symptoms (%)		
Heartburn	76	(66)
Chest pain	72	(62)
Dysphagia	48	(41)
Asthma	18	(16)
Cough	17	(15)
Vomiting	13	(11)
Anemia/gastrointestinal bleeding	11	(9)
Preoperative evaluation		
Hernia size (cm)*	6 ± 2	(range 3–14)
Endoscopic esophagitis (%)	18/116	(16)
Hypotensive lower esophageal sphincter (%)	13/111	(12)
Poor esophageal motility (%) (mean amplitude <30 mm Hg and/or ≥30% failed waves)	8	(7)

*Mean ± SD.

reveals poor esophageal motility, in which case a partial (Toupet) posterior wrap is used. If a short esophagus is identified after complete esophageal mobilization (defined as <3 cm of esophagus caudad to the hiatus without traction), an esophageal lengthening procedure (Collis gastroplasty or wedge fundectomy) is performed. Anterior gastropexy using T-fasteners is carried out selectively for patients with organoaxial volvulus to prevent recurrent volvulus postoperatively.

Data Analysis

All patients completed a questionnaire at each office visit preoperatively and postoperatively at 1 month, 6 to 12 months, and yearly thereafter. On late follow-up, those patients who did not return to the clinic were interviewed by phone. Data accrual included age, sex, BMI, height, previous abdominal operations, other medical conditions, medication use, symptoms, ASA score, and results of diagnostic studies (endoscopy, esophageal manometry, 24-hour pH testing, barium swallow). The presence of symptoms was assessed on a "yes/no" basis, including heartburn, chest pain, water brash, cough, regurgitation, nocturnal aspiration, asthma (wheezing), dysphagia, odynophagia, nausea, vomiting, bloating, and change in bowel habits. Surgical findings, technical details, and complications at the time of surgery were assessed, as well as postoperative outcomes, morbidity, and mortality. All data were recorded prospectively.

Postoperative physiologic assessment (manometry, 24-hour pH tests) and endoscopy were not performed routinely. Contrast barium esophagograms were obtained routinely at 6 to 12 months after surgery and/or to assess symptoms during follow-up. Recurrences assessed by esophagograms were defined as either migration of the intact fundoplication into the thorax or hiatal herniation with disruption of the fundoplication. Hernia size (as defined previously) was classified as small if less than 4 cm and large if 4 cm or more. Follow-up from 6 months to 2 years is defined as medium term. Complications were graded on a scale of I to IV, using the system described by Clavien et al.,[11] as follows: grade I = non–life-threatening complications; grade II = potentially life-threatening complications requiring either medical (IIa) or surgical/interventional (IIb) therapy; grade III = complications with residual and lasting disability; and grade IV = death as a result of a complication. The term "major complication" in this report is defined as any complication that is grade IIb or higher. For data analysis, chi-square/Fisher exact tests were used for comparison of discrete variables and the two-tailed *t* test for continuous data. Significance was defined as $P < 0.05$. Summary data are presented as mean ± SD.

RESULTS

Perioperative Data

Preoperatively the predominant symptoms were heartburn followed by chest pain and dysphagia (see Table 1); 11 patients (90%) had anemia and 12 patients (10%) were asymptomatic. Type II hiatal hernias (with the gastroesophageal junction located ≤2 cm from the hiatus) were found in 23 patients (20%) and type III or IV in 93 (80%). Esophagitis graded as greater than class 2 by the Savary-Miller classification was present in 16%, including two patients with Barrett's esophagus. All cases in this study were scheduled as elective procedures, although four of the hernias were incarcerated at the time of surgery. In no case was ischemic necrosis discovered.

Laparoscopic PEH repair was attempted in 119 patients and was successfully completed in 116 (97.5%). In two severely obese patients with marked hepatomegaly, the procedure was aborted because of inadequate exposure, bleeding from the liver capsule, and inability to visualize the hiatus. The third patient was converted to an open procedure to control bleeding from a hepatic vein laceration but did not undergo repair. Procedures performed in conjunction with PEH repair are shown in Table 2. Nissen fundoplication was performed in 108 patients and Toupet fundoplication in six patients; two patients had no fundoplication. Gastropexy was done in 48 patients. Prosthetic (mesh) hiatal closure with polypropylene was carried out in six patients, and in three patients a primary closure of the hiatus was reinforced with a small intestine submucosal patch (Surgisis; Cook Surgical Inc., Bloomington, IN). An esophageal lengthening procedure was performed in six patients (Collis gastroplasty in three and wedge fundectomy in three). Procedures performed for concurrent condi-

Table 2. Paraesophageal hernia: Repair techniques in 116 patients

Fundoplication		
Nissen	108	(93%)
Toupet	6	(5%)
None	2	(2%)
Other procedures		
Gastropexy	48	(41%)
Esophageal lengthening	6	(5%)
Mesh closure	6	(5%)
Onlay mesh buttress*	3	(3%)

*All small intestine submucosa patches.

tions included adhesiolysis in 19 patients, laparoscopic cholecystectomy in four patients, and partial colectomy in one patient who underwent laparoscopic right colon resection with ileocolic anastomosis for Crohn's disease.

Mean operative time was 169 ± 52 minutes, with a median of 162 minutes (range 100 to 320 minutes). During the first 20 procedures, the average operative time was 258 minutes, which decreased progressively to the current average. Intraoperative pleural tears causing minimal intraoperative pneumothorax were observed in 17 patients. None of these patients needed a chest tube, and therefore these events were not considered a complication in the analysis. Postoperative hospital stay ranged from 1 to 18 days with a median stay of 2 days. Postoperative hospitalization exceeded 10 days in three patients: one because of underlying cardiovascular disease, one because of atelectasis, and another because of severe dysphagia leading to esophageal dilatation. Table 3 summarizes the complications. Grade I and IIa complications included pulmonary embolism in two patients who were managed successfully with anticoagulation, pneumonia in two patients, three cases of atelectasis requiring pulmonary therapy, one case of hydrothorax managed by chest tube drainage, one patient with delayed gastric emptying and persistent postprandial diarrhea (suspected vagal injury), and one patient with urinary retention. Complications grade IIb and greater included two postoperative deaths (1.7%), both resulting from myocardial infarctions that occurred after discharge during the third postoperative week, one case of esophageal perforation requiring reoperation on postoperative day 1, one acute postoperative diaphragmatic herniation (slipped wrap) caused by early postoperative vomiting, also in a patient operated on during the first day after surgery, and one esophageal obstruction requiring endoscopic dilatation.

Postoperative Follow-Up

Mean follow-up has been 30 ± 25 months; five patients were lost to follow-up, and nine patients died in the late postoperative period of unrelated causes. In 96 patients (83%), postoperative follow-up exceeded 6 months (Table 4). Of these 96 patients, 73 (76%) had no esophageal symptoms. Heartburn was the most common complaint and was present in 12 patients (13%). Dysphagia was observed in 10 patients (10%); in eight of them it was present with ingestion of solids only and in two after liquids as well. Chest pain was present in seven patients. When pre- and postoperative symptoms were compared, marked improvement was noted in all symptoms evaluated ($P < 0.001$). A significant decrease in the use of antacid medications from preoperative (77%) to postoperative (11%) evaluation was also observed ($P < 0.001$).

Barium esophagograms were obtained in 66 (69%) of the 96 patients with more than 6 months of follow-up. The remainder have either refused to participate in the study or have not been evaluated in the office for more than 6 months postoperatively. Recurrent hiatal hernias were observed radiographically in 21 patients (22% overall or 32% of those undergoing contrast x-ray evaluation; Table 5), eight (38%) of whom were totally asymptomatic. Of the patients with recurrent hiatal hernias, 17 (80%) were operated on during the first half of the series. The nature of the recurrent anatomic defect was as follows: an intact fundal wrap migrated into the thorax in eight patients, PEH with dehiscence of the fundoplication in seven, and a sliding hiatal hernia (type I) in six patients. X-ray images of one of the patients with a recurrence are shown in Figs. 1 and 2. The size of the recurrent hernias was less than 4 cm in 12 patients (57%) and 4 to 7 cm in nine patients (43%). Univariate analysis of preoperative parameters, including sex, age, ASA score, BMI, and hernia size, to assess their influence on postoperative recurrence showed no statistically significant association between these variables and recurrence ($P > 0.05$). Recurrences have been found in two patients with mesh repair of the hiatus (33%) and

Table 3. Laparoscopic paraesophageal hernia repair: Complications

Complications grades I and IIa*	10 (8.6%)
Complications grade ≥IIb*	5 (4.3%)
Myocardial infarction/death	2 (1.7%)
Esophageal perforation	1 (0.9%)
Acute postoperative herniation	1 (0.9%)
Esophageal obstruction	1 (0.9%)

*Classification according to Clavien et al.[11]

Table 4. Medium-term symptomatic outcome: Ninety-six patients followed for more than 6 months postoperatively

	Preoperative	Postoperative
Symptoms present	88 (92%)	23 (24%)*
Dysphagia		
Solids only	34 (35%)	8 (8%)*
Liquids and solids	6 (6%)	2 (2%)*
Heartburn	63 (66%)	12 (13%)*
Chest pain	60 (62%)	7 (7%)*
Medication use†	74 (77%)	11 (11%)*

*$P < 0.01$.

†Proton pump inhibitors, H_2 blockers, antacids.

Table 5. Analysis of recurrent hiatal hernias: Twenty-one patients (22%)*

Type of defect		
Intrathoracic wrap (intact)	8	(38%)
Dehiscence with PEH	7	(33%)
Dehiscence with type I hiatal hernia	6	(29%)
Size of defect		
<4 cm	12	(57%)
4–7 cm	9	(43%)

*Thirty-two percent of those undergoing contrast radiography had recurrent hiatal hernias.

in 19 patients with primary nonmesh repair (21%). In those patients who underwent gastropexy, 12 (25%) of 48 had a recurrence compared to nine (13%) of 68 without gastropexy ($P = 0.08$). In evaluating symptoms among patients with recurrences, 13 (62%) were found to be symptomatic but only six patients have required medication for their complaints. No correlation was found between the presence of symptoms and the type or size of the recurrent defect. Reoperation for recurrent hernia has been performed in three patients (2.6%): two with recurrent PEH and one with acute postoperative herniation of an intact wrap. At 12 months' follow-up, this last patient was asymptomatic. None of the six patients with a recurrent sliding hernia (type I) have required reoperation.

DISCUSSION

Since the first report of laparoscopic Nissen fundoplication in 1991,[12] laparoscopic techniques have been used increasingly in the approach to patients with PEH.[13] Although many aspects of the operation are standardized, controversies remain regarding some of the various technical details. The roles of resection of the hernia sac, fundoplication, mesh closure, and gastropexy are still under evaluation. The results of this study show that despite a relatively consistent operation performed by experienced surgeons, including fundoplication and sac resection, the recurrence rate (22% overall, or 32% in those patients who underwent postoperative contrast x-ray imaging) is relatively high. Variations in technique did not seem to influence the rate of recurrence, whereas increased experience with the operation resulted in a lower rate of recurrence. Recurrence rates have varied between series depending on the extent of follow-up, but radiographic hiatal abnormalities after laparoscopic PEH repair are common, as shown in Table 6.[8,14–19] Despite the high recurrence rate in this series, a statistically significant improvement in symptoms and in medication use was observed in the group as a whole.

The proposed factors for anatomic failure of PEH repair are many. The diaphragm is a thin muscular structure providing poor support to anchoring sutures. Because most patients are elderly, the physiologic muscular changes make this area even weaker, especially in women.[20] The diaphragm is in continuous dynamic activity placing the edges of the repaired defect under increased tension. In addition, the large size of some PEHs often makes primary

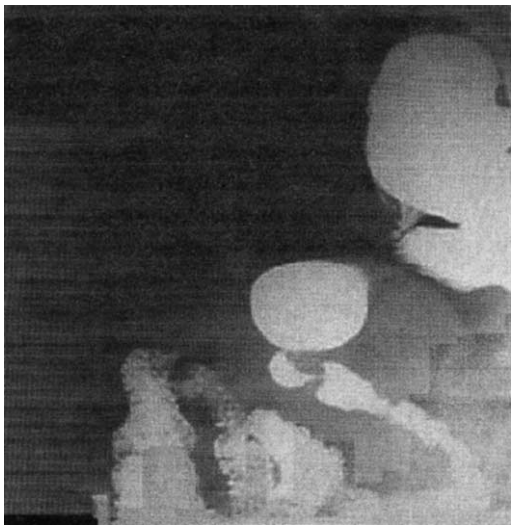

Fig. 1. Preoperative study showing typical contrast x-ray image of a type III PEH with 80% of the stomach in the chest.

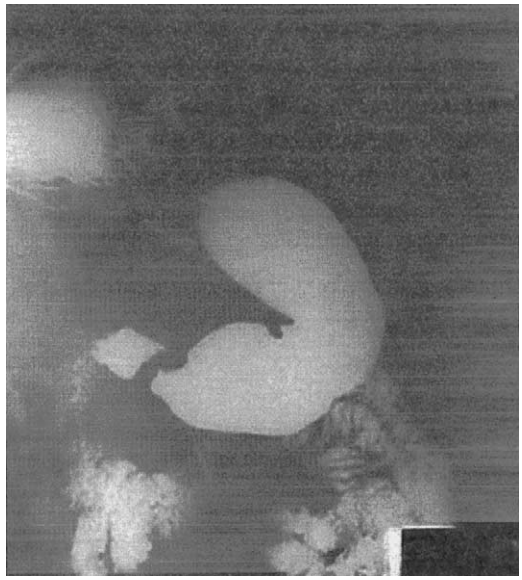

Fig. 2. Same patient as in Fig.1, now 12 months postoperative, clearly showing a recurrent large PEH with 40% gastric herniation into the chest.

Table 6. Recurrence rates after laparoscopic repair of paraesophageal hernia

Reference	Year	No. of patients followed	No. of recurrences (%)
Oddsdottir[13]	1997	53	7 (13)
Perdikis et al.[14]	1998	49	2 (4)
Gantert et al.[15]	1999	49	4 (8)
Hashemi et al.[8]	2000	21	9 (42)*
Swanstrom et al.[16]	2000	90	1 (1)
Luketich et al.[17]	2001	83	14 (17)
Mattar et al.[18]	2002	187	32 (17)*
Current series	2002	96	21 (22)*†

*Routine esophagogram performed.
†Incidence was 32% in the subset of patients who underwent contrast study more than 6 months postoperatively

tension-free repair impossible. Another factor that influences the recurrence rate after PEH repair is the presence of a foreshortened esophagus.

Because of the high recurrence rates, some investigators advocate a return to the thoracic approach because of lower recurrence rates reported for open transthoracic procedures.[8] However, the actual recurrence rates for open procedures are not well known, in part because esophagograms are often necessary to detect new hiatal abnormalities after surgery, and there are no large prospective series of conventional surgery that fulfill this requirement. Because the principal risk factors for recurrent herniation (large defect size and poor tissue characteristics) are not addressed by changing the location of the incision, the principal advantage of the thoracic approach would be a more extensive esophageal mobilization in patients with a short esophagus. However, laparoscopic techniques can now be used to lengthen the esophagus either by Collis-type gastroplasty[21] or wedge fundectomy. Additionally, the low morbidity and mortality of laparoscopic PEH repair compared to that performed by laparotomy (or thoracotomy)[10] has encouraged us to continue pursuing this approach. It is our view that the advantages of a minimally invasive approach to PEH repair outweigh the advantages of thoracic repair for most patients.

The analysis of the mechanisms for failure with inguinal hernia surgery led to the development of tension-free repair techniques involving prosthetic materials.[22,23] These operations were soon adopted by surgeons worldwide in view of the dramatic decrease in recurrence rates.[24] In the case of PEH, there has been reluctance to use prosthetic materials, despite good results in some studies,[25–27] because of the potential for esophageal or aortic erosion. An animal model of laparoscopic PEH repair has been developed in our laboratory to test new materials for prosthetic closure of this difficult area. Preliminary results suggest that small intestine submucosa (Surgisis) results in good tissue incorporation, while at the same time having a soft, noneroding surface.[28] Current studies are in progress to evaluate the long-term effectiveness of this material on recurrences.

As previously reported by the senior author (N.J.S.), many patients with recurrences are asymptomatic and are diagnosed only with the use of contrast studies.[7] In the present study, 38% of recurrences were completely asymptomatic and six patients (29%) required medical treatment. Only three of these patients have gone on to a second repair, to date, including the patient who underwent reoperation on postoperative day 1. Given our high rate of asymptomatic patients, routine barium swallow studies should be performed during follow-up.

The widely accepted management of PEH, regardless of symptoms, is surgical intervention because of the possibility of life-threatening complications when patients are managed by nonsurgical means. High rates of strangulated hernias were reported by Skinner and Belsey[2] and later by others,[29,30] but these observations have not been confirmed, and recent reports challenge this dogma.[31] In a study by Allen et al.,[32] only 4 of 23 patients who were followed without surgery for a median of 78 months became symptomatic. No patient developed strangulation, and there was only one death secondary to aspiration pneumonia after a barium study. In the present study, none of the patients had surgery on an emergency basis, and none of those with recurrent PEH hernias, who have been managed without surgery (12 patients followed for a mean of 20 months), have presented as surgical emergencies, except for the patient with an acute postoperative herniation on the day after surgery. A review of the English-language literature over the past 15 years, of clinical series reporting on more than 20 cases of PEH, revealed that only 49 (5.1%) of 962 patients presented as surgical emergencies.* If a minimally symptomatic recurrent hiatal hernia is discovered on follow-up, the appropriate management is unclear. We have adopted the position of close ongoing observation, rather than re-repair, because of the much higher rate of complications during reoperative hiatal surgery.[40]

Despite the documented 22% rate of recurrent hiatal abnormalities, laparoscopic PEH repair was associated with a low rate of complications in this relatively high-risk population. With experience, the recurrence rate decreased. There were no deaths due to technical complications, although two patients died of cardiac disease within 30 days postoperatively (mortality

*References 8,10,13–15,21,32–39.

1.7%). Major complications (grade IIb or greater) developed in 4.3% of patients overall and minor complications in 8.6%, which compares favorably with the reported incidence in other series of 10% to 37%.[13] Considering the age and the comorbid conditions of these patients, this low morbidity suggests that laparoscopic PEH repair is a relatively safe procedure. Further efforts should be made to develop an effective method of tension-free hiatal repair in order to minimize the rate of recurrent hiatal hernias.

CONCLUSION

Laparoscopic repair of PEH is safe and feasible, and most patients obtain good symptom control after repair. A relatively high incidence of recurrent hiatal abnormalities was seen after laparoscopic PEH repair; however, most of these recurrent hernias were small, asymptomatic, and have required no treatment over medium-term follow-up. Protocol esophagograms are necessary to detect recurrences that are minimally symptomatic. New techniques are needed to improve the outcome of laparoscopic PEH repair.

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Discussion

Dr. V. Velanovich (Detroit, MI): I am interested in your mesh repairs. Can you give us a little more detail about how you did the mesh repairs and if you had any mesh-related complications?

***Dr. S. Diaz*:** Most of the mesh repairs were performed at the beginning of our experience. All six mesh repairs were done with polypropylene. We have seen no complications related to the presence of the mesh; however, we are cautious about the use of it, and are concerned about the perforation, stenosis, or stricture that have been reported in other studies.

Dr. L.W. Way (San Francisco, CA): I have a couple of questions. First, you have a large experience here and you have evaluated your patients very nicely. You reported that your recurrences seemed to have been clustered in the early part of your experience. You have analyzed your operations, and you have used a variety of techniques. I wonder if you have any insight into the steps that were associated with absence of recurrence or failures to do certain things that were associated with recurrence? The big question in this operation concerns recurrences. What were the things you did differently in the second half of your experience that you did not do in the first half that led to your reduction in these problems? I would also like to make a historical comment with regard to the large number of persons who have embraced the technique of gastropexy for treatment of paraesophageal hernias, and just remind everyone that gastropexy was the standard operation in the 1950s, 1960s, and early 1970s until it was learned that anterior gastropexy had an extremely high incidence of recurrence. When we reoperated on these patients, we would find even the most vigorous gastropexy anteriorly would be represented some years later by long fibrous bands, the stomach back up near the hiatus, and these bands to the fixation points in the posterior rectus sheath. It did not really matter whether or not the patients had a gastrostomy, which some people have thought would be a more secure fixation. Anterior gastropexy has a terrible record of durability in the treatment of this, and to my mind, we cannot reinvent the wheel here, intuitively, just because it seems like the right thing to do. I would focus on a posterior gastropexy, which experience has shown is the best way to repair the stomach and gastroesophageal junction within the abdomen.

Dr. Diaz: We have not found a clear factor associated with recurrence. Because of the small number of mesh repairs, we cannot say whether there is a statistical difference in recurrence related to this part of the technique. Close to half of our patients had a gastropexy, when comparing these to patients that did not have it, there was no statistically significant difference in recurrence rates. The rationale for the gastropexy is not to prevent reherniation, but to prevent recurrent organoaxial volvulus. During gastropexy, besides using T-fasteners, currently we mark the inner abdominal wall site with electrocautery to stimulate adhesion formation on that zone.

Invited Discussion—Expert Commentator

David W. Rattner, M.D.: Dr. Soper and colleagues have made many contributions to the field of antireflux surgery. In this report of their experience with laparoscopic paraesophageal hernia repair (PEH), they confirm the high rate of radiographic recurrence that many other groups are experiencing. The study population is perhaps different from that in other reports in that 20% of patients had type II hernias—a rare defect. Although the recurrence rate for PEH was only 22%, I take issue with the authors' statement that these recurrences were not signifi-

cant. Only 8 of 21 recurrences were asymptomatic, although the reoperation rate was low. It is also interesting to note that the use of prosthetic mesh did not prevent recurrence.

One of the "take-home" messages of this report is that even the best surgeons cannot always overcome biology—that is, when the crura are flimsy and the patient is obese, a high rate of failure should be anticipated. Although I agree with nearly all of the authors' statements in their report, I must confess that in some patients one can get better purchase on their crural sutures with the use of open rather than laparoscopic techniques. The larger question, however, is whether we are operating on too many of these patients. Laparoscopic PEH repair is a challenging operation and has a mortality rate of 1.7% in this series. As the authors point out in their presentation, the risk of catastrophic complications from conservatively managed PEH is often misconstrued. Our group recently presented data based on large national databases, which show that the risk of death and the need for emergency surgery have been grossly overestimated. In fact, the risk of a patient with a minimally symptomatic PEH requiring emergency intervention is approximately 1% per year. If one accepts that the mortality rate for emergency surgery is 17% (the published rate, although the actual mortality rate in 1997 was 6%) and of elective laparoscopic PEH repair is 1.4%, a strategy of watchful waiting benefits more patients than routine elective laparoscopic PEH repair. It behooves us to consider all the odds—of surgical success, surgical mortality, recurrent hernia, and risks of watchful waiting—and individualize the facts to the patient sitting in our office when recommending surgical correction to minimally symptomatic patients with PEH. For patients with postprandial chest pain and obstructive symptoms, surgery is clearly indicated and the surgeon should use the technique that works best in his or her hands to achieve optimal results.

Helicobacter pylori Induces Apoptosis in Barrett's-Derived Esophageal Adenocarcinoma Cells

Andrew D. Jones, M.D., Kathy D. Bacon, B.S., Blair A. Jobe, M.D., Brett C. Sheppard, M.D., Clifford W. Deveney, M.D., Michael J. Rutten, Ph.D.

Helicobacter pylori may protect against the development of dysplasia in Barrett's epithelium of patients with gastroesophageal reflux disease. The aim of this study was to determine whether *H. pylori* preferentially induces apoptosis in Barrett's-derived cancer cells compared to normal cells. A Barrett's-derived adenocarcinoma cell line (OE33) was grown. *H. pylori* wild-type, isogenic *vacA*-, *cagA*$^-$, and *picB-/cagE*- mutant strains were grown on agar plates. Intact or sonicated bacteria were used to treat normal and OE33 cells for 24 hours, and Hoechst dye binding was performed to measure apoptosis. *FAS* protein expression was determined by Western immunoblotting. OE33 cells treated with intact *H. pylori* wild-type strains produced significant ($P < 0.05$) dose-dependent increases in apoptosis compared to normal esophageal cells. *H. pylori* wild-type and *vacA*- isogenic strains were more effective than *cagA*- and *picB-/cage*- isogenic strains in inducing apoptosis in OE33 cells. In OE33 cells, *H. pylori* sonicates produced lower levels of apoptosis than intact bacteria. Wild-type *H. pylori* strains increased *Fas* protein expression in OE33 cells at 18 hours. *H. pylori* induced apoptosis at a higher rate in the Barrett's-derived human esophageal adenocarcinoma cells than in normal esophageal cells. The *H. pylori*–induced apoptosis was primarily dependent on intact bacteria and the presence of the *cagA* and *picB/cagE* gene products. *H. pylori*–induced apoptosis may involve the *Fas*-caspase cascade. (J GASTROINTEST SURG 2003;7:68–76.)

KEY WORDS: *Helicobacter pylori*, Barrett's, apoptosis, esophagus, adenocarcinoma

Helicobacter pylori is a common pathogen of the human gastrointestinal tract that causes chronic inflammation of the stomach, which leads to mucosal atrophy and metaplastic changes.[1] As the rate of *H. pylori* infections has declined over the past 20 years, so too have the rates of gastric ulcers, duodenal ulcers, and gastric cancer.[2] However, during this same time period, the prevalence of Barrett's esophagus and esophageal adenocarcinoma has tripled.[3] Some investigators have postulated that there may be a relationship between the decreasing rate of *H. pylori* infection and the increasing rates of gastroesophageal reflux disease (GERD), Barrett's esophagus, and esophageal adenocarcinoma.[4,5] That is, in several case-control studies it was found that Barrett's esophagus and esophageal adenocarcinoma were less common in *H. pylori*–infected patients.[6–11] In addition, a number of studies have shown that when patients are cleared of *H. pylori* infections, their incidence of reflux esophagitis increases significantly.[12–14]

Although a few theories have been proposed for this *H. pylori* effect in Barrett's esophagus, the exact protective mechanism is still not known. Some studies have suggested that the higher pH found in chronic *H. pylori*–inflamed gastric mucosa produces a refluxate into the esophagus that is less damaging to the esophageal cells.[15] In addition, it has been reported that certain strains of *H. pylori* appear to be more protective that other strains.[16] That is, *H. pylori* strains positive for the "cytotoxin-associated gene A" (*cagA*$^+$), which is found in approximately 70% of wild-type *H. pylori*, have been postulated to have a greater protective effect in the prevention of Barrett's esophagus than the *H. pylori cagA*$^-$ strains.[4,9,16–19] These same *H. pylori cagA*$^+$ strains have also been

Presented at the Seventeenth Annual SSAT Resident and Fellow Research Conference, San Francisco, California, May 18, 2002; and at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (poster presentation).

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Published by Elsevier Science Inc.
1091-255X/03/$—see front matter
PII: S1091-255X(02)00129-4

shown to induce apoptosis to a greater extent than the *cagA*$^-$ strains in human gastric[20–31] and intestinal[32] epithelial cells. In this regard it is possible that the *H. pylori* may have direct protective effect against the development of esophageal adenocarcinoma by preferentially inducing apoptosis in cancer cells compared to normal esophageal cells. The aim of this study was to examine the direct effects of *H. pylori* on apoptosis in normal and Barrett's-derived esophageal cells.

METHODS

Cell Culture

Normal esophageal epithelial cells were isolated from surgical specimens using modifications of previously described techniques.[33–36] The Oregon Health and Sciences University Human Studies Subcommittee approved the use of all human tissues in this study. Esophageal tissues obtained after elective esophageal surgery were opened longitudinally, washed in phosphate-buffered saline (PBS) solution, and cut into 15 mm fragments. The fragments were then transferred to a plastic culture dish containing 5 ml of ice-cold Matrisperse (Collaborative Biomedical Products, BD Discovery Labware, Bedford, MA) and incubated at 4° C for 12 to 14 hours without agitation. After this time, each dish was gently shaken to separate the epithelium, or a glass slide was used to gently scrape the epithelial tissue away from the submucosa. The epithelial suspension was then washed twice in 4° C Hank's balanced salt solution (HBBS), centrifuged at $100 \times g$ for 5 minutes, then resuspended in Dulbecco's modified Eagle's medium (DMEM; Gibco/BRL, Grand Island, NY) supplemented with 4 mmol/L glutamine, 20 mmol/L HEPES, 50 U/ml penicillin, 50 μg/ml streptomycin, 5 ng/ml recombinant human epidermal growth factor (PromegaCorp., Madison, WI), and 10% fetal bovine serum (FBS; Hyclone, Logan, UT) plated in two to three 60 mm culture dishes (Falcon, Becton Dickenson Labware, Franklin Lakes, NJ) and/or eight-well Lab-Tek slides (Nunc; Fisher Scientific, San Fransciso, CA) previously coated or not with a solution of type I collagen (Vitrogen; Celltrix, Palo Alto, CA), and cultured at 37° C with 5% CO_2.

For identification of normal esophageal cells, esophageal cells were grown on glass slides or on type I collagen (Vitrogen) and fixed in 5% buffered zinc–formalin for 10 minutes. Esophageal cells grown on extracellular matrix were processed for paraffin embedding. Thick sections (5 μm) from the paraffin blocks or cells grown on glass slides were then stained for the presence of mucin using either alcian blue or periodic acid–Shiff (PAS) stains. Cultured cells were also cultured on glass slides, washed with PBS, and fixed in cold acetone for immunocytochemical detection of the epithelial marker, cytokeratin, as well as the fibroblast marker, vimentin, using commercially available antibodies and the procedures supplied by the manufacturer (Zymed Laboratories, South San Francisco, CA).

A Barrett's-derived human esophageal adenocarcinoma cell line (OE33) was purchased from the European Collection of Animal Cell Cultures (ECACC, Wiltshire, England). Both cell lines were then karyotyped to verify genetic characteristics.

Bacterial Culture

The *H. pylori* bacterial strains used in this study were the wild-type *vacA*$^+$, *cagA*$^+$ strains 60190 (49503; American Type Culture Collection, Rockville, MD) and 84-183, an isogenic *vacA*$^-$ mutant (60190:v1), an isogenic *cagA*$^-$ mutant (84-183:M22), a *cagA*$^-$ isogenic mutant (60190:M22), and an isogenic *picB-/cageE-* mutant (60190 *picB* null mutant). Both *H. pylori* 60190 and 84-183 wild-type strain contain type s1/m1 *vacA* alleles,[37] and the *vacA*$^-$, *cagA*$^-$, and *picB*$^-$/*cagE*$^-$ mutants have been well characterized and previously described.[37–39] The bacteria were grown on blood agar plates (trypticase soy agar with 5% sheep blood; PML Microbiologicals, Tualatin, OR) under microaerobic conditions using a CampyPak jar (Fisher Scientific) at 36° C. Unless noted otherwise, all bacteria were harvested at 24 hours using a sterile cotton swab and 3 ml of PBS (pH = 7.1). The bacterial suspensions were put into 12 ml Falcon round-bottom tubes, and the *H. pylori* were resuspended by gentle inversion. One milliliter of the suspension was put into a cuvette, and the *H. pylori* concentration was determined using an OD_{600}, where an OD of $1 = 1.2 \times 10^9$ colony-forming units(cfu)/ml. All final bacterial suspensions (1×10^5 to 1×10^9 cfu/ml) were adjusted with the appropriate cell culture media.

H. pylori sonicates were made by growing the bacteria on agar plates for 24 hours, then harvesting the bacteria in PBS as indicated above. The bacteria were washed twice in PBS by centrifugation at $10{,}000 \times g$ for 15 minutes, and the pellet was then resuspended in mammalian Ringer's solution (pH 7.4). The bacterial suspensions were disrupted by means of sonication (10 30-second pulses); then the sonicates were filtered through a 0.2-micron filter, and the protein content was determined using a Bio-Rad protein assay. Aliquots were frozen and stored at −80° C until needed. For control studies both live bacteria and bacterial sonicates were heated to 70° C for 30 minutes to make heat-inactivated bacteria and sonicates.

Apoptosis Assay

Intact bacteria or sonicated bacteria were used to treat normal esophageal cells and the OE33 cells for 24 or 48 hours in 12-well plates using 1.5 ml of HBSS as medium. After being treated for 24 or 48 hours, the cells were rinsed with 400 μl of HBSS with calcium, transferred to 12 mm tubes, and then centrifuged at 900 × *g*. The supernate was then aspirated off, and 500 μl of 2X trypsin was added to each well to detach the live cells. After cell detachment, 500 μl of HBSS was added to each well, the trypsin-HBSS solution was transferred to 12 mm centrifuge tubes, and the tubes were spun at 900 × *g* to form cell pellets. The supernate was then aspirated, and the cells were fixed in 100 μl of 3.75% formaldehyde. The cells were then spun again to form a pellet, and resuspended in 50 μl of Hoechst dye; an ultraviolet staining buffer used to measure apoptosis. After the stain was allowed to set, the cells were placed onto slides and sealed. The slides were then viewed under an ultraviolet microscope. The percentages of normal and apoptotic cells were counted and recorded.

Western Immunoblotting

Western immunoblotting was used to detect *Fas* protein expression. Normal and OE33 Barrett's-derived esophageal cells were grown to preconfluency in 10 cm Falcon plastic dishes and then treated with *H. pylori* at 1×10^9 cfu/ml. At specific times, the medium was removed, the cells were rinsed with 4° C lysis buffer, and then the cells were scraped into 1 ml of lysis buffer. The lysis buffer contained 1% Triton X-100, 10% glycerol, 20 mmol/L HEPES, and 150 mmol/L NaCl. The suspension was briefly centrifuged and the supernate was transferred to a prechilled 1.5 ml tube. Lysate protein was quantitated using the Bradford method with a commercially available kit (Bio-Rad Laboratories, Hercules, CA). Size fractionation of the proteins was performed using a 10% sodium dodecyl sulfate polyacrylamide (SDS) gel with 5 μg of protein added to each lane. The proteins were transferred to polyvinylidene diflouride (PVDF) membrane (Immobilon P; Millipore Corp., Bedford, MA) by electroblotting. The membrane was blocked in 3% BSA, 0.05% sodium azide, for 1 hour at room temperature, followed by overnight incubation with primary anti-*Fas* antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at 4° C in tris tween buffered saline (TTBS) (0.05% Tween-20, 20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl). The membrane was washed three times in TTBS, then incubated with a secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) for 1 hour at room temperature. The membrane was then extensively washed in TTBS, and the protein bands were visualized by chemiluminescence (Renaissance; DuPont NEN, Boston, MA) using Kodak X-Omat AR film. The film was photographed and a densitometric analysis of the bands performed using SigmaGel software (SPSS, San Rafael, CA).

Statistics

All data points are expressed as means ± standard error (SE). The differences between means were considered significant at $P < 0.05$, as calculated by means of Student's *t* test for paired cultures. Multiple cell culture comparisons were analyzed by means of analysis of variance and Duncan's multiple-range tests. Unless stated otherwise, "N" in this study represents the total number of different "individual" cell preparations isolated from different surgical specimens. All statistical calculations were made with the use of Sigma-Stat statistical software (SPSS).

RESULTS

H. pylori Induces an Apoptotic Morphology in Barrett's-Derived Epithelial Cells But Not in Normal Esophageal Cells

In the first series of experiments, we wanted to determine whether infection with live intact *H. pylori* could directly stimulate apoptosis in normal and OE33 Barrett's-derived esophageal cells. Cultures were incubated with wild-type *H. pylori* (1×10^8 cfu/ml) for 24 hours, and apoptotic bodies were assessed using the fluorescence–Hoechst DNA dye binding assay. We found that a 24-hour treatment of normal esophageal cells with wild-type *H. pylori* produced very little apoptosis (see Fig. 1, *A* and *B*). In contrast, cultures of OE33 Barrett's-derived esophageal cells infected for 24-hours with wild-type strain 60190 produced a characteristic apoptotic morphology that included nuclear chromatin condensation and apoptotic bodies (see Fig.1, *C* and *D*). Specifically, the nuclear condensation within the apoptotic OE33 esophageal cells could easily be identified because of the increased nuclear Hoechst dye fluorescence (see Fig. 1, *D*). In addition, numerous small fluorescent apoptotic bodies around the nucleus were also easily identifiable within cells of the *H. pylori*–treated OE33 cell cultures (see Fig. 1, *D*).

H. pylori Dose Dependently Increases Apoptosis in OE33 Barrett's-Derived Esophageal Cells But Not in Normal Esophageal Cells

In the next series of experiments, we wanted to determine whether live intact wild-type *H. pylori* pro-

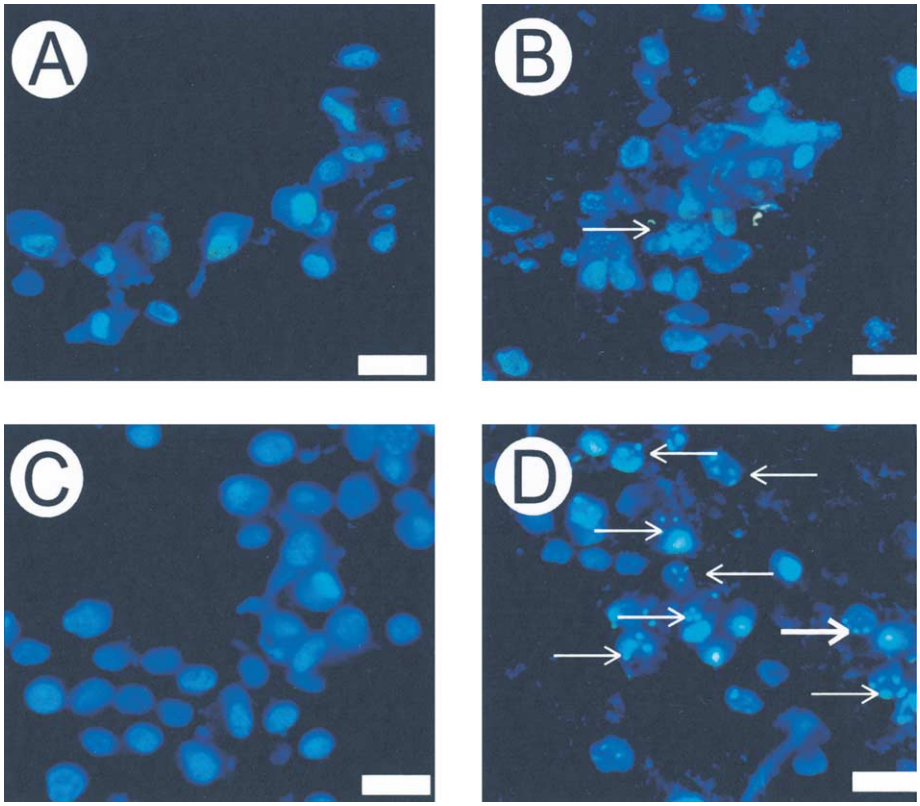

Fig. 1. A series of photographs showing Hoechst dye fluorescence of apoptotic bodies of *H. pylori*–treated normal esophageal cells (**A** and **B**) and Barrett's-derived OE33 cells (**C** and **D**). *White arrows* (**B** and **D**) point to apoptotic bodies. Bar = 20 μM.

duced a dose-dependent increase in apoptosis. When the wild-type 60190 *H. pylori* (1×10^5 to 1×10^9 cfu/ml) strain was added to cultures of OE33 Barrett's-derived esophageal cells, we found a 1.3 ± 0.05–fold increase in apoptosis over untreated control cultures at 1×10^6 cfu/ml, and a 3.1 ± 0.2–fold-increase in apoptosis over control cultures at 1×10^8 cfu/ml (Fig. 2). We could not detect any significant ($P > 0.05$) apoptosis in OE33 cell cultures using *H. pylori* at 1×10^5 cfu/ml (data not shown). *H. pylori* at 1×10^9 cfu/ml induced a 2.7 ± 0.3–fold increase in apoptosis, which was slightly less but not significantly different ($P > 0.05$) from the 1×10^8 cfu/ml *H. pylori*–treated OE33 cell cultures (see Fig. 2). Similar results in OE33 esophageal cell apoptosis were also observed with the 84-183 wild-type *H. pylori* strain (data not shown).

Compared to the OE33 Barrett's-derived esophageal cells, treatment of normal esophageal cells with wild-type 60190 *H. pylori* strains (1×10^6 to 1×10^9 cfu/ml) did not produce a dose-dependent increase in apoptosis. Only at the highest concentrations used, 1×10^8 and 1×10^9, did we detect a 0.2 ± 0.05–fold increase and a 0.31 ± 0.05–fold increase, respectively, over untreated control cultures (see Fig. 2). Heat-killed *H. pylori* produced no apoptosis in either OE33 Barrett's-derived esophageal cells or normal esophageal cells (data not shown).

H. pylori Induced Apoptosis in OE33 Barrett's-Derived Esophageal Cells Is Primarily *cagA* and *picB/cagE* Dependent

It is now known that the *H. pylori* VacA toxin and the *cag* pathogenicity island are involved in several aspects of gastric epithelial inflammation, growth, and apoptosis.[40–44] Several genes in the *cag* pathogenicity island, including the *cagA* and the *picB/cagE* gene, have been shown to be involved in apoptosis in other cell types.[23,32,45,46] For the following experiments, we therefore were interested in testing the effects of *H. pylori* *cagA*-, *vacA*-, and *picB-/cagE-* isogenic mutants on apoptosis in normal esophageal and OE33 Barrett's-derived esophageal cells.

In OE33 cell cultures, the *H. pylori* 84-183:M22 *cagA*- isogenic strain at 1×10^8 cfu/ml produced only a 1.61 ± 0.18–fold increase in apoptosis compared to a 3.61 ± 0.18–fold increase in apoptosis by the wild-type 84-183 strain (Fig. 3). The addition of the *picB-/cagE-*

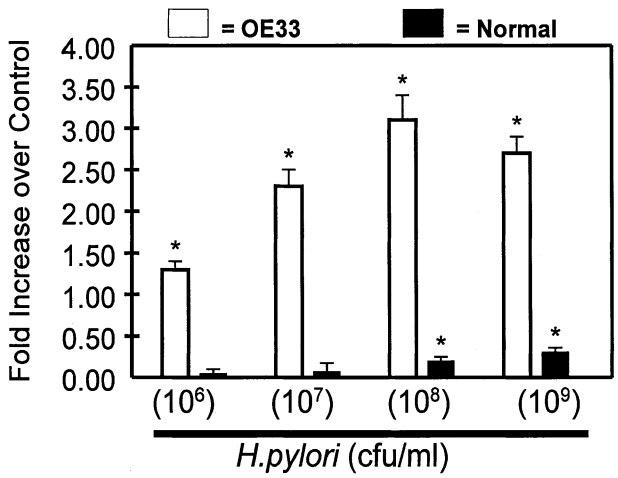

Fig. 2. Graph showing dose-dependent effects of intact *H. pylori* (wild-type 60190) on apoptosis in Barrett's-derived OE33 cells and normal esophageal cells. Data are expressed as mean ± SE; N = 8.

isogenic mutant to OE33 esophageal cells only produced a 0.70 ± 0.1–fold increase in apoptosis (see Fig. 3). The *vacA*- isogenic mutant–treated cultures had a 2.89 ± 0.10–fold increase in apoptosis, which was only approximately 20% lower than that seen with the wild-type *H. pylori* (see Fig. 3). In normal esophageal cultures, we found that all three of the isogenic mutants were less effective than the wild-type 60190 or 84-183 strains in producing apoptosis (see Fig. 3). However, there were no significant differences ($P > 0.05$) between the various isogenic strains in their ability to induce apoptosis in normal esophageal cells (Fig. 3).

H. pylori Lysates Produce Lower Levels of Apoptosis

In most cases the attachment of live intact *H. pylori* to gastric cells is necessary to produce the full biological effects of the bacteria on the gastric epithelial cells.[47] However, in other studies it has been reported that *H. pylori* bacterial lysates can also produce some biological effect.[48–50] We therefore wanted to determine whether *H. pylori* lysates could produce apoptotic effects comparable to those in the intact live bacteria on normal esophageal and OE33 Barrett's-derived esophageal cells. As shown in Fig. 4, lysates made from the *H. pylori* wild-type strain had a 1.6 ± 0.18–fold increase in apoptosis, but this was significantly lower ($P < 0.05$) than the 3.1 ± 0.2–fold increase in apoptosis observed with live intact bacteria (see Fig. 2). Lysates from the *H. pylori* 84-183:M22 *cagA*- isogenic mutant and the *picB-/cagE*- isogenic mutant were also less effective in producing apoptosis compared to the effects seen with their respective live bacteria (compare Fig. 4 to Fig. 2). Lysates made from the *picB-/cagE*- isogenic mutants also produced lower rates of apoptosis compared to intact *picB-/cagE*- bacteria (compare Fig. 4 to Fig. 2). However, the *picB-/cagE*- lysates had apoptotic rates similar to those of lysates made from the wild-type bacteria *(picB-/cagE-* = 1.9 ± 0.1–fold increase, wild-type 1.6 ± 0.18–fold increase; see Fig. 4).

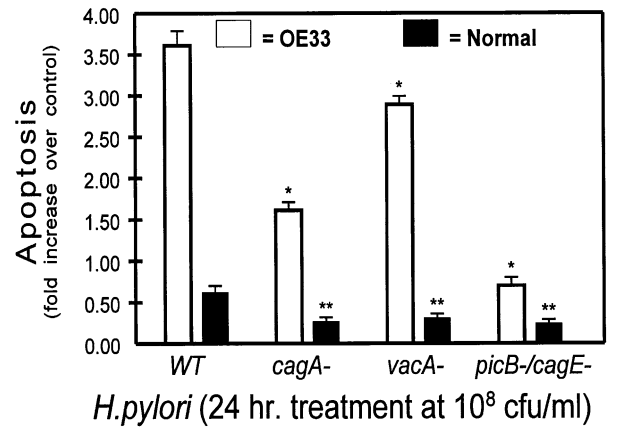

Fig. 3. Graph showing dose-dependent effects of various *H. pylori* strains on apoptosis in Barrett's-derived OE33 cells and normal esophageal cells. Compared to normal esophageal cells, *H. pylori*–induced apoptosis in Barrett's-derived OE33 esophageal cells was primarily dependent on the *cagA*⁻ and *picB-/cage*- gene products. Data are expressed as mean ± SE; N = 7. (*, ** Significantly different from wild-type (*WT*) treatment; $P < 0.05$.)

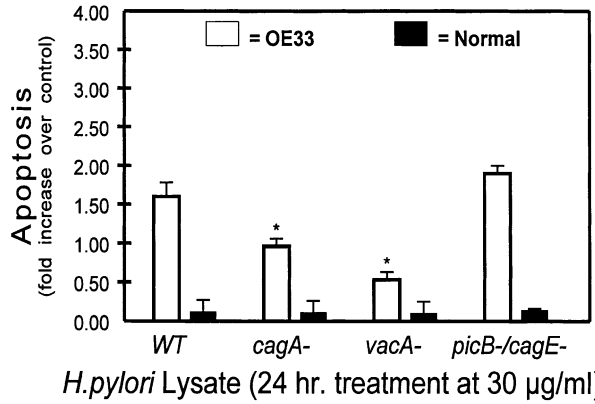

Fig. 4. Compared to live intact bacteria, *H. pylori* lysate treatment produced lower apoptosis rates on Barrett's-derived OE33 cells. There was no significant effect of the lysates on normal esophageal apoptosis. Data are expressed as mean ± SE; N = 5. (*Significantly different from wild-type (*WT*) treatment; $P < 0.05$.)

In normal esophageal cells, there was no effect of the *H. pylori* lysates on apoptosis when either wild-type or isogenic strains were used (see Fig. 4).

H. pylori Increases *Fas* Expression in OE33 Barrett's-Derived Esophageal Cells

H. pylori has been shown to increase *Fas* expression in both intestinal and gastric cells.[20–32] We therefore wanted to determine whether *H. pylori* changed *Fas* expression in OE33 Barrett's-derived esophageal cells. As shown in Fig. 5, treatment of OE33 cells with wild-type 60190 *H. pylori* (1×10^8 cfu/ml) increased *Fas* expression by 18 hours. In other preliminary data we found that wild-type *H. pylori* could increase *Fas* expression as early as 4 hours after treatment (data not shown). Heat-killed wild-type *H. pylori* produced no change in basal *Fas* expression.

DISCUSSION

As the rate of *H. pylori*–induced gastric and duodenal ulcers has decreased over the past 30 years, the rates of GERD, Barrett's esophagus, and esophageal adenocarcinoma have more than tripled. Based on these divergent trends, some investigators have postulated that *H. pylori* may protect against the development of reflux disease and the development of Barrett's esophagus.[6] The proposed mechanism by which *H. pylori* exerts its effects is by causing chronic gastritis, thereby decreasing gastric acid production and raising the pH of the esophageal refluxate.[2] Although it is debatable, some clinical studies have supported this hypothesis.[12–14] The prevalence of *H. pylori* infection also has been shown to be significantly less in persons with high-grade Barrett's dysplasia and esophageal adenocarcinoma (15%) compared to those with GERD alone (44%).[7] Based on these studies, the investigators have theorized that *H. pylori* indirectly protects against the development of reflux and its sequelae.[2]

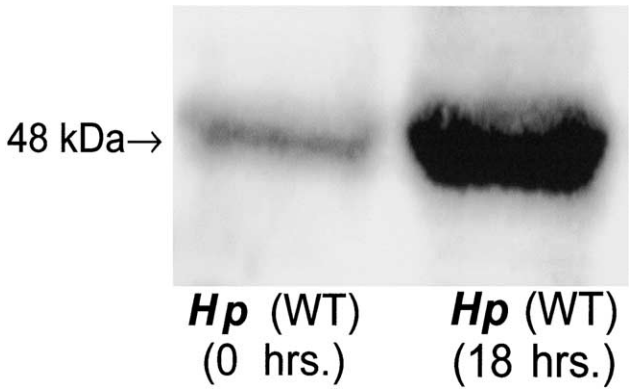

Fig. 5. Representative Western immunoblot showing effects of *H. pylori* (wild-type 60190) on *FAS* expression in OE33 Barrett's-derived esophageal cells. Note that *H. pylori* treatment increased *FAS* expression by 18 hours.

However, it is also possible that *H. pylori* may have a more direct protective role against the development of high-grade Barrett's dysplasia and esophageal adenocarcinoma. For example, it is known that wild-type *H. pylori* strains can directly induce apoptosis in human gastric[20–31] and intestinal[32] epithelial cells. We therefore hypothesized that *H. pylori* may directly protect against the development of esophageal adenocarcinoma by preferentially inducing apoptosis in the Barrett's-derived adenocarcinoma cells compared to normal esophageal cells. We found that *H. pylori* did induce significantly more apoptosis in the OE33 Barrett's-derived adenocarcinoma cells line compared to normal esophageal cells. That is, with increasing concentrations of the wild-type *H. pylori*, we found a dose-dependent increase in apoptosis. In contrast, we found minimal apoptotic changes in the normal esophageal cells treated with the same concentrations of wild-type *H. pylori*.

We were also interested in determining which components of the *H. pylori cag* pathogenicity island may be important in the apoptotic process in the Barrett's-derived adenocarcinoma cells. It now is well known that *H. pylori* has a number of virulence factors, including the vacuolating cytotoxin A (VacA) and the cytotoxin-associated gene A (*cagA*).[40,41,47] In addition, it has been shown that the protein products encoded by the *picB/cagE* gene are important in the assembly of the *H. pylori* type IV secretion system that is responsible for the injection of the CagA protein from the bacteria into the host cells.[51–53] With the use of *H. pylori vacA*-, *cagA*, and *picB-/cagE*-isogenic mutants, we found that the *vacA*- isogenic mutant was nearly identical to the wild-type strain in inducing apoptosis in the Barrett's-derived adenocarcinoma cells. However, the $cagA^-$ and *picB-/cagE*-isogenic strains produced very low levels of apoptosis. These findings suggest that the presence of the CagA protein and an intact *H. pylori* injection system are important for inducing apoptosis in these cells, whereas the presence of the VacA toxin is less important. We also found that live intact *H. pylori* are needed to produce the maximal apoptotic effects seen in the Barrett's-derived adenocarcinoma cells because the bacterial lysates of both wild-type strains and isogenic mutants produced much less apoptosis.

It also has been suggested that one possible mechanism of *H. pylori*–induced apoptosis in gastric and intes-

tinal cells is through the regulation of the *Fas* receptor caspase signaling cascade.[20–32] The *Fas* receptor (CD95/APO-1) is a member of the tumor necrosis factor superfamily and is ubiquitously expressed in human epithelial cells.[54] On binding with its ligand (*FasL*), *Fas* activates the *caspase* cascade, eventually executing the apoptotic process through fragmentation of cellular DNA.[54] Of interest are the reports that some esophageal adenocarcinoma or tumor cells overexpress *FasL*, whereas there is little expression of the *Fas* receptor.[55–58] These findings have prompted some investigators to suggest that esophageal tumor cells escape immune attack because the increased *FasL* binds to and kills the tumor-reactive T lymphocytes while the esophageal cancer cells escape the effects of their own *FasL* because of low *Fas* receptor expression.[56] In our study we also found that *H. pylori* increased *Fas* protein receptor expression over time in the Barrett's-derived adenocarcinoma esophageal cells. This *H. pylori*–induced increase in *Fas* receptor expression in the Barrett's-derived adenocarcinoma esophageal cells may represent a novel mechanism where *H. pylori* can induce apoptosis and thus reduce the rate of esophageal tumor cell proliferation (Fig. 6).

Even though several lines of evidence indicate that *H. pylori* may be protective against the formation of GERD or Barrett's esophagus, the role of *H. pylori* and it association with GERD remains controversial.[59–63] For example, several studies have reported that there is no association between the presence or absence of *H. pylori* and the occurrence of Barrett's esophagus in patients with GERD.[64–67] The discrepancy between these studies and those reports that have shown *H. pylori* to have a protective role against the development of Barrett's esophagus[9,10,68,69] may be a result of the variability of the *H. pylori* strains present in the patient population examined. In this regard some have suggested that the proposed *H. pylori* protective mechanism against the development of GERD may involve *H. pylori* strains that are different from, or less virulent than, the *H. pylori cag*+ strains associated with active gastritis.[70,71] Also, compared to the gastric mucosa, where *H. pylori* adherence and colonization have been easy to identify in patients with ulcer disease,[72] histologic studies of Barrett's esophagus have reported either the absence[10,64,66] or presence[5] of *H. pylori*. These conflicting data suggest that *H. pylori* colonization of the esophagus may be low or occur by a mechanism that is different from what has been observed in the gastric mucosa. Overall, additional studies are still needed to resolve the issue of whether those patients who are successfully treated for *H. pylori* infection are at increased risk for the development of Barrett's esophagus.[73]

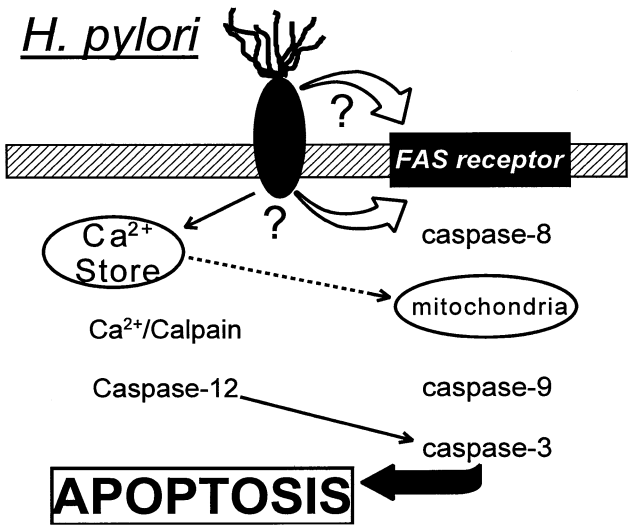

Fig. 6. Graph showing a model of two possible signaling mechanism(s) for *H. pylori*–induced apoptosis in Barrett's-derived adenocarcinoma cells. Adherence of *H. pylori* to the host cell could produce immediate changes within the cells by changing intracellular Ca^{2+} levels, as well as more prolonged activation of the *Fas*-caspase signaling pathway that would lead to apoptosis.

CONCLUSION

H. pylori induces apoptosis at a higher rate in Barrett's-derived human esophageal adenocarcinoma cells compared to normal esophageal cells. Wild-type bacteria produce more cell death than the isogenic mutant strains, and intact bacteria cause more deaths than bacterial sonicates. *H. pylori*-induced apoptosis may be directed through the *Fas*, *caspase* pathway.

We thank Dr. Timothy Cover from the Department of Microbiology and Immunology at Vanderbilt University for the H. pylori *strains, as well as for some helpful discussions at the beginning of these studies.*

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Apoptotic and Proliferative Indexes in Esophageal Cancer: Predictors of Response to Neoadjuvant Therapy Apoptosis and Proliferation in Esophageal Cancer

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Altered expression of the genes that control apoptosis and proliferation may influence the response of cancer cells to cytotoxic agents. The primary aim of this study was to determine the role of the novel antiapoptotic and cell cycle gene, survivin, in apoptotsis and proliferation in esophageal cancer and to evaluate whether the survivin, p53, and bcl-2 status were able to predict a patient's response to neoadjuvant therapy. A total of 104 patients with esophageal tumors were studied. Tumor tissue was immunostained for survivin, p53, and bcl-2 proteins. Proliferative and apoptotic activity was measured using ki-67 immunohistochemical analysis and the TUNEL method, respectively. Forty-eight patients whose pretreatment biopsies were analyzed received neoadjuvant chemoradiation therapy or chemotherapy followed by surgery. Outcome was graded as a complete response, a partial response, or no response according to the results of histologic examination and CT imaging. Expression of survivin was found to correlate significantly with the proliferative index but not the apoptotic index. Patients who received neoadjuvant treatment were more likely to achieve a complete response if their tumors had high proliferative activity, and p53 positive tumors were more likely to contain residual tumor after treatment. In conclusion, survivin expression appears to foster proliferative activity in esophageal cancer, and tumors with a high proliferative index or a functioning p53 gene are more responsive to neoadjuvant chemoradiation therapy. (J GASTROINTEST SURG 2003;7:77–87.)

KEY WORDS: Esophageal cancer, neoadjuvant therapy, survivin, p53, bcl-2, apoptosis, proliferation

In an attempt to improve the outcome for patients with esophageal cancer, treatment with preoperative (neoadjuvant) chemotherapy or combined chemotherapy and radiation therapy has been proposed.[1–4] However, the response to treatment is variable and unpredictable, and although most patients are downstaged, approximately 25% to 30% of patients experience no response or even show disease progression during treatment. For these reasons it would be helpful to have an indicator of response that could be detected in the tumors of patients before they receive treatment, so that patients who are unlikely to benefit from neoadjuvant therapy may undergo surgical resection alone. We postulated that the differences in the molecular profile of the tumors may be an indication of the varied response to treatment. This profile may provide molecular markers that predict a patient's response to neoadjuvant therapy and assist in making management decisions for patients.

Ionizing radiation and chemotherapeutic agents use the process of apoptosis (programmed cell death)

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (oral presentation).
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Supported by grants from the Royal College of Surgeons of England and Yorkshire Cancer Research.
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Published by Elsevier Science Inc.
1091-255X/03/$—see front matter
PII: S1091-255X(02)00141-5

to induce cancer cell death.[5] The development of resistance to either chemotherapy or radiation therapy may be mediated by alterations in the pathways involved in signaling and executing the process of apoptosis. Consequently mutations and alterations in the expression of genes that modulate apoptosis may dictate how individual tumors respond to chemotherapy and radiation therapy. For example, mutations in the p53 tumor suppressor gene may fail to cause cell death in cytotoxic damaged cancer cells because the mutated form of p53 is ineffective in triggering apoptosis.[6] Equally, overexpression of the bcl-2 gene may exert a potent inhibitory action on the effector phase of apoptosis once this has been signaled as a result of cellular damage caused by cytotoxic agents.[7]

One of the most interesting tumor-associated genes is survivin[8] because of its function in the regulation of both apoptosis and proliferation. Survivin is a member of the Inhibitor of Apoptosis (IAP) gene group,[9] which acts by the direct inhibition of the terminal effector proteases of apoptosis, namely, caspase-3 and caspase-7. Studies have shown that the expression of survivin inhibits cell death induced by a variety of apoptotic stimuli.[10]

Survivin is at variance with the other IAPs because it is implicated in cell cycle control. Survivin is primarily expressed in a cell cycle–dependent manner, and this expression is predominantly during the G2/M phase where it is upregulated by greater than 40-fold. Survivin localizes to the mitotic apparatus and appears to be associated with the function of the microtubules in the mitotic spindle during cytokinesis.[11]

One of the most significant features of survivin is its differential upregulation in cancer compared to normal tissues. Survivin is strongly expressed in embryonic tissues but is undetectable in most terminally differentiated normal adult tissues. However, it is apparent that there is widespread overexpression of survivin in many types of human cancer.[8] As a result of this overexpression, there have been a number of investigations into the prognostic value of survivin. The expression of survivin has been shown to predict a poor prognosis, in terms of survival and disease recurrence, for patients with colorectal cancer,[12,13] neuroblastoma,[14] and bladder cancer.[15] In addition, the downregulation of survivin in lung cancer cells results in a higher level of apoptosis after the addition of chemotherapeutic agents,[16] suggesting survivin may play a role in the cellular mechanisms of resistance to cytotoxic agents. Therefore the differential expression of survivin between tumors may be a predictive marker of response to neoadjuvant chemoradiation therapy in esophageal tumors.

In the present study we investigated the expression of survivin, p53, and bcl-2 in 104 patients with esophageal tumors together with the apoptotic and proliferative activities. A subgroup of 48 patients who received either neoadjuvant chemoradiation therapy or chemotherapy was also investigated for any associations between the molecular parameters and the outcome to treatment.

METHODS

Tissue Samples

A total of 104 patients with a diagnosis of esophageal cancer were recruited to the study (Fig. 1). Approval for the study was granted by the Research Ethics Committee of St. James's University Hospital. Participation in the study was with the informed consent of the individual patients. Thirty-nine patients underwent surgical resection alone involving either a transhiatal approach or a two-stage en bloc resection. Forty-eight patients received neoadjuvant chemoradiation therapy or chemotherapy with planned

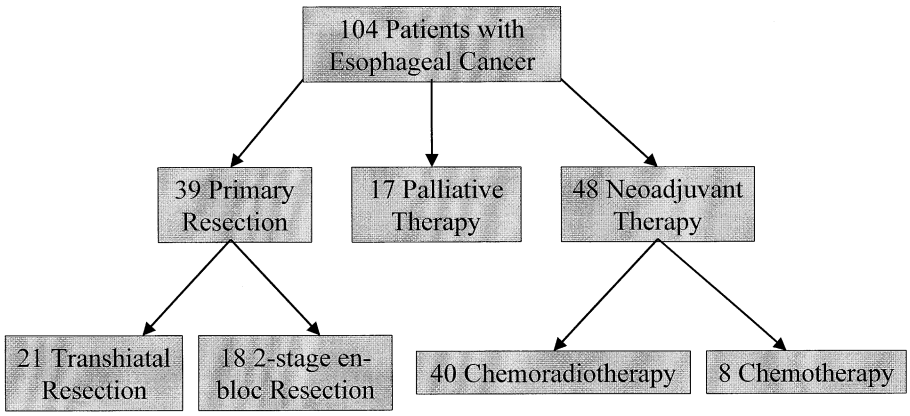

Fig. 1. Flow chart illustrating the various treatments in 104 patients with esophageal cancer.

surgical resection, and 17 patients received palliative treatment. For those patients who underwent surgical resection alone, tumor samples were obtained from the resected specimens. For patients receiving neoadjuvant or palliative treatment, endoscopic biopsy material was used. Tumor tissue was formalin fixed and embedded in paraffin, and serial sections of 5μm were taken. For morphologic analysis, tissue sections were analyzed and stained with hematoxylin and eosin.

Neoadjuvant Treatment

Forty patients were recruited into a phase II clinical trial of neoadjuvant chemoradiation therapy. Patients with esophageal cancer stage T3 NI/N0, as determined by means of a combination of endoscopy, endoscopic ultrasound, spiral CT, liver MRI, and isotope bone scanning, were included in the study. These 40 patients received a protracted intravenous infusion of 5-fluorouracil, 300 mg/m^2/day (days 1 to 42), followed by 225 mg/m^2/day (days 43 to 75) and combined with cisplatin, 60 mg/m^2/day (days 1,22,43, and 64). Concurrent radiation therapy of 45 Gy was administered (days 43 to 75) in 25 daily fractions.

Another eight patients received neoadjuvant chemotherapy consisting of a regimen of an infusion of 5-fluorouracil, 225 mg/m^2/day (days 1 to 21), with epirubicin, 50 mg/m^2/day (day 1), and cisplatin, 60 mg/m^2/day (day 1). This treatment was repeated for three cycles.

Surgery involved a radical two-stage en bloc esophageal resection with lymphadenectomy and was performed 6 weeks after the completion of neoadjuvant therapy. Restaging with spiral CT imaging was performed prior to surgery.

Criteria for Response to Neoadjuvant Therapy

Responses to neoadjuvant treatment were assessed clinically with spiral CT imaging and by analysis of resected specimens. Patients were then categorized as follows: complete response, if there was total absence of tumor in the resected specimen; partial response, if there was evidence of downstaging; and no response, if there was absence of tumor downstaging or the tumor had progressed with metastatic spread as detected by CT scanning.

Survivin Immunohistochemistry

Tumor sections were mounted on 3-aminopropyltriethoxysilanine (APES)–coated slides. Sections were dewaxed and rehydrated through a xylene and alcohol series. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in 100% methanol, and antigen retrieval was performed by heating the sections to 100° C in a microwave for 10 minutes while they soaked in 10 mmol/L citrate buffer, pH 6.0. Nonspecific binding sites were blocked with 5% swine serum (DAKO Ltd., High Wycombe, UK) diluted in Tris-buffered saline (TBS) (all incubations were carried out at room temperature). The primary antibody used for survivin immunostaining was a polyclonal affinity–purified rabbit antihuman survivin immunoglobulin G (IgG) termed SURV11-A (Autogen Bioclear, UK; 1μg/μl). The primary antibody was diluted 1:250 in TBS and incubated with sections for 12 hours. After washing in TBS, sections were incubated with a 1:200 dilution of biotinylated swine antirabbit IgG (DAKO). After another washing in Tris-HCl, pH 7.6, sections were incubated with streptavadin/biotin–horseradish peroxidase complex (DAKO) in 80 mmol/L Tris-HCl, pH 7.6. Sections were counterstained in Mayer's hematoxylin before dehydration using a sequential alcohol and xylene series.

Negative control specimens were included by the omission of the primary antibody and by antibody preadsorption with its conjugate peptide for 2 hours. Human sporadic colorectal cancers that had previously shown strong expression for survivin were used as positive control samples.

Immunostaining experiments were assessed by an experienced gastrointestinal pathologist who was blinded to the origin of the sections. Survivin protein expression within the cancer tissue was evaluated and categorized according to the percentage of cancer cells immunostained (0 = <5%; 1 = 6% to 25%; 2 = 26% to 50%; 3 = 51% to 75%; 4 = >75%) and also the intensity of the immunostaining (1+, 2+, 3+). As reported previously,[13] a weighted index score (0 to 12) was calculated for each tumor by multiplying the value of each of the categories. Tumors were then further grouped into low (weighted index score 0 to 3), intermediate (weighted index score 4 to 8), and high (weighted index score 9 to 12) expressors of survivin.

p53 and bcl-2 Immunohistochemistry

Tissue sections were prepared identically as for survivin immunostaining. Antigen retrieval for p53 was achieved by microwave treatment for 10 minutes and for bcl-2 by pressure cooking for 1 minute in 10 mmol/L citrate buffer, pH 6.0. Nonspecific binding sites were blocked with 5% rabbit serum (DAKO) diluted in TBS. The primary p53 antibody was the monoclonal mouse antihuman p53 antibody clone,

DO-7 (DAKO), at a dilution of 1:150. The primary bcl-2 antibody was the monoclonal mouse antihuman bcl-2, clone 124 (DAKO), at a dilution of 1:40. Both antibodies were incubated for 1 hour. The conjugate antibody for both procedures was a biotinylated rabbit antimouse IgG (DAKO) used at a dilution in TBS of 1:200. The remainder of the procedure was identical to that used for survivin immunostaining.

Negative control specimens were included by the omission of the primary antibody, and positive contol specimens were provided by sections of normal tonsil for bcl-2 and colorectal cancer for p53 immunostaining. Tumors were classified as positive for p53 or bcl-2 immunostaining if more than 10% of the cells were immunostained.

ki-67 Immunostaining and Assessment of the Proliferative Index

Immunostaining for the ki-67 antigen, which is expressed in the G1, S, and G2 phases of cycling cells, was performed using the standard avidin-biotin-peroxidase complex technique as described previously. Antigen retrieval was achieved by microwave treatment for 10 minutes in citrate buffer. Blocking of nonspecific binding sites was achieved by using 5% swine serum in TBS. The primary antibody was the rabbit polyclonal antihuman ki-67 antibody, MIB 1 (DAKO), which was incubated for 1 hour at a dilution of 1:200. The remainder of the procedure was identical to that described for survivin immunostaining.

The proliferative fraction of tumor cells was expressed as the ratio of cells positively stained for ki-67 to all tumor cells and presented as a percentage for each case. More than three areas were randomly selected and counted under $\times$400 magnification until more than 1000 tumor cells were counted. This was performed with the assistance of an image that was digitally captured using the Lucia G v 4.1 software program.

Detection of Apoptosis and Determination of the Apoptotic Index

Apoptotic tumor cells were detected in the tissue sections by terminal deoxynucleotydyl transferase–mediated dUTP-biotin nick end–labeling (TUNEL), using the Apop Tag in situ detection kit (Intergen Company, Oxford, UK) according to the manufacturer's instructions. The apoptotic index was expressed as the ratio of positively stained tumor cells and bodies to all tumor cells, and was presented as a percentage for each case. The method for counting apoptotic cells was identical to that described for the proliferative index.

Statistical Analysis

The SPSS v11 software package (SPSS Inc., Chicago, IL) was used for all statistical analyses. Variables associated with survivin, p53, and bcl-2 were analyzed by means of chi-square and Fisher's exact tests. Variables associated with the apoptotic and the proliferative indexes were examined using the Mann-Whitney U test. Correlation between the survivin weighted index score and the proliferative and apoptotic indexes on a per case basis was further analyzed by Spearman's rho correlation coefficient test. A $P < 0.05$ was considered statistically significant.

RESULTS

Expression of Apoptosis-Related Proteins in Esophageal Tumors

A total of 104 tumor specimens with the clinical and pathologic characteristics described in Table 1 were examined for their immunoreactivity to the apoptosis-related proteins survivin, p53, and bcl-2. Representative results of the immunostaining are illustrated in Fig. 2. Survivin expression was observed in the cytoplasm of tumor cells, and relatively weak staining was also observed in the adjacent normal esophageal mucosa, particularly in the basal cell layers of the stratified squamous epithelium. Ninety-nine of the esophageal tumors (95.1%) revealed varying levels of survivin immunostaining.

p53 immunoreactivity was identified by typical nuclear staining. Positive p53 immunostaining was identified in 73 of the esophageal tumors (70.2%). bcl-2 immunoreactivity was detected by cytoplasmic staining in 23 of the tumors (22.1%), and in general the immunostaining was relatively weak. In contrast, infiltrating lymphocytes in all tumor samples stained prominently and uniformly for bcl-2 expression. Survivin, p53, and bcl-2 expression were independent of all standard clinicopathologic variables.

Relationship Between Apoptotic Gene Expression and Tumor Cell Apoptosis

Apoptotic cells and bodies were detected in all cases of esophageal cancer. Representative results are illustrated in Fig. 2. Esophageal tumors had a median apoptotic index of 1.40% (range 0.17 to 6.55). There was no correlation between the apoptotic index and the clinicopathologic characteristics described in Table 1. There was no significant associa-

Table 1. Clinicopathologic variables*

Variable	Category	No. (%)
Age (yr)	<65	61 (58.7)
	>65	43 (41.3)
Sex	Male	76 (73.0)
	Female	28 (27.0)
Histology	Adenocarcinoma	79 (76.0)
	Squamous cell carcinoma	25 (24.0)
Histological grade	G1	14 (13.5)
	G2	39 (37.5)
	G3	51 (49.0)
Tumor depth	T1/T2	11 (28.2)
(Resections n = 39)	T3/T4	28 (71.8)
Lymph node metastases	Negative	12 (30.8)
(Resections n = 39)	Positive	27 (69.2)
Adenocarcinoma	Lower esophagus	49 (47.1)
location (n = 79)	Gastroesophageal junction	24 (23.1)
	Cardia	6 (5.8)
Survivin	Low (weighted index 0–3)	30 (28.8)
	Intermediate (weighted index 4–8)	37 (35.6)
	High (weighted index 9–12)	37 (35.6)
p53	Negative	31 (29.8)
	Positive	73 (70.2)
bcl-2	Negative	81 (77.9)
	Positive	23 (22.1)

*Clinicopathologic characteristics of patients and esophageal tumors together with the apoptotic gene immunohistochemistry scores.

tion between the apoptotic index and the weighted survivin score, p53, and bcl-2 immunostaining.

Relationship Between Apoptotic Gene Expression and Tumor Cell Proliferation

Proliferating cells were detected by intense nuclear immunoreactivity to the ki-67 antigen, as illustrated in Fig. 3. The median proliferative index was 34.2% (range 10.9% to 58.3%). No relationship was identified with the clinicopathologic variables in Table 1 and the proliferative index of the tumors. A correlation coefficient test showed a positive correlation between the proliferative index and the weighted survivin score on a per case basis (Spearman's rho correlation coefficient $r = 0.396$, $P = <0.001$; Fig. 3). No correlation was observed between p53 and bcl-2 immunoreactivity.

Correlation Between Tumor Apoptotic Gene Expression, Apoptosis, Proliferation, and Response to Neoadjuvant Therapy

According to pathologic and clinical criteria, 12 patients (25%) demonstrated a complete response, 21 (44%) showed a partial response, and 15 (31%) had no response to neoadjuvant treatment. The results of the experiments on the cohort of patients who received neoadjuvant therapy are illustrated in Table 2. No correlation was observed between survivin or bcl-2 expression and response to neoadjuvant treatment. However, p53-positive tumors were predictive of a reduced response (partial response or no response) to neoadjuvant therapy (Fisher's exact test, $P = 0.024$; Fig. 4).

In addition to this, patients who showed a complete response had tumors with higher proliferative indexes compared to patients who had a partial response or showed no response after treatment (Mann-Whitney U test, $P = 0.03$; Fig. 5). No correlation was observed between the apoptotic index and the response to treatment.

DISCUSSION

In normal adult tissues, dividing cell populations maintain a critical balance between cell proliferation and cell loss. Cell loss can be executed by the process of apoptosis, which is the evolutionary conserved and genetically regulated process of programmed cell death. The balance between cell division and cell loss is important in maintaining the integrity of human tissues. If there is increased proliferation, de-

creased cell loss, or a combination of the two, then uncontrolled cell growth occurs and this is a key contributory factor in carcinogenesis. One of the aims of this study was to describe the expression of the antiapoptotic and cell cycle gene survivin in esophageal cancer and its relationship to the apoptotic and proliferative activity within cancer tissue. We have shown that there is a strong positive correlation between the levels of survivin expression and the number of proliferating malignant cells within the tumor tissue. A number of studies have linked survivin expression to proliferative activity within human malignancy including pancreatic cancer,[17] colorectal cancer,[18] and hepatocellular carcinoma.[19]

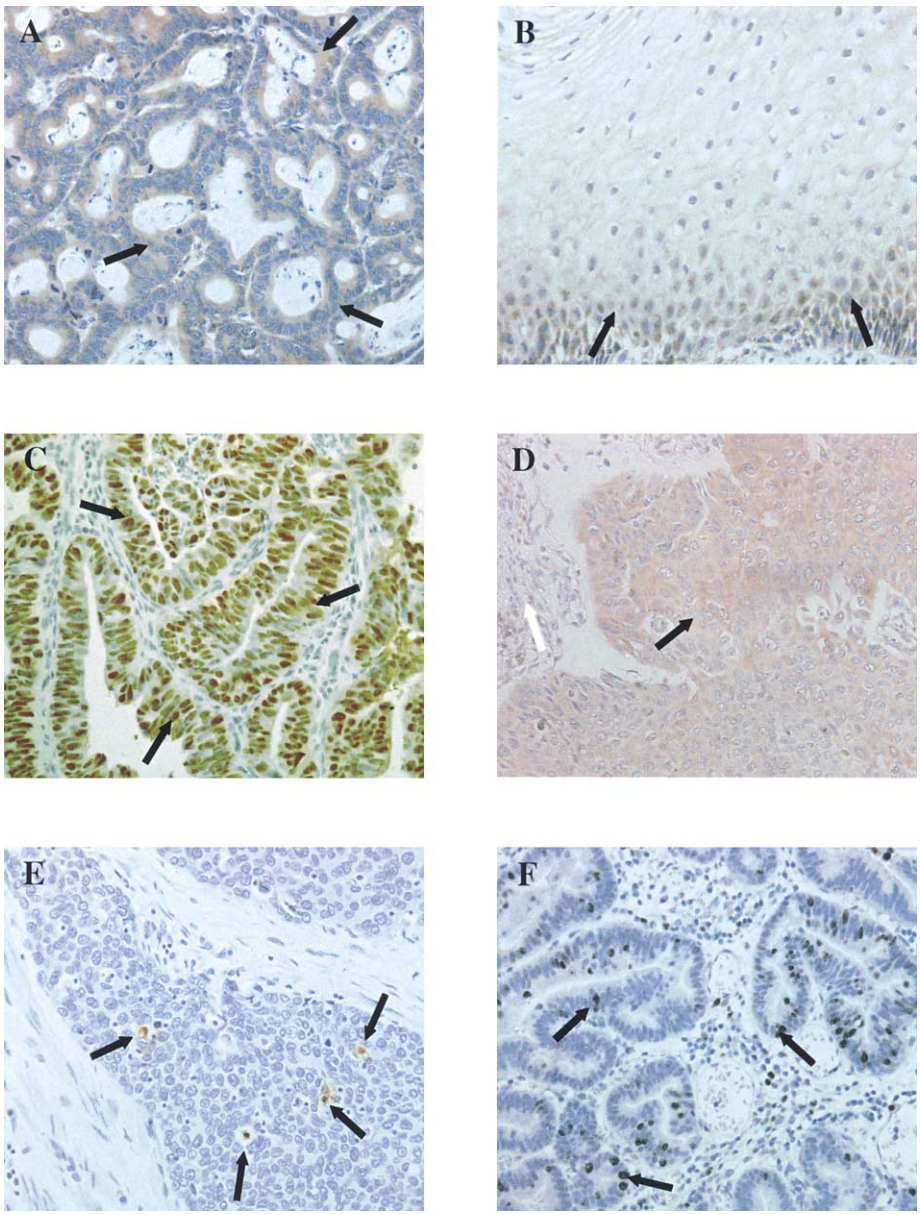

Fig. 2. Immunohistochemical staining. **A,** Adenocarcinoma demonstrating survivin expression restricted to the cytoplasm (*arrows*) of the tumor cells. **B,** Survivin immunostaining in the basal layers (*arrows*) of normal esophageal squamous epithelium. **C,** Typical p53 immunoreactivity demonstrating nuclear accumulation of abnormal p53 protein in an adenocarcinoma. **D,** Cytoplasmic staining for bcl-2 in the tumor cells (*black arrow*) and infiltrating lymphocytes (*white arrow*) of a squamous cell carcinoma. **E,** TUNEL in situ labeling for apoptosis detecting apoptotic cells and bodies (*arrows*) in a squamous cell carcinoma. **F,** ki-67 nuclear immunostaining (*arrows*) revealing proliferating tumor cells in an adenocarcinoma. (Original magnification ×200 for all photomicrographs.)

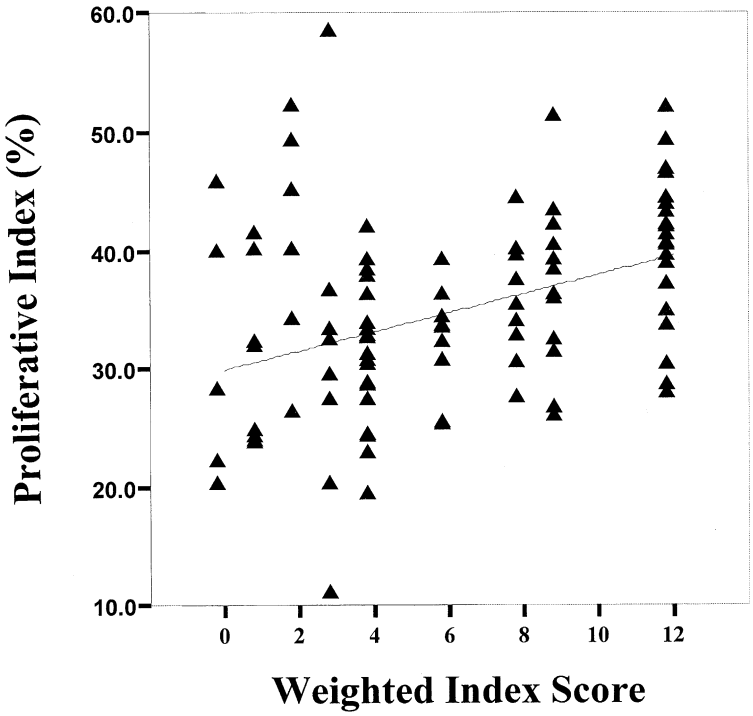

Fig. 3. Scatter plot illustrating the correlation between tumor cell proliferation and weighted survivin score on a per case basis ($r = 0.396$; $P = <0.001$).

Indeed, more evidence is coming to light that survivin, through its differential expression during the G2/M phase of the cell cycle, functions to control microtubule stability and assembly of a normal mitotic spindle during cell division. Indeed, by the very nature of its presumed function, it seems plausible that survivin expression would be necessary for cells with a high proliferative turnover such as those seen in the basal layers of the squamous epithelium of normal esophageal mucosa, as was observed in this study. Although early studies on survivin expression in cancer reported complete absence of expression in normal adult tissues, there have been a number of reports suggesting that there may be selective low expression of survivin in proliferating cells such as the basal cells in the crypts of colonic epithelium[20] and $CD34^+$ bone marrow–derived stem cells.[21]

This study failed to show a relationship between the inhibition of apoptosis and survivin expression in esophageal cancer. However, several studies have consistently shown that the expression of survivin inhibits cell death induced by various apoptotic stimuli,[10] although perhaps less potently than other IAP family members or bcl-2. Indeed, studies have illustrated an inverse correlation between apoptotic activity and survivin expression in colorectal,[12] gastric,[22] and breast cancer.[23] As a result of its dual roles in the cell cycle and apoptosis, survivin may function

Table 2. Tumor responses and molecular profiles*

Tumor response	Median apoptotic index (range)	Median proliferative index (range)	Survivin weighted index score			p53		bcl-2	
			0–3	4–8	9–12	Positive	Negative	Positive	Negative
Complete response (n = 12)	1.19 (0.4–3.78)	43.7 (23.8–52.1)	4	3	5	5	7	2	10
Partial response (n = 21)	1.04 (0.42–3.55)	32.3 (19.2–49.1)	5	9	7	17	4	3	18
No response (n = 15)	1.23 (0.4–4.54)	33.5 (19.2–52.1)	4	4	7	12	3	4	11

*Tumor response categories to neoadjuvant therapy and a summary of the molecular profile for each response group.

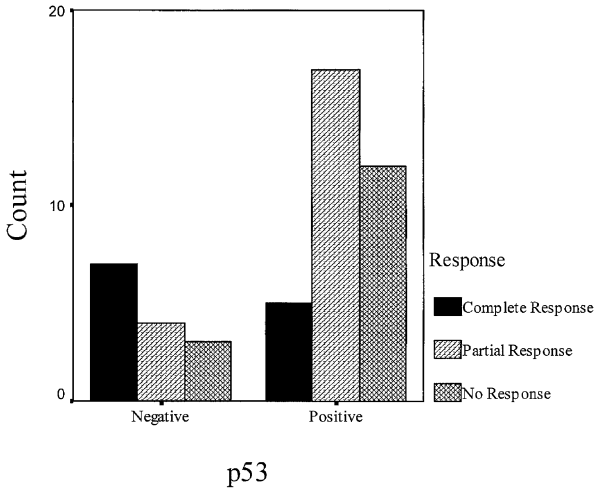

Fig. 4. Bar chart illustrating patients' responses to neoadjuvant treatment and the p53 status of their tumors. p53-positive tumors were found to be more likely to have residual tumor (*Partial Response* and *No Response*) after treatment than p53-negative tumors ($P = 0.024$).

during esophageal carcinogenesis to facilitate evasion of cell cycle checkpoints, suppress apoptotic stimuli, and ultimately foster the proliferation of transformed cells.

This study showed p53-positive immunostaining in 70.2% of esophageal tumors. p53 has a central role to play in the regulation of apoptosis through its role in cell cycle arrest, detection of DNA damage, and triggering of the apoptotic process. It is also well documented that p53 dysfunction through genetic mutation, insertion, or deletion is central in the development of human cancer.

bcl-2 is a potent inhibitor of apoptosis, which counteracts the terminal phases of apoptosis. Overexpression of bcl-2 may also be important in the development of human malignancy, and its importance is particularly well documented in lymphoma and hematogenous malignancies.[7] In this series only 22.1% of esophageal tumors were positive for bcl-2 immunostaining. This relatively low level of expression has previously been observed in adenocarcinoma, which suggests that esophageal cancer cells acquire other ways of avoiding apoptosis.[24]

Second, we aimed to elucidate whether any of the molecular parameters studied contributed to the response to neoadjuvant therapy. Positive p53 immunostaining revealed an association with the existence of residual disease after neoadjuvant treatment. This supports the theory that a tumor cell possessing a mutant p53 gene is unable to detect the damage pro-

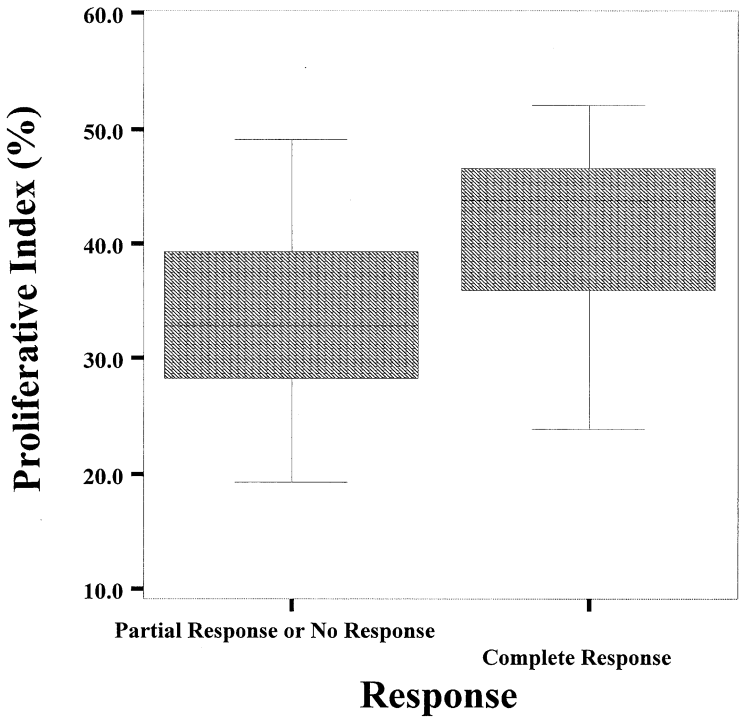

Fig. 5. Box plot (boxes show interquartile range, whiskers show range, and horizontal line represents median value) illustrating the distribution of tumor proliferative index scores and tumor response categories. Patients with a complete response had significantly higher proliferative indexes compared to those with a partial response or no response ($P = 0.03$).

duced by a cytotoxic agent and subsequently triggers the apoptotic events, which dispose of the cell. In this study, p53 immunoreactivity was considered to be attributable to the accumulation of abnormal p53 protein. Although this idea is generally accepted, it is worth noting that negative immunoreactivity does not always indicate normal p53 function, because there may be homozygous deletion, stop codon mutation, or acceleration of protein degradation. In addition, up to 30% of overexpressed p53 protein may actually possess wild-type conformation and stain positively, thus providing a false positive result. This may account for why 5 of 12 tumors that were p53 positive demonstrated a complete response after treatment and 7 of 36 tumors that were p53 negative contained residual tumor. Nevertheless, our findings are consistent with those in previous studies[25,26] and, in general, the p53 status does appear to play a significant role in determining the response to neoadjuvant therapy.

Another factor that appeared to be important in determining the response to neoadjuvant therapy was proliferation. In this study, tumors with higher levels of proliferation were more likely to receive a complete response. The explanation for why the increased level of proliferation should be so important in rendering cancer cells more susceptible to cytotoxic agents is not so clearly understood. However, one possible theory is that drugs such as 5-fluorouracil, which inhibits the DNA synthesis enzyme thymidylate synthetase, are only active in proliferating cells. Consequently the greater the level of proliferation, the more susceptible the cancer cells are to the drugs. Indeed, our finding that increased proliferation is linked to improved tumor response to neoadjuvant therapy is supported by the results of a previously reported study in which similar methods were used.[25]

Despite their association with response to neoadjuvant therapy, neither p53 nor ki-67 immunohistochemistry can accurately predict an individual patient's response to neoadjuvant therapy. It should be considered that the mechanisms of resistance to chemotherapy and radiation therapy are multifactorial and are not only influenced by the apoptotic and proliferative aspects of the tumor but also rely on the level of drug uptake and efflux, the degree of drug inactivation and activation, the altered levels or affinity of target enzymes, and the integrity of DNA repair systems. Consequently, a more comprehensive molecular profile, which takes into account all these factors, may possibly provide a more accurate prediction of response. However, it is possible to conclude that p53 status and proliferation have significant influences on the response to neoadjuvant treatment, and it may be possible to harness and enhance these properties through molecular manipulation to render cancer cells more susceptible to neoadjuvant therapies.

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Discussion

Dr. D. Shibata. (Baltimore, MD): Did you look at the tumors in partial responders and nonresponders after resection to see if there was any type of selection process? What were the gene profiles of those tumors postoperatively?

Dr. D.M. Beardsdmore: No, we did not examine the tumors after treatment. Clearly, this would be useful to elicit if tumors developed genetic aberrations during treatment that rendered them more resistant to the treatment. However, it is debatable whether any reliable genetic information can be obtained from tumors after prolonged courses of mutagenic cytotoxic therapy.

Dr. Shibata: It has been recently suggested that, in fact, survivin may be part of the p53 pathway and that wild-type p53 causes downregulation of survivin. Did you observe any correlation in your tumor samples?

Dr. Beardsmore: No, we did not observe any correlation between p53 status and survivin expression. One study on gastric cancer has revealed an association between positive p53 status and survivin expression.

Invited Discussion—Expert Commentator

Jeffrey H. Peters, M.D. (Los Angeles, CA): The third paper highlights recent interest in using genetic markers in an attempt to improve patient selection for both primary and adjuvant chemotherapy over the past several years. The largest body of such data focuses on expression levels of the thymidylate synthetase (TS) gene and its relationship to 5-FU efficacy in patients with colorectal, gastric, and in some instances esophageal carcinoma. Recent data have shown not only a more predictable response, but also a survival advantage in patients with colorectal cancer who have low levels of TS expression. Similar studies have been done with ERCCI, the gene encoding the metabolic enzyme of cisplatinum Dr. Beardsmore and colleagues have studied novel molecular markers—namely, survivin, bcl-2, and p53—as adjuncts guiding the use of adjuvant chemotherapy in esophageal cancer. They used immunohistochemical rather than polymerase chain reaction–based measurement, a more practical but much less quantifiable method of measuring the three markers, which may in part explain the significant overlap between responding and nonresponding groups of patients. That being said, complete histologic regression of the tumor was

more common in patients with increased proliferative rates and in those who were p53 negative.

Importantly, although perhaps underemphasized in the literature, it is quite clear that there is a differential sensitivity of primary, nodal, and metastatic tissue to chemotherapeutic agents. This fact has been borne out in the era of genetic testing for chemotherapy sensitivity where it is now evident that expression levels differ markedly, and nodal and/or metastatic tissue should be evaluated rather than the primary tumor.

A continued search for new markers is well justified. The emerging science of pharmacogenetics will hopefully one day soon allow us to recommend chemotherapy to those patients who are most likely to benefit and spare those who are not. In the case of 5-FU–based chemotherapeutic regimes, we are nearly there.

Effect of Aging on the Adaptive and Proliferative Capacity of the Small Bowel

Robert P. Thomas, M.D., Michele Slogoff, M.D., Farin W. Smith, M.D., B. Mark Evers, M.D.

Our society is aging at a rapid rate; the effects of aging on physiologic functions (e.g., small bowel adaptation) are poorly understood. The purpose of this study was to determine the ability of the aged small bowel mucosa to adapt after resection. Young (2-month-old) and aged (24-month-old) F344 rats underwent massive (70%) proximal small bowel resection (SBR) or sham operation; rats were killed at 9 or 16 days after surgery. The remnant small bowel and corresponding sham segments were harvested, weighed, and analyzed for DNA content and villus height. To determine whether the adaptive response after SBR could be enhanced, aged rats underwent SBR or sham operation and were treated with either neurotensin or saline solution (control). SBR resulted in adaptive hyperplasia in the remaining small bowel remnant in both young and aged rats at 9 and 16 days compared with sham animals. At 9 days, significant increases were noted in weight, villus height, and DNA content of the distal remnant in young and aged rats after SBR; the increases were similar in both young and aged rats. At 16 days, both young and aged rats displayed significant increases in remnant weight after SBR. Administration of neurotensin increased the weight of the remnant intestine in aged rats after SBR compared with saline treatment. Our findings demonstrate that aged small bowel mucosa exhibits a proliferative and adaptive capacity in response to SBR that was similar to that of the young animals. In addition, neurotensin administration enhanced the normal adaptive response of the small bowel in aged rats, providing further evidence that neurotensin may be therapeutically useful to augment mucosal regeneration in the early periods after massive SBR. (J GASTROINTEST SURG 2003;7:88–95.)

KEY WORDS: Aging, neurotensin, small bowel resection, intestinal proliferation

With the aging of the baby boomer generation, it is estimated that nearly a quarter of Americans will be at least 65 years of age or older by the year 2050.[1] This, combined with the fact that medical advances in the past century have allowed people to live longer and stay healthy for a significantly longer period of time, will result in an increase in the number of elderly patients who will require medical attention.[1] In this regard, it is estimated that 50% of patients in many general surgical practices are 65 years of age or older.[2] It is imperative that surgeons understand the changes that occur in organ systems with aging so that problems can be anticipated and prevented; however, the effects of aging on the physiologic response of many organs are not entirely known. Unlike other organs in which proliferation ceases with adolescence (most notably, the brain, liver, and kidneys), previous studies suggest an equivalent or increased proliferation in the intestinal mucosa of rats and humans associated with aging.[3–7] Clearly, studies are required to better ascertain the response of the gastrointestinal tract in aged subjects to surgical diseases or after intestinal resection.

Neurotensin (NT), a gut tridecapeptide localized predominantly to the distal small intestine, facilitates fatty acid translocation from the small bowel, and affects gastrointestinal and pancreatic secretion and gastrointestinal motility.[8,9] In addition, NT is an important trophic factor for the small bowel and colon mucosa.[10–13] Neurotensin reverses the small bowel mucosal atrophy associated with feeding rats an elemental diet and stimulates mucosal growth in de-

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (poster presentation), and published as an abstract in *Gastroenterology* 2002;123(Suppl):20.
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Supported by grants R37 AG10885, P01 DK35608, and T32 DK07639 from the National Institutes of Health and by a Jeane B. Kempner Scholars Award (R.P.T.).
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Published by Elsevier Science Inc.
1091-255X/03/$—see front matter
PII: S1091-255X(02)00128-2

functionalized self-emptying jejunoileal loops or isolated loops of small bowel (Thiry-Vella fistulas).[12,14] Izukura et al.,[15] in our laboratory, demonstrated that administration of NT to young (2-month-old) rats augments the normal adaptive hyperplasia of gut mucosa that is associated with massive (i.e., 70%) small bowel resection (SBR). Consistent with our results, de Miguel et al.[16] demonstrated that exogenous NT significantly increased villus length in both the jejunum and ileum in rats after an 80% SBR. We have found that, with aging, the ability of small bowel mucosa to respond to NT is greater when compared with the proliferative response in young rats.[17] Administration of NT increased crypt cell density in young and aged rats, but only the aged rats exhibited an increase in crypt depth and villus height. Therefore these findings further support the hypothesis that aged mucosa retains its proliferative response following trophic stimuli such as the administration of trophic hormones.

Massive SBR is more often required in either newborn infants or elderly patients because of the increased propensity of these populations to develop ischemic conditions of the intestine, which require resection.[18,19] Intestinal adaptation can occur, but this response is oftentimes insufficient to compensate for the massive amounts of intestine that are resected. The resulting clinical condition (i.e., short bowel syndrome) is associated with increased morbidity and mortality because of the marked loss of intestinal absorptive surface and may require a lifelong regimen of total parenteral nutrition.[20] Although massive SBR is more common at the extremes of age, the effects of SBR in the newborn infant and young adult have been extensively analyzed both in experimental and clinical models. However, the effects of SBR in the elderly and the effects of trophic factors in this regenerative process have not been assessed. Therefore the purpose of our present study was twofold: (1) to determine the effects of age on the ability of the small bowel mucosa to regenerate after massive SBR and (2) to determine whether administration of NT to aged rats could enhance the adaptive hyperplasia noted after SBR.

MATERIAL AND METHODS

Young (2-month-old) and aged (24-month-old) male Fischer 344 rats were obtained from the National Institute of Aging (Bethesda, MD) stock colony that is maintained under barrier-reared conditions. Aged animals are defined as having achieved the age at which one half of the population ordinarily dies. That age in Fischer 344 rats is approximately 22 months and in humans corresponds to the eighth decade of life.[21,22]

Rats were housed at a constant temperature (22° C) with 12-hour light and dark cycles and fed standard laboratory chow (Formulab Chow, Purina Mills, Inc., St. Louis, MO), ad libitum, with free access to water for an acclimation period of at least 7 days.

Experimental Design

In the first experiment, young and aged rats were fasted overnight after the acclimation period; the rats were then weighed and randomly divided into two groups. Rats were anesthetized with inhaled 5% halothane gas, and the abdomen of each was shaved and prepared with a Betadine solution (povidone iodine, 10%). Next the abdomen was opened by a midline incision, and the small intestine was measured along the antimesenteric border. Sham-operated control rats underwent transection of the small bowel at 5 cm distal to the ligament of Treitz and approximately 25 cm proximal to the ileocecal valve, without any removal of intestine, followed by reanastomosis. Another group of rats underwent a 70% proximal SBR with bowel transections at 5 cm distal to the ligament of Treitz and approximately 25 cm proximal to the ileocecal valve. In both groups, intestinal continuity was restored by end-to-end enteroenterostomy with 8-0 interrupted nylon sutures. The abdomen was closed in two layers with 4-0 chromic sutures for the abdominal musculature and 3-0 silk for the skin. After operation, all rats were given 10 ml of 0.9% saline solution subcutaneously and allowed free access to water and 5% dextrose. Beginning on the morning of postoperative day 2, rats were allowed a diet of ad libitum chow for an additional 7 or 14 days. On the morning of postoperative days 9 or 16, rats were weighed and then killed by decapitation.

In the second experiment, aged rats underwent either sham or 70% SBR as described previously. Beginning on the morning of postoperative day 2, rats were further subdivided to receive subcutaneous injections of either saline solution (control) or NT (300 μg/kg, Bachem, Inc., Torrance, CA) every 8 hours for 7 days.

Neurotensin Peptide Preparation

A stock solution of NT was prepared by first dissolving the amount of NT needed for the study in 1 ml of sterile water containing 0.1% (weight/volume) bovine serum albumin (BSA; Sigma Chemical, St. Louis, MO) and then diluted to the required concentration with saline solution containing 0.1% BSA. Equal portions of

this solution, sufficient for a single injection of all animals in a group, were stored in vials at −20° C. Sterile saline solution containing 0.1% BSA (control) was likewise divided into equal aliquots and stored at −20° C. To prolong absorption, saline or NT was mixed 1:4 (volume/volume) with 15% (weight/volume) hydrolyzed gelatin (Sigma) before administration.

Tissue Collection

In the first experiment, after an overnight fast, rats were weighed and then killed by decapitation beginning at 8:00 AM on postoperative day 9 or 16. The abdomen was opened and the distal ileum was removed. The distal remnant bowel was hung vertically with a 10 g weight to ensure constant lengths. The removed section (15 cm) was trimmed of any remaining mesentery, and the luminal contents were removed by flushing with cold saline solution and gentle manual stripping. Each segment was then blotted dry, weighed, and the mucosa carefully scraped from the underlying seromuscular layer on a chilled platform, using a glass slide. An additional 1 cm segment of full-thickness remnant bowel was removed just proximal to the 15 cm segment and fixed in 10% buffered formalin for histologic analysis.

In the second set of experiments, the last injection of either saline or NT was given at 10:30 PM on postoperative day 8. After an overnight fast, rats were weighed and then killed by decapitation beginning at 8:00 AM on postoperative day 9. The remnant distal small bowel was excised and hung vertically using a 10 g weight, as described previously. A 20 cm segment was removed, weighed, and the mucosa scraped from the underlying seromuscular layer. Additionally, a 1 cm segment of small bowel was removed, fixed in formalin, and subjected to histologic analysis.

All scraped mucosal samples were immediately frozen with liquid nitrogen and stored at −70° C until assayed for DNA and protein content.

DNA and Protein Determination

Tissues were thawed and homogenized in 1 ml of 0.9% saline solution. The DNA content was measured by the Burton modification[23] of the diphenylamine procedure with calf thymus DNA used as the standard. Protein content was determined by the method of Bradford[24] with BSA as the standard.

Histologic Analysis

Full-thickness ileal segments were fixed in neutral buffered formalin and embedded in paraffin. Eight to 10 μm sections were stained with hematoxylin and eosin. Next, a blinded observer measured the villus height of 10 random intact villi using a calipered microscope.

Statistical Analysis

Tissue weight of the small bowel segments was normalized to kilograms of body weight. DNA and protein content measurements were normalized to kilograms of body weight and centimeters of length resected. Values were expressed as mean ± SEM and analyzed by the Kruskal-Wallis test. In all instances, $P < 0.05$ was considered significant.

RESULTS

Effect of Aging on Small Bowel Adaptation After Massive Small Bowel Resection

We first determined the potential of aged small bowel mucosa to adapt after a massive SBR. Young and aged rats underwent a 70% proximal SBR or sham transection, and then the remnant or corresponding sham distal small bowel was assessed on postoperative day 9 or 16. The overall mortality rates for young and aged rats were 14% and 12%, respectively. At 9 days after either SBR or sham operation, young and aged rats demonstrated an average weight loss of 4% and 6%, respectively, compared to preoperative levels. At 16 days after surgery, young

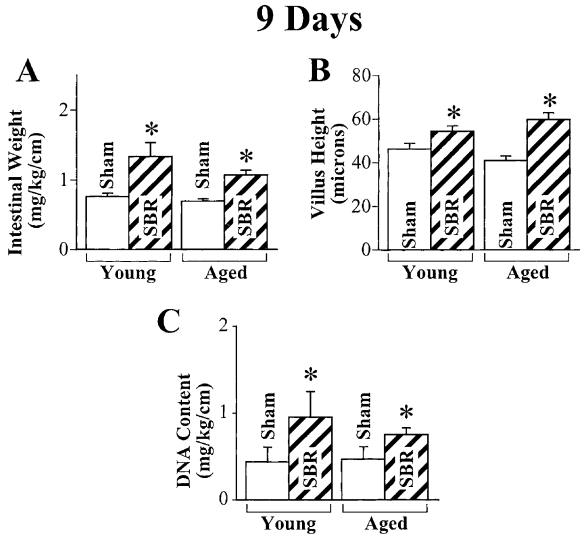

Fig. 1. Intestinal weight (mg/kg/cm) (**A**), villus height (μ) (**B**), and mucosal DNA content (mg/kg/cm) (**C**) for remnant distal small bowel or corresponding sham segments in young (2-month-old) and aged (24-month-old) F344 rats 9 days after either proximal small bowel resection (*SBR*) or sham operation, respectively. (*$P < 0.05$ vs. corresponding sham groups).

rats demonstrated a 7% increase in mean weight, whereas aged rats lost approximately 5% of their weight compared to preoperative levels. Furthermore, the aged rats that underwent operation followed by either control (saline) or NT injection for 7 days demonstrated an average decrease in weight of 8%, regardless of the type of injection.

All parameters of growth were increased in the young and aged rats 9 days after SBR; the adaptive increases were similar in both groups (Fig. 1). Specifically, the weight of the remnant small bowel increased by 75% and 53% in young and aged rats, respectively, compared with corresponding sham control rats (see Fig. 1, *A*). Villus height was increased significantly in both young and aged rats compared with sham animals (see Fig. 1, *B*). Similarly, mucosal DNA content was increased 118% and 60% in young and aged rats, respectively, compared with corresponding sham control rats (see Fig. 1, *C*). Furthermore, mucosal protein content was increased, albeit not significantly, in both age groups after SBR (data not shown). Taken together, these findings demonstrate that the small bowel mucosa in aged rats proliferates in response to SBR and that this adaptive hyperplasia is similar to the response noted in the small bowel mucosa of young rats. Our findings of weight, villus height, and DNA content are further supported by representative histologic sections demonstrating proliferation of young and aged small bowel remnants after SBR compared with the corresponding sham segments (Fig. 2).

We also analyzed the segments of distal intestine 16 days after SBR or sham operation (Fig. 3). Significant increases in the weight of the remnant intestine were noted in both young and aged rats (95% and 50% increases, respectively, compared with sham operation) (see Fig. 3, *A*). Changes in villus height (see Fig. 3, *B*) and DNA content (see Fig. 3, *C*) were less apparent with only a significant increase in villus height in young rats after SBR compared with the sham group. Although villus height was increased in the aged mucosa after SBR, the differences were not

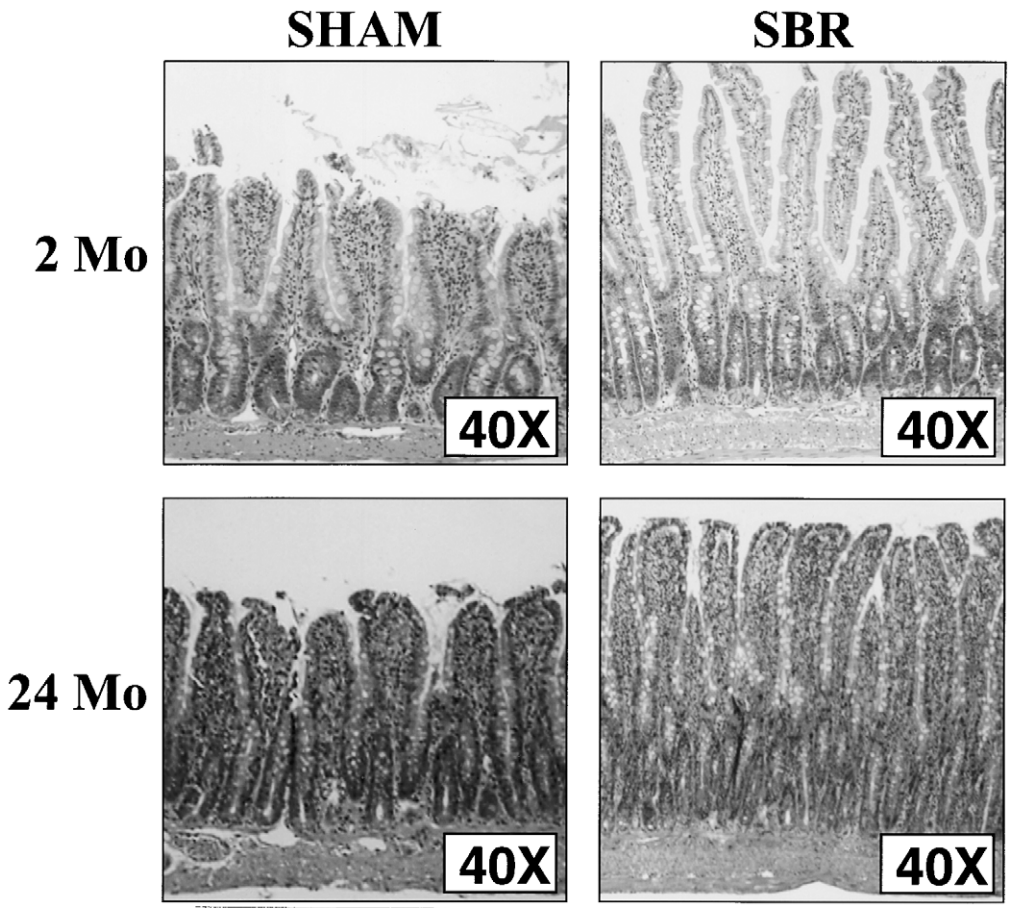

Fig. 2. Photomicrographs from representative histologic sections of full-thickness distal small bowel in young (2-month-old) and aged (24-month-old) rats 9 days after either sham operation or proximal SBR (original magnification for all sections ×40).

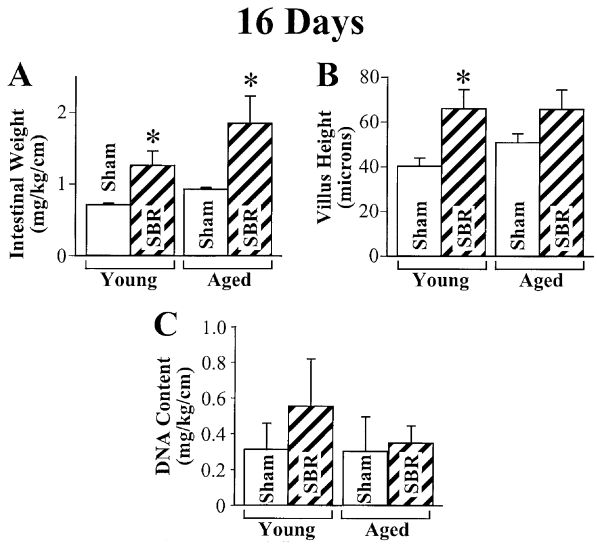

Fig. 3. Intestinal weight (mg/kg/cm) (**A**), villus height (μ) (**B**), and mucosal DNA content (mg/kg/cm) (**C**) for remnant distal small bowel or corresponding sham segments in young (2-month-old) and aged (24-month-old) F344 rats 16 days after either proximal small bowel resection (*SBR*) or sham operation, respectively. (*$P < 0.05$ vs. corresponding sham groups).

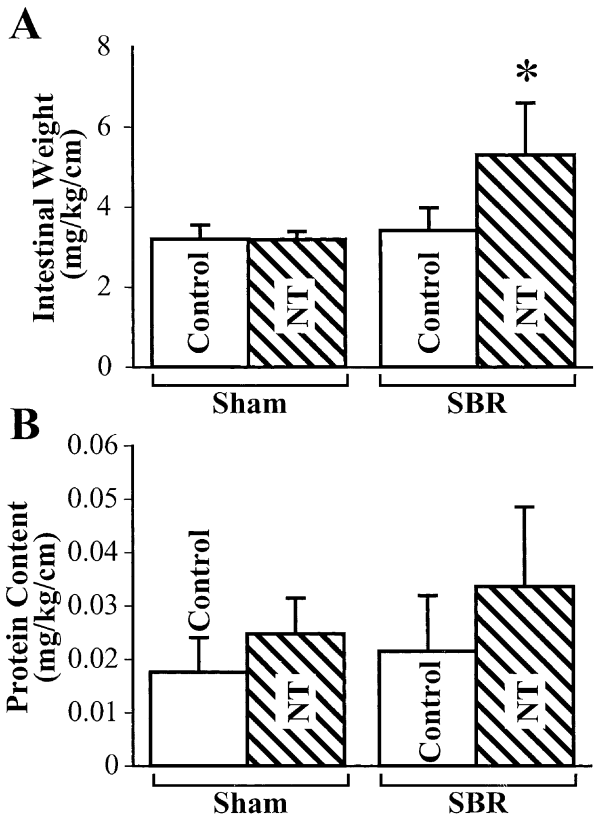

Fig. 4. Intestinal weight (mg/kg/cm) (**A**) and mucosal protein content (mg/kg/cm) (**B**) for remnant distal small bowel or corresponding sham segments in aged F344 rats treated with either saline solution (*control*) or neurotensin (*NT*; 300 μg/kg) for 7 days. (*$P < 0.05$ vs. corresponding sham groups).

significant. Similarly, there were no differences in DNA content between young and aged rats undergoing SBR.

Effect of Neurotensin on Small Bowel Adaptation After Small Bowel Resection

Previously we have shown that NT can augment the normal adaptive hyperplasia in the remnant small bowel of young rats after SBR.[15] We next determined whether NT could enhance the trophic response of the remaining mucosa in aged rats after massive SBR. Aged rats underwent either sham operation or 70% proximal SBR; the rats were then subdivided on postoperative day 2 to receive either NT (300 μg/kg three times a day) or saline solution, and the remnant or sham bowel segments were analyzed 7 days after treatment was initiated (Fig. 4). Neurotensin administration resulted in a significant increase (55%) in the weight of the remnant intestine compared to saline solution after SBR; administration of NT to sham-treated rats failed to stimulate intestinal weight (see Fig. 4, *A*). The increase in protein content did not achieve significance with NT treatment in either SBR or sham-treated aged rats (Fig. 4, *B*). Consistent with our findings of enhanced intestinal weight, representative histologic sections of remnant intestine in aged rats after SBR demonstrated an increase in the intestinal villi of rats given NT compared with saline treatment (Fig. 5).

DISCUSSION

The effects of aging on mucosal proliferation in the gastrointestinal tract are currently poorly understood. In the present study, we demonstrated that a massive SBR produces a similar adaptive hyperplasia in young and aged rat small bowel mucosa. In addition, administration of NT to aged rats after SBR can significantly augment bowel weight compared to rats given saline solution. These findings suggest that aging does not significantly alter the ability of the gut mucosa to respond to proliferative stimuli. In fact, in many respects, the response of the aged mucosa is similar to that in young animals. These findings provide important insights into the functional response of the gastrointestinal tract mucosa associated with aging.

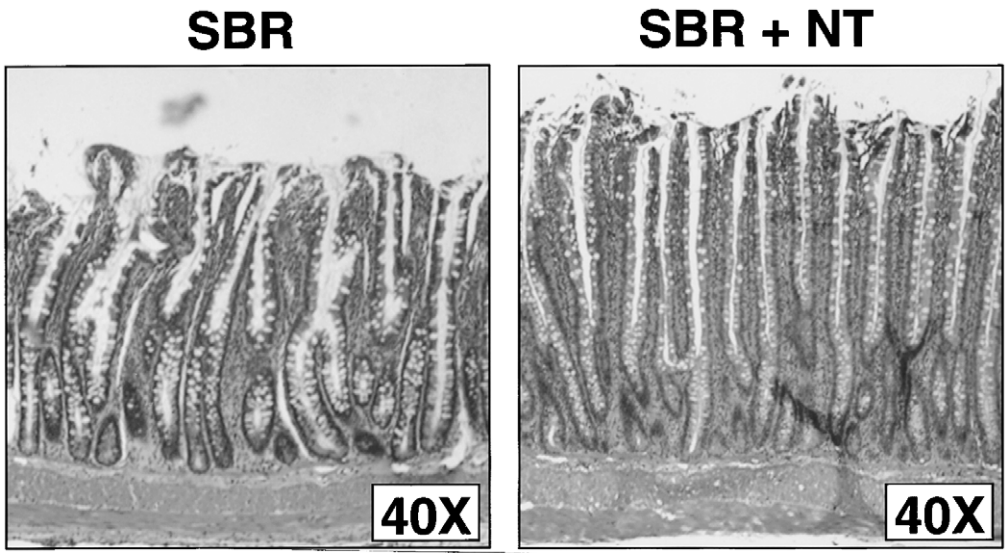

Fig. 5. Photomicrographs from representative histologic sections of full-thickness distal small bowel in aged (24-month-old) F344 rats after SBR and treated with either saline solution or NT (300 μg/kg 3 times/day) for 7 days (original magnification for all sections ×40).

Massive SBR is associated with a compensatory hyperplasia in the remaining gut mucosa.[25–28] A similar trophic response is noted in various animal models,[25,26,28–30] as well as in humans after intestinal resection.[20] Although a significant number of elderly patients will require massive SBR to treat underlying medical conditions, the ability of the aged mucosa to respond and undergo hyperplasia has not been examined. In the current study, we demonstrate that aged mucosa is capable of generating an adaptive hyperplasia that is similar to the response noted in young adult rats. Significant increases were noted in intestinal weight, villus height, and DNA content in both young and aged rats 9 days after SBR. Therefore the ability of the intestinal mucosa to respond to the proliferative signals regulating this hyperplastic response appears to be intact and functional. Similarly, Fadrique et al.[31] demonstrated that aged small bowel mucosa in Wistar rats displayed a compensatory increase in villus height (59%) and increased proliferating cell nuclear antigen labeling in bowel after 80% SBR as compared to sham operations; however, there was no comparison of the adaptive response in young and aged small bowel. Therefore our results are the first, to our knowledge, to provide evidence that the aged bowel has a similar proliferative ability after SBR as young adult bowel. These observations are in contrast to the regenerative and proliferative capacity of the aged liver, pancreas, and nervous system.[32–38] With respect to the liver, the proliferative response to partial hepatectomy in aged rats is both reduced and delayed.[32,33] Pancreatic resection in aged animals reduces B-cell function and replication, resulting in an age-related functional decline manifested as impaired glucose tolerance.[37] An age-associated decrease in the remyelination of the central nervous system in aged animals has also been demonstrated.[38] Therefore, although the process of aging appears to alter the proliferative response of other tissues, the intestinal mucosa appears to be unique in its ability to respond to trophic stimuli, which is not altered by the aging process.

Similar to our study, which showed an unaltered proliferative response in the intestinal mucosa of aged rats, other investigators have demonstrated an equivalent, or even exaggerated, proliferative response in aged mucosa. For example, Holt and Yeh[3,4] demonstrated that refeeding rats after a 72-hour fast produced a dramatic and rapid increase in the crypt proliferative zone of both the small bowel and colon of aged rats. This response resembled the aberrant proliferation noted after administration of the carcinogen dimethylhydrazine. These results suggest that increases in actual cell number (i.e., hypertrophy) are responsible for the increases in intestinal growth in aged rats. Moreover, in a previous study we examined the ability of aged rat mucosa to respond to the trophic gut hormone NT.[17] Administration of NT produced significant increases in mucosal weight, DNA, RNA, and protein content in both young and aged rats when compared with age-matched control rats. In addition, the increase in crypt depth and villus height appeared to be more pronounced in aged rats given NT compared with

young rats. Taken together, these results indicate that a variety of proliferative stimuli (i.e., fasting and refeeding, administration of trophic hormones, or massive SBR) can produce an equal or, in some instances, a more pronounced effect in the mucosa of aged animals.

Limited clinical studies would tend to support the data obtained with the use of experimental in vivo models. For example, three clinical studies have compared the histologic appearance of colonic mucosa of young and elderly human volunteers.[5–7] Cell proliferation in young persons was predominantly confined to the lower two thirds of the colonic crypts. In contrast, in elderly persons there was a shift in the major zone of DNA synthesis from the base to the middle and upper third of glands, a pattern first detected in patients with colorectal cancers.[39] These reports describe findings in the human intestinal mucosa at basal (i.e., unstimulated) conditions; however, the effects of proliferative stimuli on the response in humans have not been assessed.

Izukura et al.,[15] from our laboratory, demonstrated that administration of NT to young rats, after a massive proximal or distal SBR, augmented the normal adaptive hyperplastic response of the remaining small bowel mucosa. Consistent with these results, de Miguel et al.[16] demonstrated that NT enhanced small bowel regeneration after an 80% bowel resection in rats. Ryan et al.[40] demonstrated, in a rabbit model of midgut bowel resection, that NT significantly increased microvillus height and brush-border surface areas after administration. Given the fact that NT can augment the adaptive response after resection in young animals, we determined whether NT could enhance the proliferation noted in the aged mucosa. We found that NT increased the weight of the remnant intestine in aged rats after SBR compared with rats given saline solution suggesting that, similar to findings in young animals, NT may be useful to further increase the adaptive response of the remaining intestinal mucosa after a massive SBR. The lack of a significant effect of NT on mucosal growth in the sham-operated aged rats cannot be entirely explained, although we have shown in previous studies that the ileum is less responsive to the trophic effects of NT, which probably relates to the fact that NT peptide is predominantly localized to the distal ileum.[12,14]

CONCLUSION

We have shown that small bowel proliferative and regenerative potential is retained with aging after massive SBR and that administration of NT can further augment this response. Our results provide additional insights into the functional response of the gut mucosa to proliferative stimuli. Although SBR is more often required for patients at the extremes of age, our findings would suggest that the ability of aged mucosa to adapt should be similar to that in a younger patient. Moreover, our findings further imply that enterotrophic peptides such as NT may be useful in the early phases of bowel regeneration in both young and elderly individuals.

We thank Eileen Figueroa and Karen Martin for manuscript preparation and Tatsuo Uchida (Office of Biostatistics, UTMB) for statistical analysis of the data.

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Evaluation of Porcine-Derived Small Intestine Submucosa as a Biodegradable Graft for Gastrointestinal Healing

Sebastian G. de la Fuente, M.D., Marcia R. Gottfried, M.D., D. Curtis Lawson, M.S., Mary B. Harris, R.V.T., Christopher R. Mantyh, M.D., Theodore N. Pappas, M.D.

High-risk anastomoses in the gut may benefit from the application of a synthetic reinforcement to prevent an enteric leak. Recently a porcine-derived small intestine submucosa (SIS) was tested as a bioscaffold in a number of organ systems. The aim of this study was to evaluate the effectiveness of SIS in stimulating healing in the stomach. Twelve rats underwent surgical removal of a full-thickness gastric defect (1 cm) and subsequent repair with a double-layer patch of porcine-derived SIS. The graft was secured with interrupted sutures placed within 1 mm of the edge of the graft. After 21 days, the animals were killed and their stomachs harvested for histologic examination. Cross sections were processed for paraffin embedding and 4-micron sections were stained with hematoxylin and eosin. All animals survived, gained weight, and demonstrated no signs of peritonitis over the 3-week postoperative period. On postmortem examination, the defect was completely closed in all animals by granulation tissue and early fibrosis. Although most of the luminal surface of the grafted areas remained ulcerated, early regeneration of normal gastric mucosa was seen at the periphery of the defect. SIS may act as an effective scaffolding agent for intestinal mucosa and may offer protection in high-risk anastomoses. (J GASTROINTEST SURG 2003;7:96–101.)

KEY WORDS: Small intestine submucosa, porcine, SIS, anastomosis, perforation

Gastrointestinal anastomosis failure represents a devastating postoperative complication that increases the hospital stay and the perioperative mortality by two- and threefold, respectively.[1] Despite improved perioperative care and the advent of new operative techniques, anastomosis leakage continues to constitute a significant health problem. Several risk factors have been associated with anastomosis breakdown including the use of corticosteroids, bowel obstruction, low serum albumin levels, and the need for intraoperative blood transfusions.[2] Commonly recommended precautions to enhance complication-free healing of the anastomosis include a sufficient blood supply to the ends being anastomosed, minimal tension between edges, adequate preoperative preparation, and proper surgical technique. Because of the multifactorial nature of and difficulty in preventing anastomotic dehiscence, a variety of products have been tested to provide protection to the anastomosis site.[3–6]

Recently, porcine-derived small intestine submucosa (SIS) has been introduced as a reinforcement of soft tissue.[7] SIS is an acellular collagen-based matrix primarily composed of fibrillar collagens (types I, II, and V)[8] that enhance healing while stimulating a minimal immune response.[9] Studies have shown that SIS serves as a bioscaffold for generation of a wide variety of tissues and organs such as urinary bladder,[10,11] dura mater,[12] blood vessels,[13–15] and tendons.[16] In the present study we evaluated the effectiveness and regenerative properties of SIS in a rat model of gastric perforation. We show that in vivo implantation of SIS provides contingency and facilitates ingrowing of native gastric tissue into the matrix.

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (poster presentation).
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Published by Elsevier Science Inc.
1091-255X/03/$—see front matter
PII: S1091-255X(02)00050-1

MATERIAL AND METHODS

Surgical Manipulation and Study Design

Twelve adult Wistar rats (Charles Rivers Laboratories, Raleigh, NC) weighing 300 to 350 g were housed in cages with free access to water and food under standard laboratory conditions (room temperature 23° C; 12 hr dark-light cycles). Rats were weighed before surgery and at regular intervals after operation. Food was withheld but not water for 24 hours before surgery. On the day of surgery, anesthesia was induced and maintained with isoflurane and oxygen, and the abdominal area was prepared in the usual manner. A 3 cm midline laparotomy was performed, and the stomach was identified and gently mobilized with atraumatic forceps. A 1 cm full-thickness defect was created in the body of the stomach, which compromised approximately 40% of the area of the anterior gastric wall. After hemostasis was achieved, a two-layer SIS patch (Cook Biotech, West Lafayette, IN) was prepared and secured to the gastric wall with interrupted 5-0 Prolene sutures. Stitches were taken from the seromuscular layer and placed within 1 mm of the edge of the graft. In a subset of animals (n = 6), the operation was completed with an omentectomy. The incision was closed in two layers, and the animals were allowed to recover from anesthesia. Postoperatively animals were checked on a daily basis for signs of distress, and pain medication was administered as needed (morphine, 2mg/kg). Body weight, temperature, and food intake were monitored daily. All aspects of this research were approved by the Durham VA Medical Center Animal Care and Use Committee (Durham, NC).

Small Intestine Submucosa

The material was supplied sterile in a peel-open package and rehydrated for 10 minutes in saline solution before implantation. Soaking the material makes the sheets pliable and soft. For the present experiments, a double patch, 1.2 cm in diameter, was created from the SIS sheet by opposing two layers. Special care was taken to ensure that the luminal sides of the material were kept inward.

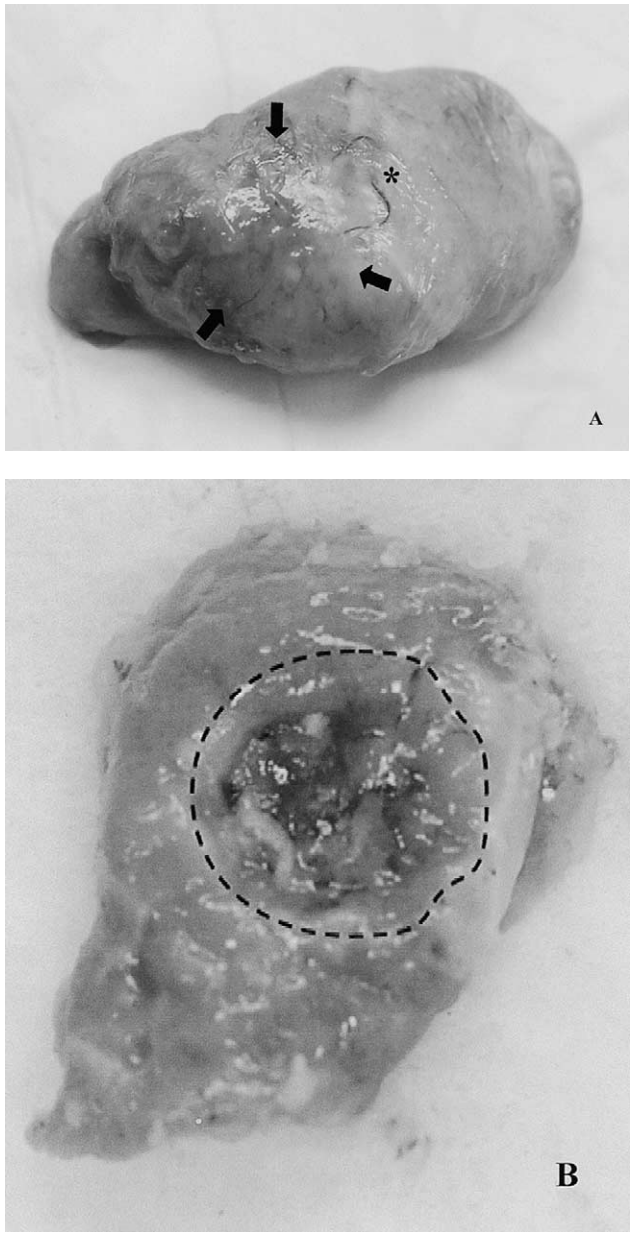

Fig. 1. Macroscopic view of external and luminal gastric wall 3 weeks after implantation. **A,** Serosal surface of gastric wall. The material was completely incorporated into the gastric wall making it difficult to distinguish between graft and normal tissue. *Arrows* denote grafted area; *asterisk* shows suture material. **B,** Luminal side of the stomach. *Broken line* denotes former defect.

Histologic Evaluation

After 21 days, the animals were killed and their abdominal cavities were macroscopically evaluated for adhesions. The grafted area surrounded by intact gastric tissue was excised and fixed in 10% neutral buffered formalin. Cross sections of the grafted area were processed for paraffin embedding, and 4-micron sections were stained with hematoxylin and eosin. All specimens were reviewed by one of us (M.G.), who was unaware of the surgical groups (i.e., with or without omentectomy).

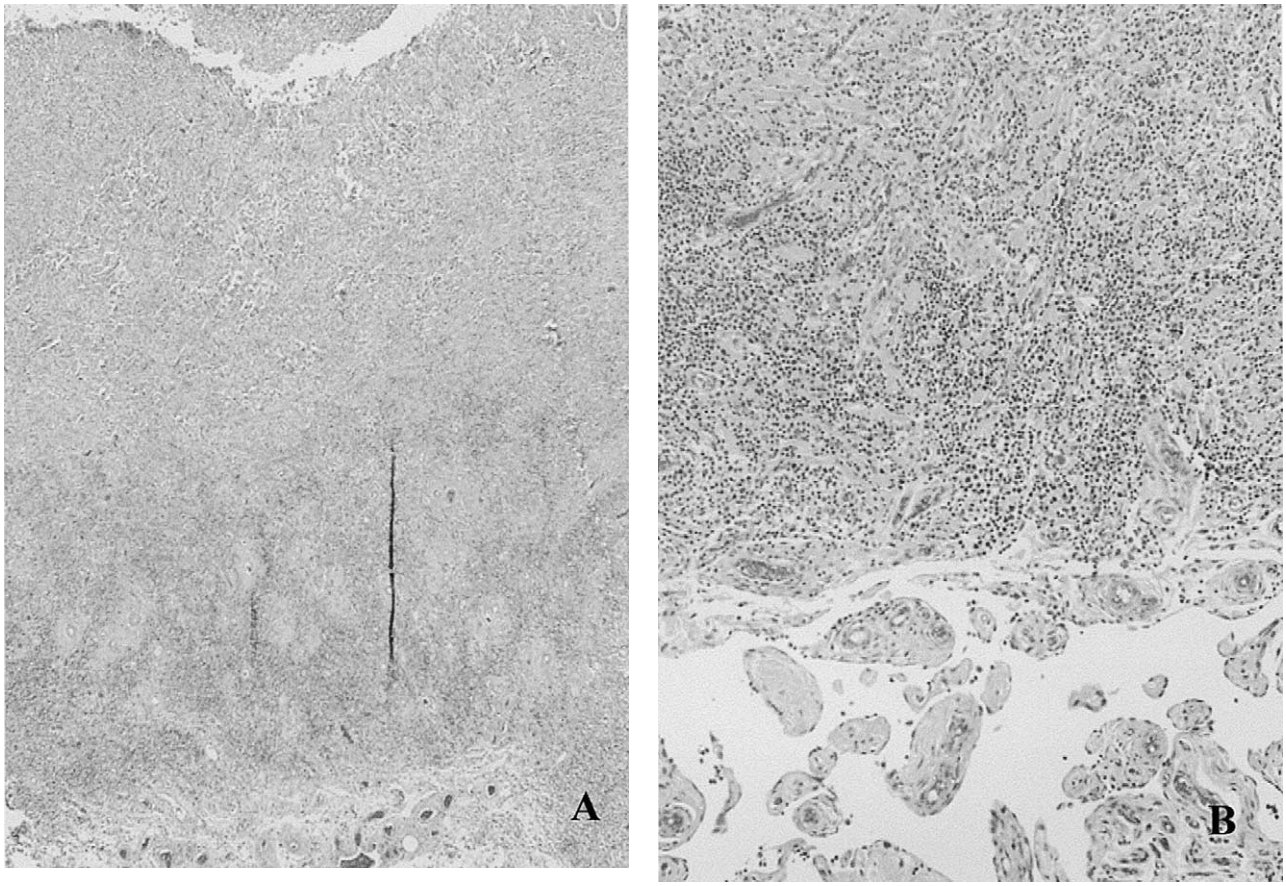

Fig. 2. Histologic examination of grafted area. **A,** Twenty-one days after the surgery, the defect was completely closed by granulation tissue and early fibrosis. Granulation tissue replaced all layers of the gastric wall including the submucosa, muscularis propria, and subserosal fat. (Hematoxylin & eosin stain; ×10.) **B,** Most of the surface of the grafted area was ulcerated. (Hematoxylin & eosin stain; ×10.) Fibrovascular adhesions were seen at the serosal surface.

RESULTS

All of the animals survived and thrived over the 3-week postoperative period. They gained weight (preoperative weight, 320.6 ± 5.5 g; 3 weeks postoperative weight, 371.5 ± 5.9 g) and had no evidence of perforation or clinical signs of leakage. In all instances, animals resumed regular food intake on the day of the surgical manipulation. At autopsy no macroscopic signs of perforation or material disruption were observed. Indeed, the graft area was incorporated into the gastric wall and was distinguished from the normal stomach only by the presence of suture material (Fig. 1). In the first group of animals in which no omentectomy was performed, the omentum was covering the grafted area in five (83.3%) of six rats. However, no adhesions were observed in the abdominal cavity or covering the stomach in four (66.6%) of six animals within the second group of animals in which an omentectomy was performed. The inferior pole of the spleen was covering the stomach in one of the remaining rats, whereas a short segment of small intestine was partially covering the graft in the other animal.

In all 12 animals, histologic examination revealed that the defect was completely closed by granulation tissue and early fibrosis. The granulation tissue replaced all layers of the gastric wall including the submucosa, muscularis propria, and subserosal fat (Fig. 2, *A*). Although most of the luminal surface of the grafted areas remained ulcerated (Fig. 2, *B*), early regeneration of mucosa was seen at the periphery of the former defect (Fig. 3). Foreign body giant cell reaction was present but limited to the areas of suture. Fibrovascular adhesions were present at the serosal surface.

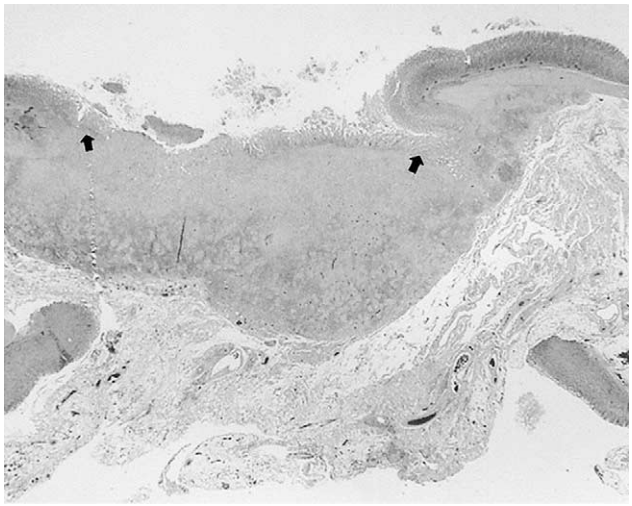

Fig. 3. Low-power overview of grafted area. The defect was completely closed by granulation tissue and early fibrosis. Granulation tissue replaced all layers of the gastric wall including the submucosa, muscularis propria, and subserosal fat. Early regeneration of the mucosa was seen at the periphery of the former defect (*arrows*). Foreign body giant cell reaction was present but limited to the areas of suturing. Fibrovascular adhesions were present at the serosal surface. (Hematoxylin & eosin stain; ×1.)

DISCUSSION

The principal finding reported here is that a double-layer patch of porcine-derived SIS served as a bioscaffold for regeneration of gastric tissue in a rat model of gastric perforation. Twenty-one days after implantation, granulation tissue had replaced the material in all layers of the gastric wall. Moreover, normal gastric mucosa was seen emerging at the periphery of the former defect.

Porcine-derived SIS is an extracellular matrix used in tissue-engineering experiments to create de novo dura mater, urinary bladder, blood vessels, tendons, and so forth.[10–16] Once extracted from the porcine intestines, the matrix is rinsed in water and peracetic acid, and freeze-dried to lyse any cells and eliminate degradation products associated with the matrix.[8] Compositional analysis has shown that the material consists predominantly of collagen types I, III, and IV,[8] with lesser quantities of proteoglycans, glycosaminoglycans,[17] glycoproteins,[18] and growth factors.[19] Interestingly, the bioactive basic fibroblast growth factor-2 (FGF-2) and transforming growth factor-β (TGF-β) are retained in the matrix following sterilization procedures,[19] and this bioactivity remains after prolonged storage at room temperature.[20] Basic FGF is normally present in the gastric mucosa and is a potent stimulator of angiogenesis,[21] accelerating healing of experimental gastric and duodenal ulcers in rats.[22,23] TGF-β is also involved in protecting the mucosa by turning off the proliferation of epithelial cells once they have left the crypts and glands.[21] Although the presence of these growth factors in the matrix might explain the findings observed in our study, information is lacking regarding the existence of other growth factors that are also

Table 1. Experimental applications of SIS matrix in the gastrointestinal tract

Reference	Species	Protocol	Findings
Chen and Badylak[31]	Dogs (n = 23)	Partial small bowel defect (7 × 3 cm)	Three animals died postoperatively due to leakage; autopsy of remaining animals at different time intervals; histologic evaluation showed mucosal layer, smooth muscle, collagen, and serosa; architecturally, layers were not organized
	Dogs (n = 4)	Small intestine interposition of SIS tube	One animal had partial obstruction; three had leakage from anastomotic site within the first week after surgery
Badylak et al.[32]	Dogs (n = 15)	5 × 3 cm esophageal defect or complete circumferential segmental defect (5 cm)	Scaffolds used for repair of defect reabsorbed completely within 60 days; histologic examination showed skeletal muscle, organized collagenous connective tissue, and squamous epithelium; segmental defect repair showed stricture within 45 days
Kini et al.[33]	Humans (n = 14)	Gastrojejunostomy reinforced with 10 × 2.5 cm SIS membrane in patients undergoing gastric bypass	Three patients developed stenosis at the anastomotic site; follow-up 87 days

crucial in maintaining epithelial integrity (e.g., epidermal growth factor, TGF-α, and trefoil factors).

On xenotransplatation of SIS, cellular infiltration and neovascularization are observed, and the matrix is rapidly remodeled into host tissue with site-specific structural properties.[24] The rate of degradation of the scaffold has been reported to range from 4 to 12 weeks[25,26]; as much as 90% of the material may be reabsorbed by as early as 4 weeks after implantation.[25–27]

The relatively fast absorption and healing rate observed in this project must be put into context—that is, all experiments were performed in normal rats with no signs of hypoalbuminemia or nutritional deficits. Animal studies have shown that perioperative malnutrition is associated with compromised anastomosis healing. For example, rats fed a protein-depleted diet have significantly lower colonic anastomosis bursting pressures than animals exposed to a standard diet.[28] Parenteral nutrition and immunosuppressant drugs administered perioperatively to rats also affect intestinal healing.[29,20] Multivariate analysis of patients undergoing bowel resection or bypass anastomosis of the small or large intestine have shown that use of corticoids, bowel obstruction, peritonitis, chronic obstructive pulmonary disease, serum albumin levels, and intraoperative blood transfusion are risk factors associated with anastomosis dehiscence.[2] In our study, animals received standard rat chow throughout the study, and no evidence of weight loss or malnutrition was observed perioperatively. Further studies examining the healing effectiveness of SIS under such conditions are warranted.

Despite increased clinical interest concerning the use of prosthetic materials in gastrointestinal surgery, few studies have assessed the potential uses of SIS in the gut (Table 1). Of the studies that we are aware of, one has used SIS for small bowel restoration[31] and one for esophageal repair.[32] Recently, an SIS membrane was used to reinforce a gastrojejunostomy in patients undergoing gastric bypass surgery.[33] Taken together, these findings show that SIS may act as an effective scaffolding agent for restoration of gastrointestinal tissue and may offer protection in high-risk anastomoses.

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Bariatric Surgery for Severely Obese Adolescents

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A 1991 National Institutes of Health Consensus Conference concluded that severely obese adults could be eligible for bariatric surgery if they had a body mass index (BMI) $\geq$35 kg/m^2 with or $\geq$40 kg/m^2 without obesity comorbidity. It was thought at that time that there were inadequate data to support bariatric surgery in severely obese adolescents. An estimated 25% of children in the United States are obese, a number that has doubled over a 30-year period. Very little information has been published on the subject of obesity surgery in adolescents. Therefore we reviewed our 20-year database on bariatric surgery in adolescents. Severely obese adolescents, ranging from 12 to less than 18 years of age, were considered eligible for bariatric surgery according to the National Institutes of Health adult criteria. Gastroplasty was the procedure of choice in the initial 3 years of the study followed by gastric bypass, which was found to be significantly more effective for weight loss in adults. Distal gastric bypass (D-GBP) was used in extremely obese patients (BMI $\geq$60 kg/m^2) before 1992 and long-limb gastric bypass (LL-GBP) was used for superobese patients (BMI $\geq$50 kg/m^2) after 1992. Laparoscopic gastric bypass was used after 2000. Thirty-three adolescents (27 white, 6 black; 19 females, 14 males) underwent the following bariatric operations between 1981 and June 2001: horizontal gastroplasty in one, vertical banded gastroplasty in two, standard gastric bypass in 17 (2 laparoscopic), LL-GBP in 10, and D-GBP in three. Mean BMI was 52 $\pm$ 11 kg/m^2 (range 38 to 91 kg/m^2), and mean age was 16 $\pm$ 1 years (range 12.4 to 17.9 years). Preoperative comorbid conditions included the following: type II diabetes mellitus in two patients, hypertension in 11, pseudotumor cerebri in three, gastroesophageal reflux in five, sleep apnea in six, urinary incontinence in two, polycystic ovary syndrome in one, asthma in one, and degenerative joint disease in 11. There were no operative deaths or anastomotic leaks. Early complications included pulmonary embolism in one patient, major wound infection in one, minor wound infections in four, stomal stenoses (endoscopically dilated) in three, and marginal ulcers (medically treated) in four. Late complications included small bowel obstruction in one and incisional hernias in six patients. There were two late sudden deaths (2 years and 6 years postoperatively), but these were unlikely to have been caused by the bariatric surgical procedure. Revision procedures included one D-GBP to gastric bypass for malnutrition and one gastric bypass to LL-GBP for inadequate weight loss. Regain of most or all of the lost weight was seen in five patients at 5 to 10 years after surgery; however, significant weight loss was maintained in the remaining patients for up to 14 years after surgery. Comorbid conditions resolved at 1 year with the exception of hypertension in two patients, gastroesophageal reflux in two, and degenerative joint disease in seven. Self-image was greatly enhanced; eight patients have married and have children, five patients have completed college, and one patient is currently in college. Severe obesity is increasing rapidly in adolescents and is associated with significant comorbidity and social stigmatization. Bariatric surgery in adolescents is safe and is associated with significant weight loss, correction of obesity comorbidity, and improved self-image and socialization. These data strongly support obesity surgery for those unfortunate individuals who may have difficulty obtaining insurance coverage based on the 1991 National Institutes of Health Consensus Conference statement.
(J GASTROINTEST SURG 2003;7:102–108.)

KEY WORDS: obesity, surgery, adolescent, bariatric

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (oral presentation).

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Published by Elsevier Science Inc.

1091-255X/03/$—see front matter
PII: S1091-255X(02)00125-7

Surgery for severe obesity has been shown to be efficacious in adults[1–5] and was supported in a 1991 National Institutes of Health (NIH) Consensus Conference for adults who had a body mass index (BMI) ≥35 kg/m^2 with or ≥40 kg/m^2 without obesity comorbidity.[6] An estimated 25% of children in the United States are obese, a number that has doubled over a 30-year period.[7,8] Very few studies have documented the efficacy of bariatric surgery in adolescents,[9–12] so insurance coverage may be difficult to obtain. Therefore we reviewed our 20-year bariatric surgery database for adolescents.

METHODS

Severely obese adolescents, ranging from 12 to less than 18 years of age, were considered eligible for bariatric surgery according to the NIH adult criteria. Gastroplasty was the procedure of choice during the initial 3 years of the study followed by gastric bypass, which was found to be significantly more effective for weight loss in adults.[1–4] Distal gastric bypass (D-GBP) was used in extremely obese patients (BMI ≥60 kg/m^2) before 1992, and long-limb gastric bypass (LL-GBP)[13] was used for superobese patients (BMI ≥50 kg/m^2) after 1992. Laparoscopic gastric bypass was used after 2000. Extensive preoperative discussion was undertaken with both the parent(s) and the adolescent, including a screening psychological questionnaire.

Comorbid conditions associated with obesity before and after surgery were analyzed, including sleep apnea syndrome as a respiratory disturbance index ≥10 hypopneic and/or apneic episodes per hour of sleep, type II diabetes mellitus as a fasting blood sugar ≥150 mg/dl or primary care physician–prescribed oral hypoglycemics or insulin, degenerative joint disease as complaints of pain in the weight-bearing joints (i.e., hips, knees, ankles, or lower back), gastroesophageal reflux symptoms of heartburn (without confirmatory studies of esophageal 24-hour pH, manometry, or endoscopy), pseudotumor cerebri documented by an elevated cerebrospinal fluid pressure of ≥200 cm H_2O with a normal cerebral MRI or CT scan except, for a dilated sella turcica, systemic hypertension (systolic blood pressure ≥150 mm Hg and/or diastolic blood pressure ≥90 mm Hg with a wide blood pressure cuff or primary care physician–prescribed antihypertensive medications), polycystic ovary syndrome in postmenarche girls with hirsutism and oligomenorrhea, and urinary incontinence, as a history of difficulty controlling urine or the need to wear a perineal pad.

Thigh-length intermittent venous compression boots placed before the induction of anesthesia have been used since the start of the bariatric surgical program in 1980. These boots were used until the patient was fully ambulatory. Early ambulation on the evening of surgery has been mandated since the inception of the program, except in patients with obesity hypoventilation or sleep apnea syndromes who required postoperative mechanical ventilation. Low-molecular-weight heparin (enoxaparin, 40 mg subcutaneously, 30 minutes before surgery) was instituted in 1992.

Patient follow-up examinations were scheduled at 2 weeks after surgery, at 3, 6, 12, and 18 months, and yearly thereafter. Approval was obtained from the Virginia Commonwealth University Institutional Review Board for collecting the data in a secure database and reporting on the results of analyses. Major efforts were made to contact patients who had not returned for follow-up visits, including contacting next of kin or the referring physicians and using a search service. The National Death Registry was also contacted, using patients' social security numbers, to be certain that no unknown deaths had occurred among the group of patients who were lost to follow-up. The database was last queried in May 2002.

RESULTS

Between 1981 and January 2002, a total of 33 adolescents (27 white, 6 black; 19 females, 14 males) underwent bariatric surgery. We have performed bariatric surgery in approximately 3100 adult patients; thus our adolescent patients represent only 1% of our bariatric surgery population. The procedures included one horizontal gastroplasty, two vertical banded gastroplasties, 17 standard gastric bypass procedures (two laparoscopic), 10 LL-GBPs, and three D-GBPs. The mean preoperative BMI was 52 ± 11 kg/m^2 (range 38 to 91 kg/m^2), and the mean age was 16 ± 1 years (range 12.4 to 17.9 years). Preoperative comorbid conditions included type II diabetes mellitus in one patient, hypertension in ten, pseudotumor cerebri in two, gastroesophageal reflux disease (GERD) in five, sleep apnea in six , polycystic ovary syndrome in one, and degenerative joint disease in ten. There were no operative deaths or anastomotic leaks. Early complications included one pulmonary embolism, one major wound infection, four minor wound infections, three stomal stenoses (endoscopically dilated), and four marginal ulcers (medically treated).

Weight loss in these patients was analyzed by means of analysis of variance and was found to be significant at 1, 5, 10, and 14 years after surgery (Table 1). However, five patients had regained all or most of their lost weight at 5 to 10 years after surgery. Excluding these patients yielded the following: 77 ± 17% excess weight loss (EWL) and a BMI of 29 ± 5 kg/m^2 at 5

years; 75 ± 9% EWL and a BMI of 30 ± 4 kg/m^2 at 10 years; and 61 ± 15% EWL and a BMI of 31 ± 2 kg/m^2 at 14 years after surgery. One patient who underwent horizontal gastroplasty maintained her weight loss for 15 years but regained the weight subsequently. Upper gastrointestinal series in three patients who had unsuccessful gastric bypass surgery showed no evidence of staple line disruption; one of these patients had a markedly dilated gastrojejunal anastomosis. One patient was converted from a standard gastric bypass to LL-GBP because of late weight gain at 5 years after a standard gastric bypass; this patient subsequently required a gastric transection because of staple line disruption at 12 years after the original surgery. One patient was converted from a D-GBP to standard gastric bypass because of severe protein-calorie malnutrition with correction of the malnutrition. Late complications included one small bowel obstruction necessitating adhesiolysis and six incisional hernias with subsequent herniorrhaphies that were treated with polypropylene mesh. None of these patients had any evidence of impaired physical or sexual maturation.

Two patients died suddenly at 2 years and 6 years postoperatively; these deaths were deemed unlikely to be directly related to the bariatric surgical procedures. The first of these was a 365-pound patient who had been in heart failure and had undergone diuresis of 45 kg before an LL-GBP procedure when she was 16 years old. As a result of severe venous stasis disease, a Greenfield vena caval filter had been placed because of the high risk of a fatal pulmonary embolism in this patient population.[14] This patient, a sophomore in college, had lost 42 kg and had undergone a panniculectomy and herniorrhaphy 1 year after her gastric bypass. She was doing well in school and had been in no distress and talking with her roommate 30 minutes before she was found dead. No autopsy was performed. The second patient was a mentally retarded African-American male who had undergone LL-GBP when he was 14 years old and weighed 355 pounds. At 5 years after surgery, he weighed 400 pounds and was in an extended care facility where he was found dead 6 years after surgery; no autopsy examination was performed.

Comorbid conditions were resolved at 1 year with the exception of hypertension in two patients, symptoms of gastroesophageal reflux in two, and pain from degenerative joint disease in seven (Table 2). One patient who regained all of his lost weight had a recurrence of his hypertension and degenerative joint disease pain 14 years after surgery. Another patient had a recurrence of gastroesophageal reflux after she regained her weight. Self-image was greatly enhanced; eight patients have married and have children, five have completed college, and one is currently in college.

DISCUSSION

This study confirms the efficacy of gastric bypass surgery for severely obese adolescents. The average preoperative weight and BMI in this group of patients was equivalent to that seen in our adult population undergoing obesity surgery.[1,2] However, this represents only 1% of our patients who have undergone gastric surgery for severe obesity. Obesity in childhood has become an epidemic, including the development of severe obesity.[7,8] Furthermore, these adolescents suffered from many of the same severe comorbid conditions seen in our adult patients. Most of the patients had three or more comorbid conditions. However, the frequency and severity of type II diabetes mellitus appears to be less than in our adult patients; the two patients with diabetes were effectively managed with an oral agent, and none required insulin. This suggests that diabetes progresses with prolonged severe obesity to develop. However, in some adolescent populations, type II diabetes has become the predominant form of diabetes, consistent with the obesity epidemic in childhood.[15] As in adult patients,[5] the diabetes resolved after bariatric surgery–induced weight loss. In a recent report, the hospital discharges among young persons 6 to 17 years of age, in whom obesity was listed

Table 1. Weight loss after obesity surgery in adolescents

	Preoperative	1 yr	5 yr	10 yr	14 yr
No. of patients	33	31/32	20/24	14/18	6/9
Weight (kg)	150 ± 40 (range 100–303)	105 ± 34*	95 ± 30*	101 ± 30*	114 ± 56*
			81 ± 13†	85 ± 15†	91 ± 7†
BMI (kg/m^2)	52 ± 11 (range 38–91)	36 ± 10*	33 ± 11*	34 ± 8*	38 ± 16*
			29 ± 5†	30 ± 4†	31 ± 2†
% EWL		58 ± 20	63 ± 32	56 ± 34	33 ± 68
			77 ± 17†	75 ± 9†	61 ± 15†

*$P < 0.0001$ compared to preop.
†Excluding five patients at 5 years and 10 years and 1 patient at 14 years who had regained their lost weight.

Table 2. Effect of surgically induced weight loss on obesity comorbidity

	DM	HTN	SAS	GERD	PC	PCOS	DJD
Preoperative	2	11	6	5	3	1	11
1 yr postoperative	0	2	0	2	0	0	7

DM = type II diabetes mellitus; HTN = systemic hypertension; SAS = sleep apnea syndrome; GERD = gastroesophageal reflux disease; PC = pseudotumor cerebri; PCOS = polycystic ovary syndrome; DJD = degenerative joint disease.

as either a primary or secondary diagnosis, doubled for diabetes, tripled for gallbladder disease, and increased fivefold for sleep apnea, and the cost of hospital care increased from $35 million to $127 million based on 2001 dollars.[16]

Some of these adolescents were incapacitated by problems associated with their comorbidity. The patients with sleep apnea had difficulty staying awake in class and completing their homework assignments, and were failing in their course work. Previous studies have shown resolution of sleep apnea after gastric bypass surgery.[17,18] After surgically induced weight loss, this problem resolved and two of the six patients with sleep apnea went on to graduate from college. One patient was in severe congestive heart failure and required diuresis of 45 kg in the pediatric intensive care unit prior to her surgery. The three patients with pseudotumor cerebri suffered from constant, severe headaches that markedly impaired the quality of their lives. As in our adult population, this complication of obesity resolved with surgically induced weight loss.[19,20] Resolution or improvement in hypertension and gastroesophageal reflux was seen in most of the adolescents with these problems, as reported in the adult populations.[21–24] It was nice to perform one operation and have it take care of three or four chronic medical conditions.

This study brings the reported number of children and adolescents who have undergone gastroplasty or gastric bypass surgery for obesity to 132.[9–12] However, one of these studies is from 1980, before the current use of a Roux-en-Y gastric bypass utilizing a 1 cm anastomosis and a 15 ml gastric pouch.[9] There were two operative deaths in that series, which included 11 mentally retarded patients with Prader-Willi syndrome. As did our one patient with mental retardation in whom the operation failed, these patients with Prader-Willi syndrome also failed to lose weight. There are two recent reports of a malabsorptive partial biliopancreatic bypass for patients with Prader-Willi syndrome with more encouraging results.[25,26] The risk of protein-calorie malnutrition in this malabsorptive procedure,[27] however, is a cause for concern in patients with severe mental retardation. There have been no operative deaths in this report or in more recent series.[10–12] Early complications such as wound infections, stomal stenosis, and marginal ulcers were easily treated medically. Late complications necessitating additional surgery occurred in 21% of the patients, including lysis of adhesions for small bowel obstruction in one and incisional herniorrhaphies in six patients. With the availability of laparoscopic gastric bypass surgery, the risk of incisional hernia has decreased from approximately 25% to 1%.[28–32] There were two sudden deaths 2 and 6 years, respectively, after surgery. The first occurred in the patient who had been in congestive heart failure before surgery with severe venous stasis disease and had had a Greenfield inferior vena caval filter placed at the time of surgery. The preoperative electrocardiogram was normal without a prolonged QT interval, which occurs with greater frequency in severely obese patients and can be the cause of sudden death.[33] The second death was in the retarded patient who had regained all of the weight he had lost and more. No autopsy was performed in either patient, so the cause of death will never be known, but it is unlikely that the deaths were related specifically to the bariatric surgery. Weight regain occurred in five of the patients. However, the weight loss remained significant and stable in the remaining patients up to 14 years after their bariatric procedures. It is certainly possible that there may have been weight regain in the four patients whom we were unable to locate for follow-up evaluation 5 years after their operations.

In addition to their significant medical comorbidity, these adolescents are socially stigmatized by their peers. Although we did not perform standardized quality-of-life assessments, all of the patients reported a significant improvement in their self-images and their social interactions within 1 year after surgery. Eight of the patients have married; five have completed college, and one is currently in college. There was no evidence of impaired physical or sexual maturation in any of these patients.

Unresolved issues include the timing of bariatric surgery in childhood and adolescence. Is it necessary for the epiphyses to be closed? None of our patients showed any evidence of impaired physical or sexual maturation. Perhaps some severely obese children should be offered surgery in elementary school, such as those with severe sleep apnea or those who are being psychologically abused by their peers. What is the opti-

mal procedure? Is it gastroplasty, adjustable gastric banding, gastric bypass (and the lengths of the intestinal limbs), or a malabsorptive biliopancreatic diversion with or without a duodenal switch? One would suspect that malabsorptive procedures should be used with great caution in this population. One of our patients required revision of a D-GBP because of severe protein-calorie malnutrition. However, late weight regain after a gastric bypass is a concern in this study, and perhaps the duodenal switch procedure may provide longer lasting benefit. There is also a problem with long-term patient follow-up in this and other studies of bariatric surgery, making outcomes-based evaluations difficult. There is also a need for more objective data on the effects of surgically induced weight loss on quality-of-life issues.

CONCLUSION

Bariatric surgery can be performed safely in severely obese adolescents. It is associated with a significant and, in most instances, a long-lasting weight loss with correction or improvement in obesity comorbidity, as well as self-image and socialization. The data from this and other studies strongly support obesity surgery for these unfortunate individuals who may have difficulty obtaining insurance coverage based on the 1991 NIH Consensus Conference statement.

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Discussion

Dr. M.T. Dayton (Salt Lake City, UT): I think this is an important study. Is there any evidence, given the common dietary indiscretion of teenagers, that this patient population has more difficulty keeping weight off. Do they, for example, drink a lot of sugar-rich drinks? Do they, as a population, have more difficulty than the adult population adhering to the strict diet that these patients need to adhere to?

Dr. H. Sugerman: Our data show that their weight loss is equivalent to that of adults we have studied. We did not put those findings into this report, but I think it is a good question. So, in fact, they do just as well as adults who have the same operation, and most of them, in our experience, find that they have difficulty with sugar drinks. Most of them find that they develop dumping symptoms with regular sodas. But it is a potential problem in terms of nibbling through this operation with chips, both in the teenagers and in adults.

Dr. R.E. Brolin (New Brunswick, NJ): I, too, would like to congratulate you and your colleagues on an excellent and extremely important presentation. I have a brief comment and then a question. Although you presented a great deal of objective data, I think the aspect that you cannot overlook is the patient testimonials and the significance of those testimonials. There is just no doubt that these operations in this age group change lives. It brings kids who are ostracized and ridiculed by their peer groups into the mainstream; they go to the prom, they graduate from college, they get married, they behave like "normal" people, and it is truly a very gratifying age group in terms of the results of these operations. My question has to do with the child who was retarded and had a less than optimal outcome. Did this child have Prader-Willi syndrome? Do you believe that these operations should be performed in that challenging group of children?

Dr. Surgerman: We do not think this child had Prader-Willi syndrome. He gained his weight actually late in his childhood. I personally don't think that this operation should be done in mentally retarded children. However, there have been two papers on biliopancreatic bypass in patients with Prader-Willi syndrome with some good results, but I think it is a fairly radical procedure in that group of patients. So I think this is one group in whom surgery for obesity should be restricted.

Dr. M.P. Callery (Boston, MA): One wonders whether adolescence can create an abyss in medical care for some young persons and their pediatricians or family practice primary caregivers. Do these caregivers actually know that this type of surgical care and follow-up is available? What is the level of attention with respect to bariatric surgery in the pediatric surgical community?

Dr. Sugerman: There are really two questions here. One is, what are we doing to prevent this problem in the first place? I mean, this is a huge problem, and we discussed it in our consensus conference earlier this week. Enormous efforts are going to have to be made, by the government, by everybody, in terms of preventing this problem, not only in our adolescents but also in our adults. Coca-Cola machines should be taken out of the elementary schools, where the schools are profiting from having these soda machines in the schools, among other things. On the other hand, I think that an increasing number of pediatricians and primary care physicians, both for adolescents and for adults, are beginning to appreciate the value of this surgery. So I think it is one of the reasons why there has been a progressive growth, not just because of the availability of laparoscopic surgery; when a pediatrician sees one of these patients for follow-up whose constant headaches are gone or the diabetes is gone, or hypertension is gone, it is very gratifying for them. We are now getting more referrals from pediatricians than we ever have, and I think that is going to continue.

Dr. B.D. Schirmer (Charlottesville, VA): I have one question. What was the incidence of postoperative metabolic complications in terms of vitamin B_{12} and iron deficiencies, and were these different from these complications in adults? Were there any adolescents who you thought might have some impaired growth because of the weight loss?

Dr. Sugerman: We did not see any evidence of impaired growth because of weight loss. In terms of iron deficiency anemia and B_{12} deficiency, they are the same as in our adult patients. We try to treat our patients prophylactically to prevent both of those complications, and the frequency is the same in both populations in terms of compliance and complications.

Dr. Mark A. Talamini (Baltimore, MD): I have one question about the laparoscopic band procedure that has recently been approved by the FDA. Do you think the laparascopic band procedure might have some role in this age group, particularly with regard to the concerns that you have addressed?

Dr. Sugerman: As you may or may not know, we were one of the seven centers evaluating the laparascopic band. We had major concerns about our results with this procedure in terms of weight loss and problems with esophageal dysmotility, dysphasia, and esophageal dilatation, so we are looking for more data from other centers. The advantage is the improvement in morbidity and mortality. Short term, the disadvantage is that weight loss is nowhere near as good, especially in African-American patients. We stopped using the band procedure 3 years ago, and we have removed half of the bands that we placed and converted most of those patients to gastric bypass. So we are not using the banding procedure at this time.

Dr. L.F. Rikkers (Madison, WI): Do you have any thoughts regarding the late weight gain in some of the patients? Is this due to the rearranged anatomy or is it a change in eating patterns far out from the time of surgery?

Dr. Sugerman: Both. We find that in the vast majority of the patients who are regaining weight—and you have to realize it is not much—it is maybe 6 or 8 pounds a year, that is, 60 to 80 pounds in 10 years. It is primarily by nibbling through the procedure with high-fat, soft-calorie foods, which Dr. Brolin has pointed out, mostly consisting of potato chips and corn chips that will crumble up and go right through this. So part of it is trying to get the patients to come back on a yearly basis and talk to dietitians and try to have them work with the operation instead of against it. Occasionally, however, we have patients who have a markedly dilated gastrojejunal anastomosis, and unfortunately, when we have reoperated on these patients—and there is a publication from Kentucky—to reduce the size of, it does not seem to have solved the problem. We are wondering if we can do something endoscopically to make the anastomosis smaller. Rarely is it from a staple line disruption with a gastric bypass. If that is the case, then the procedure needs to be redone. If the patient's anatomy is normal and he or she has regained weight and now has major obesity comorbidity, we then will take and move the Roux limb way down to provide a malabsorptive procedure; however, when that is done, there is a risk of protein-calorie malnutrition and its associated problems. We presented those data at this meeting approximately 2 years ago.

Dr. L.W. Traverso (Seattle, WA): The pediatric surgeons have not yet yielded to the demand. However, they have begun to compile their outcomes nationwide, both for pyloric stenosis and pediatric gastroesophageal reflux disease, and their next tool is going to be morbid obesity in adolescents. My question to you is, do you know of any other national outcomes database collection that is being done for these adolescent patients?

Dr. Sugerman: In terms of surgery, no, I do not; the only pediatric center that I am aware of that has begun to perform bariatric surgery is Cincinnati Children's Hospital under the direction of Victor Garcia. Maybe there are other centers, but I think they have been very reluctant to start using this operation. But perhaps that is changing now. I think they need to pick it up.

Importance of Response to Neoadjuvant Chemotherapy in Patients Undergoing Resection of Synchronous Colorectal Liver Metastases

Peter J. Allen, M.D., Nancy Kemeny, M.D., William Jarnagin, M.D., Ronald DeMatteo, M.D., Leslie Blumgart, M.D., Yuman Fong, M.D.

The purpose of this study was to compare the treatment and outcome in patients referred for staged resection of synchronous colorectal liver metastases. The records of patients who had undergone colon or rectal resection and were then referred for evaluation of clinically resectable synchronous liver metastases between January 1995 and January 2000 were reviewed. Comparisons were made between patients who did not receive neoadjuvant chemotherapy and had exploratory operations after recovery from colon resection and patients who did receive chemotherapy before liver resection. A total of 106 patients were treated during the 5-year period. Neoadjuvant chemotherapy was given to 52 of the patients; in 29 of them the disease did not progress, but in 17 patients the disease progressed while they were receiving treatment. Median follow-up was 30 months. Patient- and tumor-related variables were similar between groups. Five-year survival was statistically similar between patients who did and those who did not receive neoadjuvant chemotherapy (43% vs. 35%, $P = 0.49$). Patients within the neoadjuvant group whose disease did not progress while they were receiving chemotherapy experienced significantly improved survival as compared to patients who did not receive chemotherapy (85% vs. 35%, $P = 0.03$). In the setting of synchronous colorectal liver metastases, the response to neoadjuvant chemotherapy may be a prognostic indicator of survival and may assist in the selection of patients for conventional or experimental adjuvant therapies. (J GASTOINTEST SURG 2003;7:109–117.)

KEY WORDS: Colorectal hepatic metastases, liver neoplasm, liver resection, prognosis

Surgical resection for patients with isolated colorectal liver metastases has been shown to result in 5-year survival rates of approximately 30% and is currently considered the only curative option for these patients.[1–3] To increase the likelihood of long-term survival, patients should be selected for resection who have tumor- and treatment-related characteristics that are suggestive of a less aggressive tumor biology. A recent study from our institution identified five tumor- and treatment-related factors that were independent predictors of survival.[4] These factors included the nodal status of the primary lesion, the number of metastases, the size of the metastases, the preoperative carcinoembryonic antigen (CEA) level, and the disease-free interval.

A short disease-free interval has been associated with decreased survival in many studies.[2,5] Patients who have synchronous metastases represent a group of patients with the shortest disease-free interval—essentially zero—and have been shown to be at increased risk for recurrence and disease-specific death after metastatic resection.[1] This distinctly worse prognosis for patients with synchronous metastases has been thought to be the result of either a delay in

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (oral presentation).

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1091-255X/03/$—see front matter
PII: S1091-255X(02)00121-X

the detection of the primary tumor or a more aggressive tumor biology.

Patients who are seen for evaluation of clinically resectable synchronous metastases, after having undergone colon resection, are treated in one of two ways. These patients either have exploratory operations for metastatic resection shortly after recovery from resection of the primary lesion or they are given "neoadjuvant" chemotherapy before surgical exploration for liver resection. The current study is an examination of the patient- and tumor-related factors that are used in making this treatment decision at a hepatobiliary cancer referral center. This study attempts to define clinical practice and to determine whether the response to chemotherapy can be used as one of the criteria to better select patients for resection.

METHODS

The records of all new-patient visits for evaluation of colorectal liver metastases were reviewed from a 5-year time period (January 1995–January 2000). Patients evaluated for metachronous liver metastases were excluded, as were patients with synchronous metastases whose disease was thought to be unresectable based on the results of radiographic imaging. Patients with radiographically resectable synchronous metastases who had not undergone colon resection and who were scheduled for simultaneous colon and liver resection were also excluded. Patients undergoing simultaneous colon and liver resection are usually patients with smaller volume metastatic disease that can most often be resected with segmental or subsegmental resection.[6] Although this did not equate with a survival advantage, we do believe that these patients represent a more biologically favorable and distinct subgroup, and therefore we did not include them in our analysis.

Metastases were considered synchronous if they were detected intraoperatively during colon resection or on radiographic imaging in the perioperative period. We arbitrarily chose 1 month postoperatively as the limit for categorizing metastases as synchronous. Patients who were discovered to have liver metastases more than 1 month after resection of the primary tumor were defined as having metachronous lesions and were not evaluated in this study.

Recommendations regarding the use of preoperative chemotherapy were made by one of four attending surgeons, in conjunction with the medical oncology service. Reasons listed by the four surgeons for recommending neoadjuvant chemotherapy included the presence of multiple small tumors, significant postoperative complications from the primary resection, the presence of significant comorbid conditions, and patient preference.

Patients who received more than two cycles of chemotherapy before undergoing exploratory operations for liver resection were considered to have received neoadjuvant chemotherapy. All patients within the neoadjuvant group received systemic 5-fluorouracil (5-FU)–based chemotherapy. Additional agents used included leucovorin, irinotecan, and oxaliplatin. None of the patients received preoperative hepatic arterial infusion as a neoadjuvant treatment. Response to chemotherapy was determined from serial imaging studies and was based on diameter changes within the lesions. The metastatic disease was categorized as progressing, regressing, or remaining stable while the patient was on chemotherapy. This determination was made only when direct comparison of radiographic studies was possible.

Comparisons were made between patients who had and those who had not received neoadjuvant chemotherapy in attempt to identify how patients were selected for such therapy at our institution. Patient-, tumor-, and treatment-related variables were analyzed. The association between the use of neoadjuvant chemotherapy and patient-, tumor-, and treatment-related variables were assessed using the chi-square test. Disease-specific survival probabilities were estimated using the Kaplan-Meier method and data were compared by means of the log-rank test.

RESULTS

Over the 5-year period, 1016 new patients with the diagnosis of colorectal liver metastases were evaluated by the hepatobiliary service (Fig. 1). Synchronous metastases were present in 330 patients (32%), and half of these patients (n = 167) were shown to have radiographically resectable lesions. Within the group of patients with radiographically resectable synchronous metastases, the majority were scheduled for a staged resection (n = 106; 63%) and these 106 patients represent the group of interest for this study.

Within the group of 106 patients with radiographically resectable synchronous metastases, there were 61 men and 45 women. The median age was 62 years. The prevalence of comorbid disease was low, with nine patients (8%) having diabetes mellitus, 18 patients (17%) having a history of clinically significant coronary artery disease, and three patients (3%) having cirrhosis. Neoadjuvant chemotherapy was given to 52 of the 106 patients, and the patient-related variables listed previously were similar between the neoadjuvant and non-neoadjuvant groups (Table 1). In addition, patient selection for neoadjuvant therapy

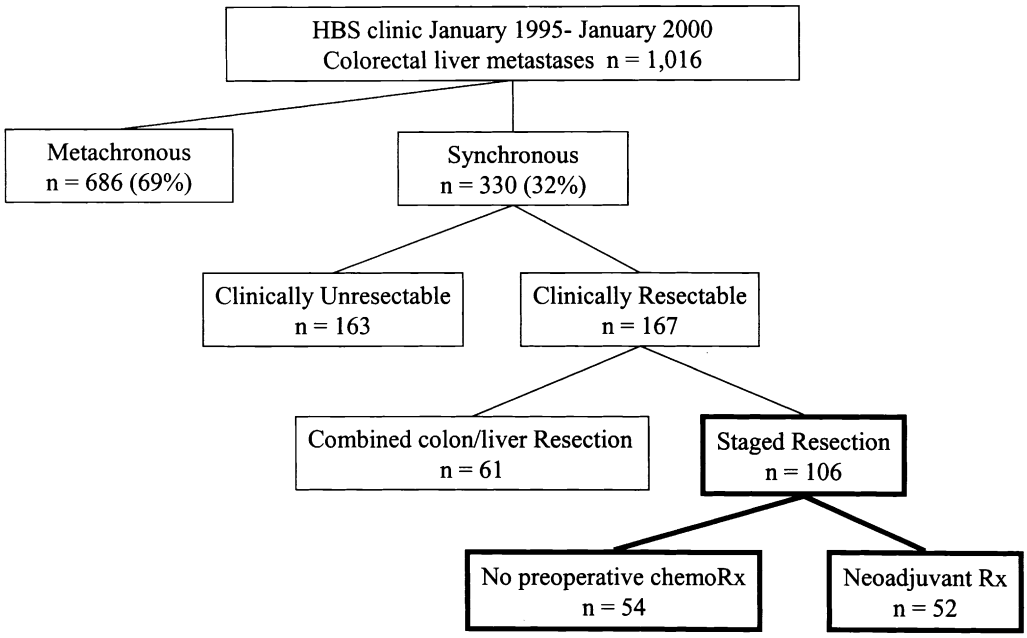

Fig. 1. Flow diagram representing all patients presenting to the hepatobiliary service for evaluation of colorectal liver metastases.

was similar among the four surgeons. Patient, primary lesion, and metastatic tumor characteristics were similar in both treatment groups for all four surgeons.

The tumor- and treatment-related variables with regard to the primary lesion were also similar in the two groups of patients (i.e., those who did and those who did not receive neoadjuvant chemotherapy; see Table 1). Nearly all of the patients (99 [93%] of 106) had primary lesions that invaded through the bowel wall (pathologic T3), with 16 of these patients having tumors that had either invaded adjacent organs or perforated into the peritoneal cavity (pathologic T4). Metastatic disease was found within the regional nodal basin in 74 (70%) of 106 patients. The operation performed for the primary lesion was a colectomy in 71 patients, a low anterior resection in 32 patients, and an abdominal perineal resection in three patients.

Tumor- and treatment-related factors with regard to the metastatic disease were also similar between patients who did and those who did not receive neoadjuvant chemotherapy (see Table 1). The number of metastases detected within the liver on imaging studies was greater than one in 74% of patients, and ranged from one to 14 lesions. Approximately one third of the patients (34%) presented with at least a single metastasis that was greater than 5 cm in diameter. Resection was performed in 87% of patients who did not receive neoadjuvant therapy and in 83% who did receive neoadjuvant chemotherapy (P = 0.53). Most of the patients (63%) who were resected underwent a hepatic lobectomy or extended resection, with the remaining 37% undergoing segmental or subsegmental resections. Adjuvant chemotherapy was given to 63 (70%) of the 90 patients resected, which was also similar between the two groups.

Patients who received neoadjuvant chemotherapy were taken to the operating room for attempted resection at a median of 8 months after beginning chemotherapy (range 2 to 24 months). Patients who did not receive neoadjuvant chemotherapy were taken to the operating room for attempted resection at a median of 6 weeks (range 4 to 14 weeks) after resection of the primary lesion in the colon. All patients who received preoperative chemotherapy underwent surgical exploration for resection because none of them became radiographically unresectable while they were receiving chemotherapy. All patients within the neoadjuvant group received systemic 5-FU–based chemotherapy, and in addition, 12 patients received pelvic radiotherapy for a primary lesion in the rectum. Response to chemotherapy could be adequately assessed in 46 of the 52 patients. Hepatic metastases were found to progress while patients were on chemotherapy in 17 patients (37%), remained stable in 17 patients (37%), and showed regression radiographically in 12 patients (26%).

The median length of follow-up for all patients was 30 months, and for patients alive at the time of last follow-up the median was 35 months. At the time of last follow-up, 27 patients were without evidence of disease,

Table 1. Comparison of patient and tumor (primary and metastatic) characteristics between patients who did and did not receive neoadjuvant chemotherapy

Factor		No neoadjuvant therapy (n = 54) No. (%)	Neoadjuvant therapy (n = 52) No. (%)	*P* value
Patient				
Sex	Male	28 (52%)	33 (63%)	NS
	Female	26 (48%)	19 (37%)	
Age (yr/mean)		63	59	NS
Diabetes	Yes	5 (9%)	4 (8%)	NS
	No	49 (91%)	48 (82%)	
Cardiac disease	Yes	7 (13%)	11 (21%)	NS
	No	47 (87%)	41 (79%)	
Primary				
T-stage	1 or 2	4 (7%)	4 (8%)	NS
	3 or 4	50 (93%)	48 (92%)	
Node positive	Yes	35 (65%)	39 (75%)	NS
	No	19 (35%)	13 (25%)	
Type of resection	LAR	17 (31%)	15 (29%)	NS
	APR	0	3 (6%)	
	Other	37 (69%)	34 (65%)	
Metastases				
No. of metastases	1	15 (28%)	13 (33%)	NS
	>1	39 (72%)	39 (67%)	
Size >5 cm	Yes	16 (30%)	20 (38%)	NS
	No	38 (70%)	32 (62%)	
Resected	Yes	47 (87%)	43 (83%)	NS
	No	7 (13%)	9 (17%)	
Type of resection	<1 lobe	15 (32%)	10 (23%)	NS
	≥Lobe	32 (68%)	33 (77%)	
Postresection chemotherapy	Yes	34 (72%)	29 (67%)	NS
	No	13 (28%)	14 (33%)	

APR = abdominoperineal resection; LAR = low anterior resection (rectosigmoid).

32 patients were alive with disease recurrence, 38 patients had died of disease, and nine patients had died of other causes. There were no patient- or primary tumor-related variables associated with improved survival (Table 2). Improved survival was associated with two treatment-related variables: the ability to perform complete resection and the response to neoadjuvant chemotherapy. Patients who received neoadjuvant chemotherapy, as a group, experienced similar overall survival as compared to patients who did not receive neoadjuvant chemotherapy (Fig. 2). The subgroup of patients with disease that did not progress while they were receiving chemotherapy, however, experienced significantly improved survival (Fig. 3).

DISCUSSION

Patients who have synchronous colorectal liver metastases experience decreased survival compared to patients whose metastases are discovered in a metachronous fashion. A study by Scheele et al.[1] clearly demonstrated the overall poor prognosis of patients presenting with synchronous metastases. In this study of 434 patients undergoing resection of colorectal liver metastases, the presence of synchronous metastases significantly decreased 5-year survival from 43% to 30%. Patients with synchronous metastases also experienced an increased rate of disease recurrence, which led them to recommend that patients with synchronous metastases undergo resection in a staged fashion to allow improved detection of micrometastatic disease.

Not only do patients with synchronous metastases have an overall worse survival, but the tumor- and treatment-related variables associated with survival have been reported to be different in this group of patients.[7,8] Nodal status, number of metastases, size of metastases, preoperative CEA level, and margin status have not consistently been shown to be associated with survival in patients with synchronous metastases.

Table 2. Association between patient, tumor (primary and metastatic), and treatment characteristics and disease-specific survival (all patients)

Factor		n	Disease-specific survival (5-year)	*P* value
Patient				
Sex	Male	61	48%	NS
	Female	45	43%	
Age (yr)	<60	52	36%	NS
	≥60	54	49%	
Primary				
Node positive	Yes	74	55%	NS
	No	32	66%	
Metastases				
No. of metastases >1	Yes	78	40%	NS
	No	28	44%	
Size >5 cm	Yes	37	36%	NS
	No	69	42%	
Resected	Yes	90	47%	0.001
	No	16	14%	
Treatment				
Neochemotherapy	Yes	52	52%	NS
	No	54	38%	
Progression on	Yes	17	38%	0.03
Chemotherapy (n = 46)	No	29	87%	

In a recent study by Sugawara et al.,[7] factors associated with survival in 304 patients undergoing hepatectomy for metastatic colon cancer were compared between patients with synchronous and metachronous lesions. In the group of patients with metachronous disease, the nodal status, CEA level, resection margin, metastatic tumor size, and number of metastases were associated with survival. In the group of patients with synchronous metastases, only the resection margin was found to be associated with survival.

This inability to identify factors associated with improved survival in this subset of patients with an

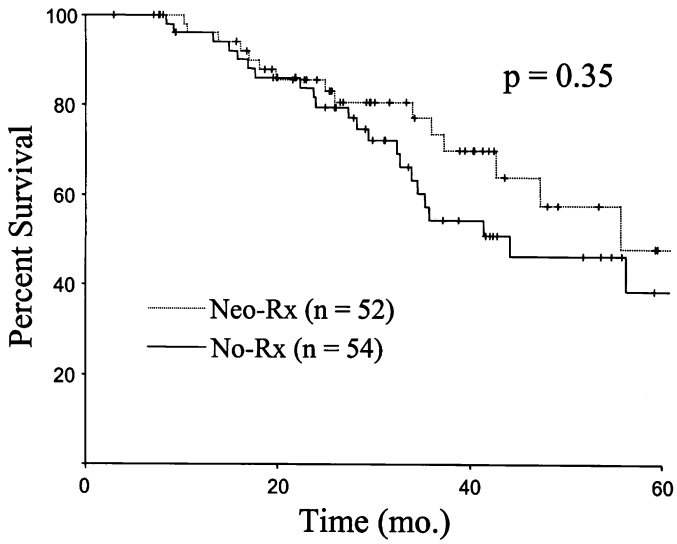

Fig. 2. Kaplan-Meier survival analysis comparing patients who received (*Neo-Rx*) or did not receive (*No-Rx*) neoadjuvant chemotherapy.

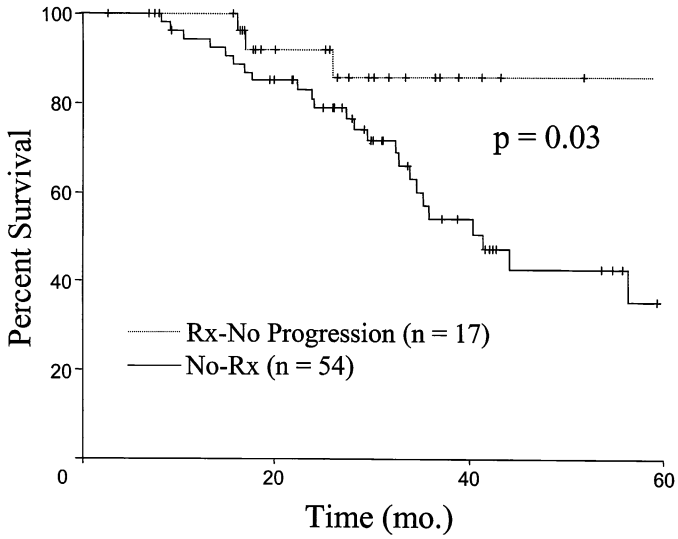

Fig. 3. Kaplan-Meier survival analysis comparing patients who did not progress (*Rx-No Progression*) while receiving neoadjuvant chemotherapy and patients who did not receive neoadjuvant chemotherapy (*No-Rx*).

overall poor prognosis makes appropriate patient selection more difficult. Because of this, the optimal surgical approach to patients with synchronous metastases has been controversial. Initially this controversy centered around whether or not these patients should even undergo resection, particularly when liver resections in general were associated with high morbidity and mortality.[6,9,10] More recently, however, controversy has centered over the timing of resection and whether or not it is safe to perform a simultaneous colon and hepatic resection. Multiple studies, including one from our own institution, have demonstrated that in selected patients simultaneous resection is safe and can be performed without a significant increase in morbidity.[11–13] As noted earlier, however, concern remains over the possibility of increased rates of undetected micrometastatic lesions in patients undergoing simultaneous resection, and long-term survival rates after simultaneous resection are yet to be reported.[1,9]

Regardless of the preceding controversy, most of the patients referred to our institution with synchronous metastases were evaluated after having had the primary tumor resected. Within this group of patients we identified two different treatment strategies. The first was to take the patient to the operating room immediately after recovery from the colon resection (median interval 7 weeks), and the second was to give the patient a course of chemotherapy before resection was attempted (median interval 8 months). In this study we compared patients who received neoadjuvant chemotherapy with those who did not, in an attempt to identify patient- or tumor-related differences used in the selection of treatment. Second, we compared factors associated with survival for the group as a whole, and compared survival between patients who did and those who did not receive neoadjuvant chemotherapy.

We could not identify any patient-, tumor-, or treatment-related factors that were different between patients who did and those who did not receive neoadjuvant chemotherapy. Patient age, incidence of comorbid disease, T and N stage of the primary lesion, the number of hepatic metastases, and the size of hepatic metastases were similar. More important, the percentage of patients who had resections was also similar in the two groups (neoadjuvant, 83%; non-neoadjuvant, 87%). Also, none of the patients who received neoadjuvant chemotherapy became radiographically unresectable, and all underwent surgical exploration for resection. This supports the notion that one should not be in a hurry to resect metastases. In this group of patients at high risk for occult disease, the delay produced by treating them with neoadjuvant chemotherapy is unlikely to allow resectable disease to become unresectable.

Similar to other studies mentioned earlier, we did not find any patient- or tumor-related variables to be associated with survival. Disease-specific survival was associated with the ability to perform complete resection but was not associated with the delivery of neoadjuvant chemotherapy. Within the group of patients who received neoadjuvant treatment, however, patients who had disease that regressed or remained stable while they were on chemotherapy experienced significantly improved 5-year survival. We doubt

that the actual 5-year survival for patients who did not progress while on chemotherapy will be as high as 85%; however, we are encouraged by the fact that 35% of the patients were without evidence of disease at last follow-up, and within this group the median follow-up was 30 months.

Response to neoadjuvant chemotherapy has been associated with improved outcome in other types of malignancy such as breast cancer, esophageal cancer, and soft tissue sarcoma.[14–16] Neoadjuvant chemotherapy has been used in selected patients with soft tissue sarcoma, and the response to neoadjuvant therapy has been shown to be associated with survival. A recent study by Eilber et al.[14] found that recurrences diminished and survival improved in patients who demonstrated a pathologic response to neoadjuvant chemotherapy. In this study of 496 patients with moderate- or high-grade extremity sarcoma, the 5-year survival for patients who responded to neoadjuvant chemotherapy was 80% as compared to a 5-year survival rate of 62% for patients who did not respond to chemotherapy.

The response to neoadjuvant chemotherapy has been reported to improve resectability, even in patients who present with radiographically unresectable colorectal liver metastases.[17,18] Bismuth et al.[18] reported on a series of 330 patients who initially presented with clinically unresectable lesions because of either location, size, or number of metastases. Fifty-three patients were clinically downstaged with the use of neoadjuvant chemotherapy and eventually underwent a resection. Within this select group of patients whose disease responded to chemotherapy and thus allowed resection, there was an overall 5-year survival rate of 40%.

In summary, this study identified the response to chemotherapy as a factor associated with improved survival in patients with synchronous colorectal liver metastases who presented for staged resection. A number of molecular determinants for response to chemotherapy have been identified for colorectal cancer.[19,20] Whether such markers can be used to select patients likely to respond to neoadjuvant therapy, and thus allow selection of patients with resistant tumors for immediate surgery, is unknown and deserves clinical testing. At present, in the setting of synchronous colorectal liver metastases, neoadjuvant chemotherapy should be considered and may assist in the selection of patients for additional adjuvant therapy.

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Discussion

Dr. J.M. Becker (Boston, MA): You chose to exclude the patients who had simultaneous colon resection and resection of liver metastases. Presumably these patients would have a better prognosis. My question is if the patients were added back into the no neoadjuvant therapy group, would that alter the overall data analysis and results?

Dr. P. Allen: We excluded those patients because we recently evaluated this group in a retrospective study comparing simultaneous vs. staged resection. In that study we found that these patients tended to have fewer peripheral lesions, and most of the time they underwent segmental or subsegmental resection. In addition, most of these patients had primary lesions located in the right colon. For these reasons we believe that this group represents a group of patients with a better disease biology—with a smaller disease burden that can be removed with less extensive operative procedures. They probably would have more favorable outcomes.

Dr. A. Bilchik (Santa Monica, CA): Can you be a little bit more specific about the neoadjuvant and adjuvant regimens given? How were the results affected by the use of regional chemotherapy and are these patients a subset of those reported by your group in a recent issue of *The New England Journal of Medicine*?

Dr. Allen: One of the greatest weaknesses of this study, being retrospective in nature, is the heterogeneity of the chemotherapy given to these patients. Many of these patients received their chemotherapy at outside hospitals, and thus the agents used and the dosing regimens were not standardized.

If we look at patients who received adjuvant therapy after resection and compare that factor between patients who did and those who did not receive neoadjuvant chemotherapy, we found no differences between groups. In addition, the method of delivery, whether systemic or pump, was also similar between groups.

Dr. Bilchik: Is this a subgroup of the patients described by your group in a report in *The New England Journal of Medicine*?

Dr. Allen: None, or at the most one or two patients, would have overlapped based on the timing of the two studies and the very small numbers of patients within this study who received pump chemotherapy.

Dr. G. Porter (Halifax, Nova Scotia, Canada): Along the same lines, obviously there was some sort of a decision-making process in terms of who received neoadjuvant chemotherapy and who did not. I presume some of it, or much of it, was medical oncology related. Your institution has published very useful clinical risk scores for post-treatment colorectal cancer. I wonder whether you applied that to these patients. Specifically, do both groups have the same clinical risk scores?

Dr. Allen: Yes, and actually we compared all seven of the risk factors that we have found to be associated with survival. When we first looked at these data, we had anticipated that one or more of these factors would fall out, but none did. There are certainly many factors outside of those listed that play a role in the decision-making process for both patient and physician. Much of this is probably patient driven. Some of the patients who come to the clinic do not want to receive chemotherapy and want to have their lesions removed as soon as possible; other patients would prefer to wait before undergoing another operation, and these patients are given chemotherapy. Dr. Blumgart, the chief of the service, prefers to treat persons who have many small lesions with neoadjuvant chemotherapy. The aspect that we were most encouraged by was that none of the patients who were treated with neoadjuvant chemotherapy progressed to a point where they were radiographically unresectable, and the percentage of patients who were resectable was similar in the two groups.

Dr. J.P. Hoffman (Philadelphia, PA): I want to dig a little deeper into this analysis. First, I did not see any statistical significance or numbers on the curves. Are they statistically different? Second, are we looking at the sort of thing we see with patients who respond to chemotherapy vs. those who do not respond to chemotherapy? Essentially is this a responder vs. nonresponder type of conclusion? Third, for those who did have a response before resection with the neoadjuvant therapy, was that same therapy then continued and was it continued longer in those patients because their cells were actually known to be responsive to that chemotherapy?

Dr. Allen: To address the first question, as displayed in Table 2, patients who responded to neoadjuvant chemotherapy had significantly improved survival when compared to patients who did not receive neoadjuvant chemotherapy. We believe that this is just a marker of a biologically better behaving tumor that responded to chemotherapy. This is similar to what has been demonstrated in other forms of malignancy. Patients whose tumors respond to chemotherapy tend to do better. The third question, with regard to whether people who responded to chemotherapy received chemotherapy for a longer period of time, I am not sure exactly whether patients who responded had a longer interval before their resections. I think that they probably did. One patient whose disease remained stable during chemotherapy was followed for a period of 24 months, so you might imagine that this patient is going to do well after resection. Thus I think that those patients probably were indeed followed longer.

Invited Discussion—Expert Commentator

Robin S. McLeod, M.D. (Toronto, Ontario, Canada): The study from Memorial Sloan-Kettering Cancer Center is interesting and important because decisions regarding treatment of synchronous liver metastases are often difficult. Some of the decisions to be made are whether the patient should receive radiation or neoadjuvant therapy preoperatively or whether one should simply go ahead and resect the primary lesion first. Then it is often difficult to know whether and when to resect the liver metastases: at the time of the resection of the primary lesion, a short time after, or wait and see if the liver tumor has progressed. This study provides some insight into what might be another prognostic variable that might be helpful in the selection of patients. However, it does have its limits given that it is a retrospective review and although an enviable series, it still has relatively small numbers of patients in each of the three groups—particularly the "Rx-No Progression" group where there were only 16 patients. Thus one must be cautious in accepting the results. Nevertheless, these investigators do show a significant difference in outcome, after a median of 30 months, in the Rx-No Progression group. This is interesting because it is controversial whether the response of the primary tumor to neoadjuvant treatment predicts outcome in patients. In fact, in another report from Memorial Sloan-Kettering, the response to neoadjuvant therapy was not shown to predict outcome in patients with rectal cancer. Thus, these data are interesting and may indicate a subgroup of patients with a good prognosis. Certainly, as the authors have pointed out, it would be incorrect at this time to suggest that the neoadjuvant therapy has led to the improvement in outcome.

Diagnostic Accuracy of Endoscopic Ultrasound–Guided Fine-Needle Aspiration in Patients With Presumed Pancreatic Cancer

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Endoscopic ultrasound (EUS)–guided fine-needle aspiration (FNA) of the pancreas allows the diagnosis of pancreatic cancer to be established without exploratory surgery. We reviewed our recent experience with EUS-FNA in patients with presumed pancreatic cancer and report the diagnostic accuracy and complications of this procedure. Data were reviewed from all patients who presented with CT evidence of a pancreatic mass or a malignant biliary stricture and underwent EUS-FNA at our institution between November 1, 1999, and October 1, 2001. Based on the findings of contrast-enhanced, multislice CT scanning, patients were categorized as having resectable, locally advanced, or metastatic disease. EUS-FNA was performed in 233 patients. A final diagnosis of cancer was established in 216 patients (93%), 15 patients (6%) were found to have benign disease, and the final diagnosis remains unknown in two patients (1%). The sensitivity, specificity, and accuracy of EUS-FNA for diagnosis of a pancreatic malignancy were 91%, 100%, and 92%, respectively. For the 216 patients subsequently proven to have cancer, the results of EUS-FNA were diagnostic in 197 (91%); 96 (90%) of 107 patients with resectable disease, 62 (97%) of 64 with locally advanced disease, and 39 (87%) of 45 with metastatic disease. Four patients (2%) developed a clinically apparent complication that required hospital admission, including two patients who required surgery for duodenal perforation. There were no EUS-related deaths. We conclude that EUS-FNA can safely and accurately establish a cytologic diagnosis in patients with both early-stage and advanced pancreatic cancer. This enables consideration of all treatment options including protocol-based therapy (J GASTROINTEST SURG 2003;7:118–128.)

KEY WORDS: Endoscopic ultrasonography, fine-needle aspiration, pancreatic cancer

The pancreas has a complex regional anatomy, making it difficult to obtain a cytologic diagnosis of malignancy without exploratory surgery. CT-guided percutaneous fine-needle aspiration (FNA) has been used for biopsy of the pancreas at many institutions. However, CT-guided FNA has been reported by some to be associated with a risk of peritoneal dissemination of cancer cells and has a false negative rate of 20%.[1,2] Endoscopic retrograde cholangiopancreatography (ERCP) brush cytology has an even higher false negative rate of at least 30%.[3] In the 1980s, endoscopic ultrasonography was developed to overcome the limitations of transabdominal ultrasonography and CT scanning when imaging the pancreas.[4] Endoscopic ultrasound (EUS)–guided (EUS-FNA) was first reported in 1992.[5] The specificity of EUS in detecting malignancies increases with the use of EUS-FNA.[4] EUS-FNA overcomes the limitations of

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (oral presentation).

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Supported by The Lockton Fund for Pancreatic Cancer Research at The University of Texas M. D. Anderson Cancer Center.

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Published by Elsevier Science Inc.
1091-255X/03/$—see front matter
PII: S1091-255X(02)00150-6

CT-guided FNA in sampling small tumors. Although EUS-FNA has been shown to have a relatively low rate of complications,[4,6] its role as a diagnostic and staging procedure has generated considerable debate.

We examined the accuracy and complications of EUS-FNA in the subpopulation of patients whose clinical presentation and radiographic imaging were suggestive of a malignant tumor of the pancreas or the periampullary region. The ability to diagnose pancreatic cancer with EUS-FNA as opposed to exploratory surgery allows pancreatic cancer to be treated like most other solid tumors—that is, the diagnostic phase is separated from the treatment phase. With such a treatment algorithm, patients can be counseled about the potential benefits of referral to a center that performs a high volume of pancreatic resections and of entry into clinical trials that use novel therapies.

METHODS

Data from all patients who presented with presumed pancreatic or periampullary cancer and who underwent EUS-FNA at our institution between November 1, 1999, and October 1, 2001, were retrieved from a prospective pancreatic tumor database. Because we sought to examine the utility of EUS-FNA in establishing a cytologic diagnosis of carcinoma, we restricted our study population to patients with a presumed pancreatic or periampullary malignancy. We therefore included only patients who had a low-density mass in the pancreas on CT images or a malignant biliary stricture on percutaneous or endoscopic cholangiography. Patients without a mass on CT who did not have an abrupt (malignant-appearing) stricture of the intrapancreatic portion of the common bile duct were excluded. Patients with ampullary tumors that were seen endoscopically and those with obvious cystic lesions of the pancreas were also excluded. In patients with malignant cells identified on FNA, the final diagnosis was confirmed by histopathologic examination of surgical specimens and/or patient follow-up. In patients with FNA results that were inconclusive for malignancy or with no malignant cells identified, the final diagnosis was established by repeat biopsy (percutaneous or intraoperative) or patient follow-up.

All patients were staged by high-quality, contrast-enhanced, multislice CT scans prior to EUS. For purposes of this study, patients were categorized as having resectable, locally advanced, or metastatic disease based on CT images, as previously described.[7] Potentially resectable disease was defined by the following CT criteria: (1) absence of extrapancreatic disease; (2) no evidence of tumor extension to the superior mesenteric artery or celiac axis, as defined by the presence of a normal fat plane between the tumor and these arterial structures; and (3) a patent superior mesenteric vein–portal vein confluence. The third criterion is based on the assumption that resection and reconstruction of the superior mesenteric vein or superior mesenteric vein–portal vein confluence are possible. Patients with extrapancreatic metastatic disease underwent EUS-FNA of the pancreas (and therefore are included in this report) only if the distant metastases were too small to biopsy.

EUS was performed under monitored anesthesia care or, rarely, under conscious sedation with an additional topical anesthetic. All procedures included an upper gastrointestinal endoscopic examination with the use of a Pentax EG-2940 video upper endoscope (Pentax Precision Instrument Corp., Orangeburg, NY). In some cases, a radial-scanning Olympus GF-UM20 echo endoscope (Olympus America, Inc., Lake Success, NY) was used to obtain additional anatomic detail. All EUS-FNA procedures were performed with a Pentax FG-36UX linear-array echo endoscope equipped with a needle for FNA biopsy. Ultrasound frequencies of 7.5 or 12 MHz were used. FNA was performed with multiple passes of a 22-gauge Wilson-Cook needle (Wilson-Cook, Winston-Salem, NC) via a transduodenal or, occasionally, a transgastric route. The mean number of passes was 2.8 (range 1 to 6), with samples obtained from several different areas of the lesion.

The aspirated tissue was smeared on slides and either air dried and stained by the Diff-Quik method or immediately fixed in modified Carnoy's solution and stained by the Papanicolaou method. All slides were immediately evaluated by a cytopathologist. Results of the FNA biopsies were classified as "no malignant cells identified" (benign or nondiagnostic specimen), "inconclusive for malignancy" (atypia or suspicious for carcinoma), or "malignant cells identified" (positive for carcinoma)[8–11] (Fig. 1). A benign aspirate contained pancreatic ductal and/or acinar cells without atypia. In a nondiagnostic specimen, the cytologic material contained benign ductal and/or acinar cells but was relatively paucicellular, reflecting inadequate sampling. Cases of atypia showed some of the cytologic features seen in carcinoma (described below); however, either the number of atypical cells was too low to support a diagnosis of malignancy or sufficient features for malignancy were not present and a reactive process thus could not be excluded. Cases called suspicious for carcinoma showed greater atypia or a greater number of atypical cells, but the changes were not enough for an un-

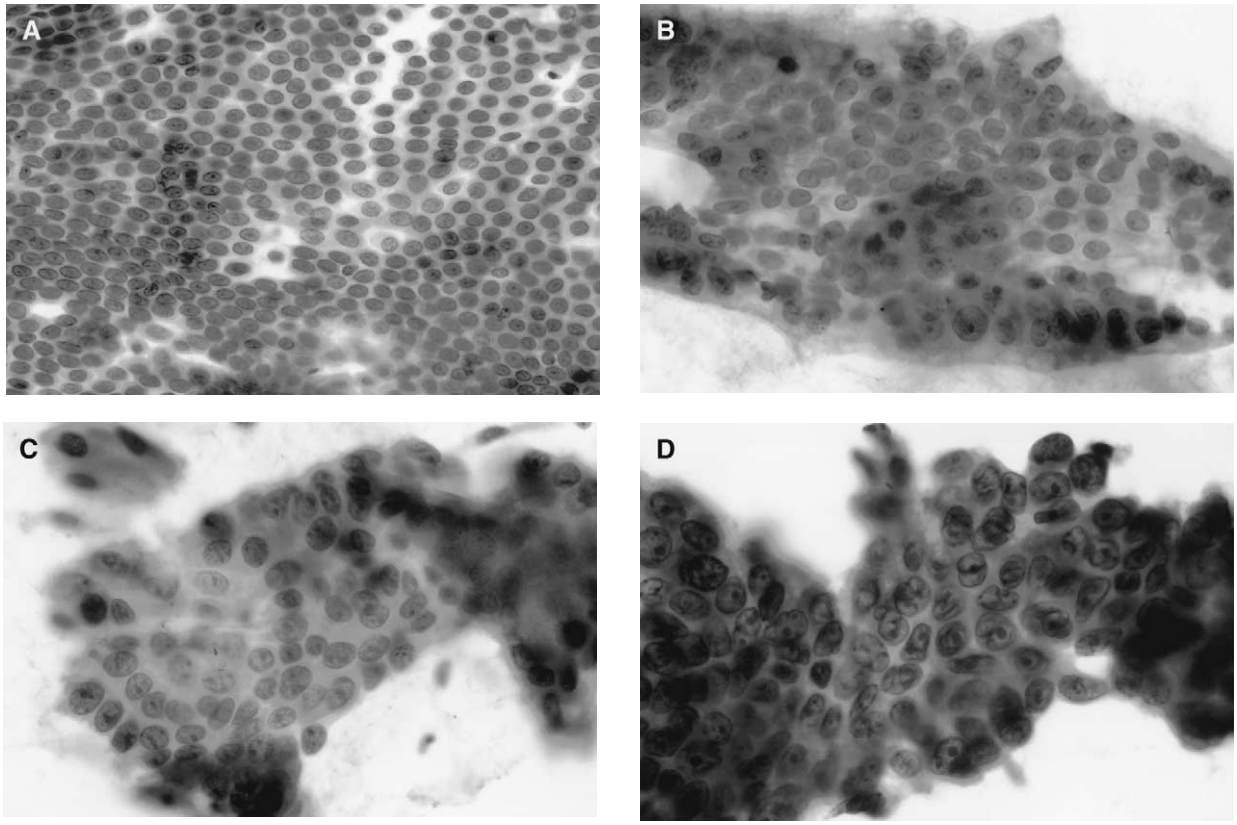


Fig. 1. Representative examples of cytologic findings. **A,** Benign ductal epithelium. Cells are evenly spaced and uniform in nuclear size and chromatin content/distribution. No nuclear irregularity, overlapping, or crowding is seen. **B,** Atypia. Cells show increased nuclear size, with anisonucleosis seen focally. There is a slight increase in cell crowding. However, changes are subtle, and there is little to no nuclear irregularity. **C,** Suspicious for carcinoma. Changes have increased in degree from the example of atypia. There is less even spacing of nuclei, nuclear crowding is greater, and the three-dimensional aspect has increased. Nuclear irregularity is more notable. Particularly if the quantity of this kind of cell group is low in the aspirate, a conclusive diagnosis of malignancy cannot be rendered. **D,** Adenocarcinoma. Cells show markedly irregular nuclear contours, hyperchromasia, chromatin clumping, and nucleolar prominence. Nuclear crowding is extreme, and three-dimensional aspect dominates this cell group.

equivocal diagnosis of malignancy. FNA results positive for carcinoma included some, though not necessarily all, of the following features: nuclear enlargement with anisonucleosis, increased nucleus/cytoplasm ratios, nuclear crowding, irregularly distributed nuclei, irregular nuclear contours, chromatin clumping, hyperchromasia, and an increased number of mitotic figures. The diagnosis of neuroendocrine carcinoma was confirmed by immunohistochemical staining.

RESULTS

EUS-FNA was performed in 233 patients with presumed pancreatic or periampullary carcinoma. A total of 222 patients underwent one attempt at EUS-FNA, and a second procedure was performed in 11 patients. Malignant cells were identified in 197 (85%), results were inconclusive for malignancy in 12 (5%; suspicious in 7 and atypical in 5), and no malignant cells were identified in 24 (10%; nondiagnostic in 16 and benign in 8) (Table 1 and Fig. 2). A final diagnosis of carcinoma was established in 216 (93%) of the 233 patients (see Table 1 and Fig. 3), a benign diagnosis was established in 15 patients (6%), and two patients (1%) were lost to follow-up without confirmation of their final diagnoses.

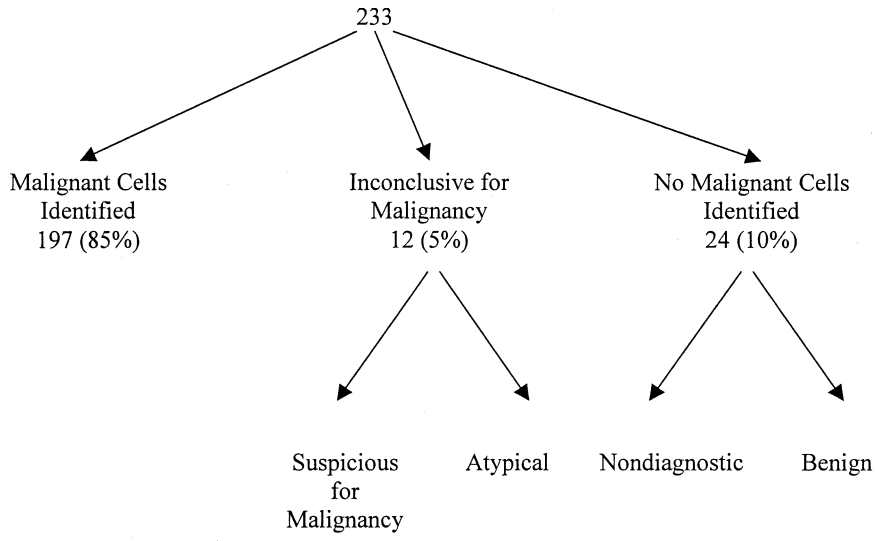
The FNA results were diagnostic of malignancy (true positive) in 197 (91%) of the 216 patients with a final diagnosis of carcinoma (Table 2 and Fig. 3). The FNA results were diagnostic of carcinoma in 96 (90%) of 107 patients with resectable disease, in 62 (97%) of 64 with locally advanced disease, and in 39 (87%) of 45 with metastatic disease. Of the 197 pa-

Table 1. Comparison of EUS-FNA results and final diagnoses in 233 patients

	No. of patients by EUS-FNA result			
Final diagnosis	**Malignant cells identified**	**Inconclusive for malignancy**	**No malignant cells identified**	**Total**
Carcinoma	197	10	9	216 (93%)
Benign	0	1	14	15 (6%)
Lost to follow-up	0	1	1	2 (1%)
Total	197 (85%)	12 (5%)	24 (10%)	233

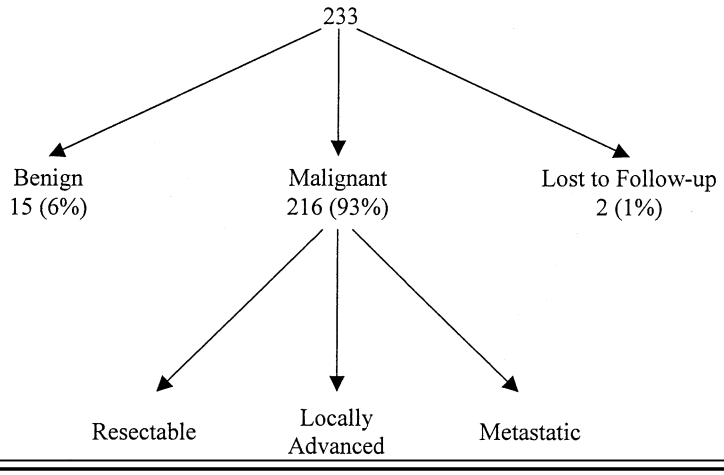
tients with malignant cells identified by FNA, 178 had pancreatic adenocarcinomas (88 resectable, 61 locally advanced, and 29 metastatic), one had a mucinous cystadenocarcinoma (a small, focal cystic tumor not appreciated as such on preoperative imaging), 13 had neuroendocrine carcinomas (7 resectable, 1 locally advanced, and 5 metastatic), one had an adenocarcinoma of the intrapancreatic portion of the common bile duct (metastatic), and four had metastases to the pancreas (from renal cell carcinoma, colon adenocarcinoma, osteogenic sarcoma, and small cell carcinoma of the lung).

The FNA results were inconclusive or not diagnostic of malignancy (false negative) in 19 patients (9%) with carcinoma (Table 3). In 11 (10%) of the 107 patients with resectable primary tumors, malignancy could not be confirmed by FNA; four had cytologic findings suspicious of malignancy, three had atypical cytologic findings, and four had nondiagnostic cytologic findings. In all 11 of these patients, the histopathologic diagnosis was established at laparotomy. The final diagnoses were adenocarcinoma of the pancreas in six patients, mucinous cystadenocarcinoma in one, focal intraductal papillary mucinous neoplasm in



Final Diagnosis	**7**	**5**	**16**	**8**	**Total (%)**
No. Malignant (%)	6 (86)	4 (80)	9 (56)	0	**19 (53)**
No. Benign (%)	0	1 (20)	6 (38)	8 (100)	**15 (42)**
Lost to follow-up	1 (14)	0	1 (6)	0	**2 (5)**

Fig. 2. EUS-FNA results in 233 patients.



EUS-FNA Result	107	64	45	Total (%)
No. True-Positive (%)	96 (90)	62 (97)	39 (87)	**197 (91)**
No. False-Negative (%)	11 (10)	2 (3)	6 (13)	**19 (9)**

Fig. 3. Final diagnoses in 233 patients.

one, neuroendocrine carcinoma of the pancreas in one, and adenocarcinoma of the intrapancreatic portion of the common bile duct in two.

Two (3%) of the 64 patients with locally advanced disease had false negative FNA results (see Table 3); both had nondiagnostic cytology. The two patients had adenocarcinoma of the pancreas. In one patient, cytologic evaluation of bile duct brushings obtained endoscopically subsequently established a diagnosis of carcinoma. The other patient eventually developed ascites, the cytologic evaluation of which was positive for adenocarcinoma.

Six (13%) of 45 patients with metastatic disease had false negative FNA results (see Table 3); one had cytology that was suspicious for malignancy, two had atypical cytology, and three had nondiagnostic cytology. The final diagnoses (4 adenocarcinomas of the pancreas and 2 adenocarcinomas of the intrapancreatic portion of the common bile duct) were established by CT-guided FNA in two, US-guided FNA in one, bile duct brushing at the time of ERCP in one, EUS-FNA of a liver metastasis in one, and intraoperative biopsy in one.

Among the 19 patients with false negative FNA results, six (32%) had a biliary stricture without a pancreatic mass on CT imaging. In contrast, among the 197 patients with true positive FNA results, only 14 (7%) had a biliary stricture without a pancreatic mass (1 additional patient had a noncontrast CT secondary to renal failure; the presence or absence of a mass could not be confirmed).

The final diagnosis could not be confirmed in two patients with nonmetastatic disease because of the lack of follow-up. One had suspicious findings on FNA, and the other had nondiagnostic findings. Both elected to have their follow-up at other institutions, so their final diagnoses could not be confirmed. One patient subsequently died, but a postmortem examination was not performed.

The final diagnosis was benign in 15 (6%) of the 233 patients (see Fig. 3). The FNA results were atypical in one, nondiagnostic in six, and benign in eight of these patients; there were no false positive FNA results. A benign diagnosis was established after surgery and pathologic evaluation of resected specimens or intraoperative biopsies in 5 of the 15 patients including the one patient with atypical cytologic findings. This patient, who presented with obstructive jaundice, had a common bile duct stricture identified by ERCP. Cytologic evaluation of bile duct material from brushings obtained at the time of ERCP and two EUS-FNA procedures failed to diagnose a malignancy over the course of 16 months. During this time, there was no evidence of local tumor or meta-

Table 2. Final diagnoses in 197 patients with true positive FNA results

Final diagnosis	No. of patients by stage of disease			
	Resectable	Locally advanced	Metastatic	Total
Adenocarcinoma of pancreas	88	61	29	178
Mucinous cystadenocarcinoma	1	0	0	1
Neuroendocrine carcinoma	7	1	5	13
Adenocarcinoma of common bile duct	0	0	1	1
Metastasis to pancreas	0	0	4	4
Total malignancies	96	62	39	197

static disease by radiographic studies. Ultimately the patient underwent a hepaticojejunostomy for persistent symptoms; the underlying etiology of her disease was believed to be consistent with autoimmune pancreatitis. The mean and median follow-ups for the remaining 10 patients were 14 months and 11.5 months, respectively (range 2 to 31 months). Eight of these 10 patients have been evaluated at our institution within the past 9 months; all 10 are still under clinical and radiographic surveillance. Five patients have less than 12 months of follow-up; therefore malignant diagnoses have not been completely excluded. Eight of the 10 patients have a history of conditions predisposing them to pancreatitis or biliary strictures, including heavy alcohol use, gallstone pancreatitis, open or laparoscopic cholecystectomy for symptomatic cholelithiasis, cholangitis, or steroid-responsive autoimmune pancreatitis.

Complications of EUS-FNA requiring hospital admission occurred in 4 (2%) of 233 patients. Two patients had duodenal perforations, one of which was recognized before completion of the FNA and one after completion of the FNA. Although the former patient did not undergo FNA because the perforation was immediately appreciated, FNA would have been attempted in the absence of this complication, and therefore the patient is included in this study (diagnosis of adenocarcinoma was confirmed by subsequent CT-guided biopsy). Both patients with perforations required laparotomy for repair. Neither had evidence of duodenal obstruction on CT imaging or during endoscopy. During laparotomy, one patient was found to have liver metastases that had not been seen on CT imaging. The second patient underwent primary repair of the perforation (at the junction of the first and second portions of the duodenum) followed by neoadjuvant chemoradiation and subsequent pancreaticoduodenectomy. A third patient experienced transient abdominal pain without an elevation in the amylase level after EUS-FNA; this required overnight hospitalization. A fourth patient developed pancreatitis after EUS-FNA and required overnight hospitalization. There were no EUS-related deaths.

Fifty-five patients who underwent EUS-FNA during the period of this study were excluded from this analysis; 18 underwent EUS-FNA for extrapancreatic masses or lymph nodes but without an obvious pancreatic mass or biliary stricture, 22 had pancreatic masses that were clearly cystic in nature, nine had ampullary or duodenal tumors that were identified and biopsied endoscopically, in addition to the EUS-FNA, four had a known diagnosis of cancer and were suspected of having a recurrence (diagnoses

Table 3. Final diagnoses in 19 patients with false negative FNA results

Final diagnosis	No. of patients by stage of disease			
	Resectable	Locally advanced	Metastatic	Total
Adenocarcinoma of pancreas	6	2	4	12
Mucinous cystadenocarcinoma	1	0	0	1
Focal IPMN	1	0	0	1
Neuroendocrine carcinoma	1	0	0	1
Adenocarcinoma of common bile duct	2	0	2	4
Total malignancies	11	2	6	19

IPMN = intraductal papillary mucinous neoplasm of the pancreas.

were gastrointestinal stromal tumor, lymphoma, metastatic melanoma, and metastatic renal cell carcinoma), and two were referred only for EUS-FNA and did not undergo formal evaluation by either the medical or surgical oncology service.

DISCUSSION

In contrast to other reports of EUS-FNA of the pancreas, we confined our study to patients whose clinical presentation and radiographic imaging were suggestive of a neoplasm of the pancreatic head or periampullary region. Our rationale was twofold. First, EUS-FNA remains controversial when used as a diagnostic procedure in patients whose clinical presentation and radiographic findings pose a diagnostic dilemma (i.e., uncertainty about the likelihood of neoplasm vs. pancreatitis).[12] In such patients a negative FNA result does not exclude the possibility of an underlying malignancy, and may complicate treatment disposition, especially if high-quality imaging is not completed prior to FNA. Therefore such patients were not included in this study. Second, a tissue diagnosis of carcinoma is necessary to separate the diagnostic phase from the treatment phase in patients with pancreatic and periampullary malignancies, as is done with all other solid tumors. This separation facilitates the referral of patients to high-volume centers and increases the number of patients who are eligible for protocol-based therapy, especially those that use systemic therapy or chemoradiation prior to surgery.

Previous investigators have suggested that patients whose CT scans demonstrate a pancreatic mass should not be subjected to EUS-FNA if the mass is resectable.[13] Instead such patients should go directly to surgery.[14] We agree with the general philosophy that in the absence of a plan for preoperative chemotherapy or chemoradiation, radiographically resectable pancreatic neoplasms do not require a preoperative tissue diagnosis. Thus the routine use of EUS-FNA for resectable tumors may not seem justified. However, most pancreatic resections are not performed at high-volume referral centers. Indeed, a review of 10,530 Medicare patients undergoing pancreatic resection nationwide revealed that 59% of these patients underwent[15] pancreatic resection at hospitals performing five or fewer such procedures per year. Operative mortality rates were inversely correlated with volume; the mortality rate was 16.3% at centers where less than one pancreatic resection per year is performed, 14.6% at centers where one or two per year are performed, and 11.0% at those hospitals where three to five pancreatic resections per year are performed. Given the high mortality rates seen at the low-volume centers where most patients undergo pancreatic resection, many physicians (including some surgeons) are not willing to proceed with a pancreaticoduodenectomy in the absence of a tissue diagnosis of malignancy. Despite improvements in radiographic imaging, such diagnostic uncertainty often results in therapeutic indecision. In turn, such indecision often leads to exploratory surgery, at which time surgeons frequently attempt intraoperative biopsy (leading to unnecessary complications) or incorrectly judge a primary pancreatic tumor to be resectable or unresectable.

In contrast to the diagnostic and staging evaluation of other solid tumors, in which the diagnostic phase is distinct from the treatment phase, diagnosis and treatment are often a continuum with pancreatic and periampullary malignancies. Patients rapidly transition from excellent health to painless jaundice to the operating room; their first chance to seek a second opinion or explore options for protocol-based therapy is often after they have already undergone an unsuccessful attempt at surgical resection or while they are recovering from complications of an ill-advised intraoperative pancreatic biopsy. The advent of EUS-FNA allows pancreatic cancer to be treated like other solid tumors; that is, the diagnostic phase is separate from the treatment phase.[12] Patients with suspected pancreatic or periampullary cancer can be accurately staged with contemporary CT, biliary obstruction can be relieved with endobiliary decompression, and the diagnosis can be established endoscopically with EUS-FNA. Patients can then be counseled about available treatment options, including protocol-based therapy, and about the potential benefits of referral to a regional center with expertise in pancreatic surgery.[15–17]

Our study, which represents the largest single-center experience with EUS-FNA of the pancreas, was designed to determine the diagnostic accuracy of EUS-FNA in patients whose clinical presentation and radiographic imaging results strongly suggested the presence of pancreatic or periampullary cancer. Other studies have occasionally combined true positive FNA biopsies with results suspicious for malignancy in reporting sensitivity and accuracy. In the present study, both sensitivity and accuracy were calculated based only on patients with malignant cells identified on FNA; patients with FNA biopsies suspicious for malignancy were not considered true positive. In our study population, EUS-FNA had a sensitivity of 91% and an accuracy of 92% for diagnosing a pancreatic or periampullary malignancy. It is important to note that for resectable tumors, the sensitivity of EUS-FNA was 90% (96 of 107). Furthermore,

even when there was no visible mass on CT scans, 70% of the tumors (14 of 20) were correctly diagnosed as malignant by EUS-FNA.

Consistent with previous investigators, we reported no false positive results, yielding a positive predictive value of 100% for pancreatic or periampullary malignancies (Table 4). Voss et al.[6] reported one patient with false positive findings whose FNA cytology showed acinar involution with atypical cells and trabecular patterns; after pancreaticoduodenectomy, the specimen demonstrated histologic changes consistent with pancreatitis with no evidence of malignancy. The specificity reported in our study was also 100%. Fourteen of the 15 patients with benign diagnoses had no malignant cells identified on cytologic examinations. The remaining patient had atypical cells on EUS-FNA; on reevaluation, this cytologic specimen showed rare cell groups of probable mesothelial origin demonstrating significant nuclear irregularity. Observation of these cell groups led to a report of atypia. Such benign inflammatory changes may be interpreted as atypical but will rarely fulfill the criteria for classification as suspicious for malignancy.

In the present study, 19 (9%) of the 216 patients with cancer had false negative findings on EUS-FNA. Therefore the negative predictive value in our study was 44%. The value of a negative EUS-FNA finding has limited clinical impact on decision-making in this select population at such high risk for having cancer. As previously reported, a negative EUS-FNA result cannot alone determine subsequent management.[19] Patients whose clinical history and radiographic images suggest a malignancy of the pancreas or periampullary region should not be dismissed as having benign disease because of a negative FNA result. Our data clearly demonstrate that false negative FNA results will occur in approximately 9% of such patients. It is important to note that one third of such patients had a biliary stricture without a pancreatic mass, implying that small tumors, especially those arising in the bile duct, pose the greatest difficulty for successful EUS-FNA.

The reported overall complication rate of EUS-FNA ranges from 1% to 5% (see Table 4). The spectrum of complications reported in the literature includes infection of cystic lesions,[20,23] duodenal or peripancreatic hematomas,[6] abdominal pain,[6] fever,[6,22] prolonged sedation after anesthesia,[22] and pancreatitis.[13] Infectious complications rarely occur with FNA of solid lesions (1% to 5%), in contrast to cystic lesions (14%).[23] Unlike with ERCP, the risk of pancreatitis is quite low with EUS-FNA.[24] Although there remains concern over the peritoneal spread of malignant cells after CT-guided FNA, that technique has a complication rate of less than 1%[25]; the higher complication rate with EUS-FNA is secondary to the added risk of endoscopy.[18] The complication rate in our series was 2%. Two of the four complications were duodenal perforations. Both patients underwent immediate laparotomy. Duodenal obstruction was not a contributing factor in either patient; to what extent the primary tumor limited duodenal compliance cannot be quantified.

Table 4. Comparison of studies of EUS-FNA of pancreatic masses

Reference	Year	No. of patients	Sensitivity (%)	Specificity (%)	Predictive value		Accuracy (%)	Complications (%)
					Positive (%)	Negative (%)		
Chang [18]	1995	20	91	100	—	—	94	—
Cahn et al.[19]	1996	50	88*	—	—	—	—	—
Bhutani et al.[20]	1997	47	64	100	100	16	—	2†
Williams et al.[21]	1999	144	72	100	100	38	76	<1†
Voss et al.[6]	2000	90	75	88	98	26	74	5‡
Powis and Chang[4]	2000	164	83	90	100	80	85	2§
Erickson et al.[22]	2000	95	88	—	—	—	—	1‖
Mortensen et al.[14]	2001	22	82	—	—	—	—	5¶
Present study	2002	233	91	100	100	44	92	2

*Sensitivity in diagnosing pancreatic malignancies only.
†Infection of cystic lesion in one patient in each of these studies.
‡Small peripancreatic (3 patients) or duodenal hematomas (1 patient); abdominal pain and fever without hyperamylasmia (1 patient).
§Perforation (1 patient), bleeding (1 patient), fever (1 patient).
‖Prolonged sedation after procedure (1 patient).
¶Acute pancreatitis (1 patient).

CONCLUSION

EUS-FNA is a reliable technique for establishing a diagnosis of cancer in patients with presumed pancreatic or periampullary malignancy. We report a very high sensitivity, specificity, positive predictive value, and accuracy for EUS-FNA of the pancreas. EUS-FNA is a relatively safe procedure, with a complication rate of approximately 2%. When used in combination with standard imaging modalities (multislice CT) and, when necessary, endobiliary decompression, EUS-FNA enables patients with pancreatic cancer to be accurately staged and diagnosed without exploratory surgery. This allows pancreatic cancer to be treated like all other malignant solid tumors: the diagnosis is established preoperatively, giving patients and physicians time to carefully review all available treatment options, including protocol-based therapy.

We thank Melissa G. Burkett, Adrienne Denny, and Susan Hogue for assistance in the preparation of this manuscript.

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Discussion

Dr. L.W. Way. (San Francisco, CA): I question a premise in your argument here. The value of the study, as you have identified it, is to help facilitate referral to a high-volume center where there are volume-outcome relationships. It seems to me, implicit in your argument, however, is that there is no volume-outcome relationship for EUS as a diagnostic tool. You performed 233 of these over a 2-year period, meaning that there were three or four or five cases a week being managed by a few people, which would make them exceedingly rehearsed in this, and I wonder whether the generalizations that you are making can be said to be typical of what can be possible in community practice from where you are going to get these referrals?

Dr. C.P. Raut: Yes, that is a reasonable concern. However, the gastroenterologists who performed these EUS procedures had only recently completed their fellowship training and had not been doing these for 10 or 15 years. We expect that EUS will become a regular part of the diagnostic algorithm for many solid tumor malignancies of the gastrointestinal tract and will, therefore, be incorporated into the training program for all gastroenterologists. As with any new procedure or device, demand will (to some extent) drive supply. Surgeons and medical oncologists should be encouraged to request EUS procedures.

Dr. M. Larvin (Derby, UK): One of the problems in a high-volume pancreatic cancer center is that patients often come with a biliary stent already in place. You did not state how many of the patients in this series had stents. It makes the EUS much more difficult in the experience of most practitioners.

Dr. Raut: I do not have those numbers, but there was a small percentage who had come to the institution prior to our evaluation, who had stents in place.

Dr. K.S. Kirkwood (San Francisco, CA): Did you find it to be difficult to perform EUS in the presence of a stent?

Dr. Raut: I cannot comment further on this issue.

Dr. C.J. Yeo (Baltimore, Maryland): Some of the results you have shown to us are pretty remarkable and certainly not comparable to those from other institutions. The lack of any false positive findings is a little bit unusual, because certainly there are false positive results in other studies. I would also caution about the issue of getting the information from EUS and using it to stage patients. I am a true fan of the M.D. Anderson Cancer Center and the preoperative chemoradiation program; I think it is very important, and the rationale for proceeding with these biopsies should be understood as such. But it is very important that we recognize, in general practice, where the preoperative chemoradiation program is not in place, that using EUS to obtain a tissue diagnosis is unnecessary, and you correctly pointed that out. It is important to underscore that. I have seen patients, and I am sure that others in the room have also, who have come without a malignant diagnosis but having had an EUS that has been nice enough to provide us with a TNM staging. Without proving cancer, the EUS calls a T4N2M0 lesion, and the patient has been told that he or she has incurable cancer without a confirmed diagnosis of malignancy. So certainly the general practice out there is not as sophisticated as you are reporting. Have you looked at your results from T stage and M stage? I recognize that many of your patients go through preoperative chemoradiation, but have you been able to correlate that in any way to response to chemoradiation therapy, to ultimate margin positivity at surgery, or otherwise to outcome after resection?

Dr. Raut: We did not use the EUS findings for staging. Our T staging was based on high-quality, multislice CT scans. However, I agree that our institution favors the use of neoadjuvant therapy, and all eligible patients are treated with protocols approved by the Institutional Review Board.

Dr. J.A. Drebin (St. Louis, MO): This is a great paper with really beautiful data, and as Dr. Yeo noted, M.D. Anderson is one of the few places that can provide it. I was impressed, though, that more than 90% of your patients were diagnosed on the basis of a CT scan—that is, you are only going to help narrow down that last 10%, and you have shown that the serious morbidity occurs in at least 2% of patients. If you could make the CT scan a little bit more accurate, the EUS would be unnecessary for those 200 or so patients in whom it contributes nothing. Have you looked at combinations of CT scan and PET scan, or are there any aspects of CT scanning that might improve your sensitivity?

Dr. Raut: No, we have not compared data from CT scans to those from PET scans. Part of the problem with our false negative results was that approximately one third of them occurred in patients who did not have CT evidence of a mass; one third of the patients actually had only a malignant stricture without a mass. It is in that subgroup where our sensitivity was lower (approximately 70%) because of a higher rate of false negative findings.

Invited Discussion—Expert Commentator

L. William Traverso, M.D. (Seattle, WA): Note that not all of these patients had "pancreatic cancer"; some had neuroendocrine, metastatic, or mucinous cystic tumors.

Most of the patients at M.D. Anderson Cancer Center were being worked up for preoperative chemoradiation. A histologic diagnosis was mandatory, and EUS and a 22-

gauge needle were used to obtain samples by FNA in 233 patients. Fully 15% of these patients had an inconclusive or benign result. In other words, 15% of the patients with highly suspected pancreatic cancer could not be confirmed by EUS to have pancreatic cancer. In my experience, approximately one third of the patients who have all the signs, symptoms, and imaging findings of pancreatic cancer cannot have the diagnosis confirmed with this EUS technology. That is my problem with EUS. It does not help me as often as I believe it should.

Readers of this article would benefit from knowing if there was a dedicated cytopathologist for these cases. Also, the experience of the person or persons performing EUS needs to be outlined. Lesions in the uncinate process are more challenging for the endoscopist, and an analysis of their data by number of passes made with the needle, tumor location in the uncinate process, or tumor size would help this report provide insight into the usefulness of this technology.

Of the 233 patients, 216 (93%) were later confirmed to have pancreatic malignancy by other means such as the surgical specimen or by clinical follow-up. In these 216 cases of confirmed cancer by other means, EUS-FNA was found to have obtained the diagnosis in 91% of these 216 patients (i.e., a true positive rate of 91%). More important from the clinician's viewpoint is that of 233 patients, 85% were confirmed to have a malignant diagnosis. That is still not too bad, particularly if the patient has avoided an open biopsy or the complications of CT-guided biopsy such as bleeding or seeding of the needle tract. There were complications with EUS, however; four patients developed a complication requiring admission—two with duodenal perforation and two with abdominal pain. Remember, if the diagnosis was confirmed, then the patient eventually underwent surgery months after neoadjuvant treatment. Most of my patients have surgery soon after EUS, and on several occasions I have found blood staining in the tissue planes connected to, but remote from, the biopsy site—down the root of the mesentery or around the gallbladder. I am suspicious that the security for needling the tumor from within the duodenum to prevent seeding of malignant cells is a false one. The tissue planes could be seeded by this technology. Also, my patients with observed blood staining of the tissue planes were asymptomatic. I am also concerned that there is talk of increasing the size of the needle to obtain a better specimen and achieve a higher yield of malignant cells with a technology that has a low negative predictive value. I would urge caution with this newer larger needle size in promoting more potential for blood spreading through the tissue planes, with even more potential for seeding. From this standpoint, I would urge the authors to include in their survival analysis the variable of the use of FNA by EUS (particularly the disease-free survival curves).

Lymphoplasmacytic Sclerosing Pancreatitis: Inflammatory Mimic of Pancreatic Carcinoma

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Lymphoplasmacytic sclerosing pancreatitis (LP) is a rare cause of benign mass lesions of the pancreas that can resemble adenocarcinoma. This study evaluates and classifies a series of patients with LP. Patients with benign pancreatic disease were identified from a prospective pancreatic database, and these cases were reviewed to identify patients with LP. Patients were subdivided into two groups: (1) classic LP, which included those patients who had all four of the characteristic histologic features of LP, including lymphoplasmacytic infiltration of the pancreas, interstitial fibrosis, periductal inflammation, and periphlebitis; and (2) intermediate LP, which included patients with at least two of these histologic findings. Patient demographics, pathologic and clinical features, and outcome were analyzed. From 1985 to 2001, a total of 1287 pancreatic resections were performed at our institution, of which 159 were for benign disease. Of these, 31 had pathologic features consistent with LP, and all of these patients had a presumed preoperative diagnosis of pancreatic carcinoma. Most of these patients presented with jaundice (n = 21) or abdominal pain (n = 7). In 29 of 31 patients, curative resection was possible. Of these, 28% (8/29) developed recurrence after resection: seven with jaundice and one with recurrent pancreatitis (median time to recurrence, 11 months; median follow up, 38 months). All patients with recurrent jaundice appeared to have biliary strictures at the time of direct cholangiography and no patient had malignancy. A review of the pathology reports identified 19 patients with classic LP and 12 patients with intermediate LP, and there was no difference between these two groups. LP is a rare cause of pancreatitis that is difficult to differentiate from carcinoma preoperatively. Patients with classic and intermediate LP appear to demonstrate a similar clinical behavior. Nearly one third of patients have a progressive course after resection, with 25% developing recurrent jaundice; thus close follow-up is mandatory for all patients. (J GASTROINTEST SURG 2003;7:129–139.)

KEY WORDS: Autoimmune pancreatitis, sclerosing pancreatitis

Lymphoplasmacytic sclerosing pancreatitis (LP) is a rare cause of benign pancreatic disease that can clinically resemble pancreatic adenocarcinoma. LP is also called autoimmune pancreatitis, sclerosing pancreatitis, and primary inflammatory pancreatitis. Characteristic features include diffuse lymphoplasmacytic infiltration of the pancreas, interstitial fibrosis, periductal inflammation, and periphlebitis. Although this process can result in diffuse inflammation of the pancreas, many patients with LP present with a mass lesion resembling pancreatic carcinoma and therefore undergo pancreatic resection.

LP has been associated with primary sclerosing cholangitis as well as other autoimmune diseases including ulcerative colitis, Sjögren's syndrome, retroperitoneal fibrosis, and Riedel's thyroiditis.[1–5] Recently the association between this entity and autoimmune disorders has been strengthened by the finding of increased levels of IgG4 in patients with LP.[6] Although the association of LP with autoimmune disease is clear, there are also many patients who present with LP who have no symptoms of autoimmune disease, either before or after the diagnosis of LP. Thus patients with this disease represent a wide clinical spectrum.

Because our knowledge of LP is based primarily on case reports, the risk of recurrence in patients undergoing resection for LP is not well defined. Clearly, if the primary etiology of LP is a diffuse au-

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (oral presentation).

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1091-255X/03/$—see front matter
PII: S1091-255X(02)00148-8

toimmune process, the incidence of recurrence of symptoms after pancreatic resection may be significant. We sought to evaluate the incidence of LP among patients referred to a tertiary cancer center to evaluate the rate of recurrence after resection. Other objectives included evaluation of presenting symptoms, preoperative imaging findings, intraoperative findings, and definition of a pathologic classification system to assess correlation of pathologic findings with outcome.

METHODS

Since 1985, all patients subjected to pancreatic resection at Memorial Sloan-Kettering Cancer Center (MSKCC) have been entered into a prospective pancreatic database. Patients undergoing pancreatic resection for benign disease were identified from this database. In addition, the pathology database was searched to locate patients with LP undergoing biopsy only.

Pathologic review of the original specimen was performed by one attending pathologist (D.K.) in all patients with benign pancreatic disease. All cases of acute or chronic pancreatitis were re-reviewed to evaluate them for the presence of any of the four pathologic features of LP—namely, dense lymphoplasmacytic infiltration of the pancreas, interstitial fibrosis, periductal inflammation, and periphlebitis (Fig. 1). Although lymphocytes and plasma cells were present within the pancreas in all cases, this finding alone is not very specific for LP. The pattern of lymphoplasmacytic infiltration within the pancreas was markedly different from the pattern seen in chronic pancreatitis, which typically consists of a sparse, nonperiductal inflammatory infiltrate.

Cases with findings consistent with LP were then divided into two types. First, those patients with all four of the typical findings of LP were designated

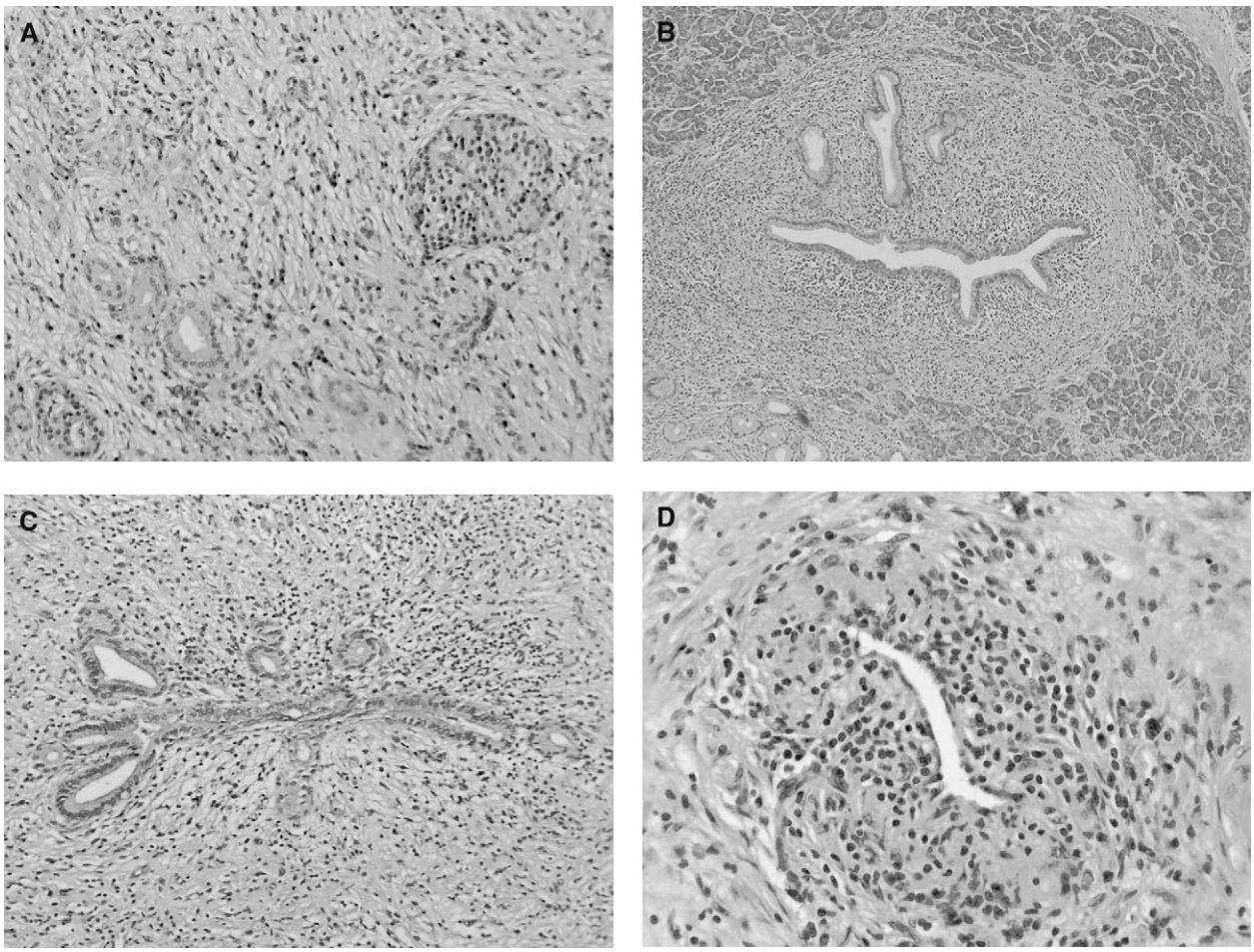

Fig. 1. Pathologic features of LP. **A,** Dense interstitial fibrosis with residual islets and intralobular ducts. **B,** Ductocentric inflammation, that is, inflammatory infiltrates concentrated around the duct with a relatively uninvolved acini. **C,** Inflammation of a pancreatic duct branch. **D,** Periphlebitis.

"classic" cases of LP. Second, patients with at least two findings suggestive of LP, but without all of the classic findings, were labeled as cases of "intermediate" LP.

Charts from all patients with classic or intermediate LP were reexamined to review the preoperative history, presenting symptoms, results of imaging studies, and operative findings. Imaging findings were recorded based on the original reading of the specific radiologic study. In addition, follow-up data regarding recurrence of symptoms after resection, such as jaundice or cholangitis, recurrent pancreatitis, or a new diagnosis of autoimmune disease, were obtained by contacting patients and/or referring physicians.

Statistical analysis was performed using the SPSS statistical software package (version 10.1, Chicago, IL). Comparisons between groups were tested using chi-square analysis or Student's *t* test as appropriate. Univariate analysis of DFS (DFS) was performed using log-rank analysis for categorical variables. Differences were considered significant at $P < 0.05$.

RESULTS

From 1985 to 2001, a total of 1287 pancreatic resections were performed at Memorial Sloan-Kettering Cancer Center. Of these patients, 159 (12%) had benign disease at pathologic evaluation. Pathologic re-review was performed in all of these cases. Of the 159 patients with benign disease, 57 had acute or chronic pancreatitis on subsequent pathologic evaluation. Of these resected patients, 29 had two or more of the pathologic features of LP. In addition, two patients were identified from the pathology database who were subjected to surgical exploration and found to have unresectable LP tumors; these 31 patients form the study group.

Patient Demographics and Clinical Presentation

There were 21 males and 10 females who had a median age of 62 years (range 17–87 years). The majority of these patients (n = 21) had jaundice, although most patients with jaundice also had associated abdominal pain (n = 9) and/or weight loss (n = 10). Fewer patients presented with abdominal pain alone (n = 8). Of the two remaining patients, one experienced weight loss and had an abdominal mass, whereas one patient had a 10-year history of abnormal liver function tests with prior normal endoscopic retrograde cholangiopancreatography (ERCP) and biopsies, until presenting with a pancreatic head mass on CT scan.

Six patients had a medical history that was suspicious for autoimmune disease (Table 1). Two patients had a history of a single episode of pancreatitis more than 8 years prior to the diagnosis of LP. No other patient had a history of pancreatitis. Only one patient had a history of jaundice that resolved spontaneously prior to presentation.

Preoperative Studies

Imaging tests included CT scans in 30 patients, MRI in 11, ERCP in 22, and ultrasonography in 19. Preoperative imaging suggested a pancreatic mass in most of these patients (19 [61%] of 31). A biliary stricture without an associated pancreatic abnormality was found in 7 (23%) of 31 patients, whereas three patients (10%) presented with an irregular pancreas with fullness in a portion of the gland. Two patients had no abnormal findings on preoperative axial imaging but presented with jaundice from a distal bile duct obstruction.

Preoperative fine-needle aspiration was performed in six patients, which revealed atypia, reactive ductal cells, and acinar cell carcinoma in one patient each. In the remaining three patients, fine-needle aspiration was inconclusive. All 31 patients had a presumed preoperative diagnosis of pancreatic carcinoma based on clinical presentation and/or radiologic findings.

Surgical Findings

Intraoperatively, 25 of 31 patients (81%) were found to have a pancreatic mass. Twenty-nine pa-

Table 1. Patients with a preoperative history of possible autoimmune disease

Patient	Sex	Age (yr)	Disorder	Recurrence after resection
1	F	51	Bell's palsy, Lyme disease, fibromyalgia	Yes
2	M	56	Interstitial nephritis	No
3	M	68	Parotiditis	No
4	F	64	Rheumatoid arthritis	No
5	F	41	Sjögren's syndrome, Graves' disease, adrenal insufficiency	Yes
6	M	63	Ulcerative colitis, granulomatous sialoadenitis of the submandibular gland, retrosternal goiter	No

Table 2. Features of patients with classic and intermediate LP

Pathology	n	Autoimmune disease*	Preoperative mass†	Intraoperative mass*	Recurrence*
Classic LP	19	4	15	16	4
Intermediate LP	12	2	3	9	4

*$P > 0.05$.
†$P = 0.01$, chi-square analysis.

tients were subjected to pancreatic resection, which included pancreaticoduodenectomy in 23 patients and distal pancreatectomy in four. In two patients, total pancreatectomy was performed because of a diffusely fibrotic pancreas.

At the time of operation, two patients were found to have unresectable disease due to superior mesenteric artery and portal vein encasement. Gastrojejunostomy was performed in one patient with unresectable disease due to gastric outlet obstruction. Both patients underwent intraoperative biopsies of the pancreas, which resulted in sufficient tissue to fully evaluate them for the pathologic features of LP.

Pathologic Classification: Patients With Classic vs. Intermediate Lymphoplasmacytic Pancreatitis

At the time of the original operation, only six patients were diagnosed pathologically with LP. Of the remaining 25 patients, 15 had been designated as cases of "chronic pancreatitis with fibrosis or sclerosis," eight were as designated "chronic pancreatitis," and two patients were reported to have "inflammation of the bile duct or pancreas." All six patients who were correctly diagnosed with LP underwent resection after 1998, attesting to our relatively recent understanding of LP as a pathologic and clinical entity.

Of the 31 patients with LP, 19 had classic LP, whereas 12 patients had intermediate LP. The clinical presentation, perioperative course, and outcome were similar between these two groups (Table 2). The only difference between the two groups was that a greater number of patients with classic LP presented with a pancreatic mass or fullness preoperatively (n = 15) compared to patients with intermediate LP (n = 3; $P = 0.01$, chi-square test). There was no difference in the number of patients with recurrent symptoms between classic and intermediate LP.

Outcome and Recurrence

Eight (28%) of 29 resected patients developed recurrence of symptoms: seven with jaundice and one with recurrent pancreatitis. The median time to recurrence was 11 months for resected patients (range

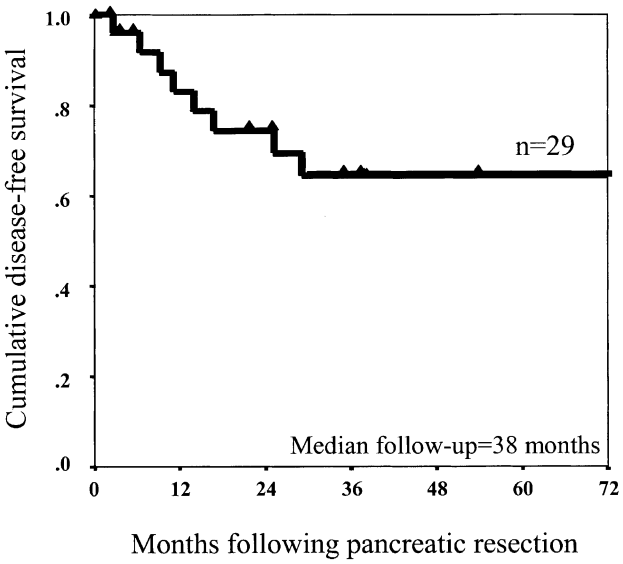

Fig. 2. Disease-free survival in 29 patients undergoing pancreatic resection for LP.

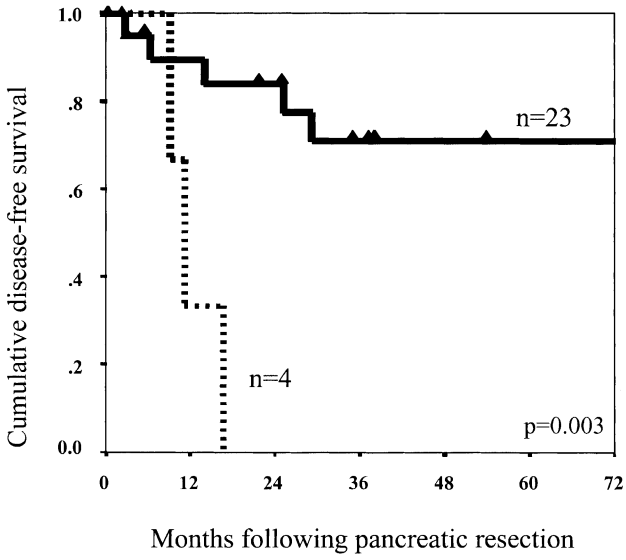

Fig. 3. Recurrence of symptoms according to type of resection: Distal pancreatectomy vs. pancreaticoduodenectomy. _____ Distal pancreatectomy; _____ pancreaticoduodenectomy

Table 3. Patients with recurrence of LP after resection

Patient	Initial operation	Time from initial surgery (mo)	Signs/symptoms of recurrence	Notes	Treatment
1	Distal	7	Retrobulbar mass	Lacrimal gland lymphoid infiltration on biopsy	Endoscopic biliary stent
		9	Jaundice	Single dominant stricture in pancreatic head	Bolus steroids
2	Distal	11	Jaundice/infiltrating pancreatic head mass	Multiple intrahepatic biliary strictures; diffuse pancreatic head enlargement	Percutaneous biliary drainage
		20	Small bowel mass	Similar histologic appearance as pancreatic lesion	Resection
3	Distal	17	Jaundice/abdominal pain	Single dominant stricture at biliary anastomosis; pancreatic head mass on CT	Endoscopic biliary stent Pain control
4	PD	29	Jaundice	Multiple intrahepatic biliary strictures	Percutaneous biliary drainage
5	PD	6	Jaundice	Multiple intrahepatic biliary strictures	Percutaneous biliary drainage
6	PD	3	Jaundice	Multiple intrahepatic biliary strictures	Endoscopic biliary stent
7	PD	25	Jaundice	Single dominant stricture at biliary anastomosis	Percutaneous biliary drainage
8	PD	14	Pancreatitis	Pancreatic duct strictures (×2) on MRCP	Symptomatic treatment

Distal = distal pancreatectomy, PD = pancreaticoduodenectomy.

3 to 29 months; median follow-up 38 months), with a two-year DFS of 74% (Fig. 2). The only factor associated with recurrence was the type of resection, with patients undergoing distal pancreatectomy having recurrences at a higher rate than patients undergoing pancreaticoduodenectomy (Fig. 3, $P = 0.003$, median DFS, 11.2 months, distal pancreatectomy; median DFS not reached, pancreaticoduodenectomy). Of note, there was no association of recurrence according to gender, autoimmune disease, presentation, preoperative mass, or intraoperative mass.

All patients with recurrent jaundice were evaluated with direct cholangiography, either ERCP or percutaneous transhepatic cholangiopancreatography (PTC). All seven patients appeared to have biliary strictures: three with a single dominant stricture in the extrahepatic biliary tree and four with multiple intrahepatic strictures (Table 3; Figs. 4 and 5). Two patients with recurrent jaundice were found to have an associated pancreatic mass on cross-sectional imaging. All patients with jaundice were managed with percutaneously or endoscopically placed stents. Attesting to our early experience with this disease, only two patients received bolus steroids at the time of re-

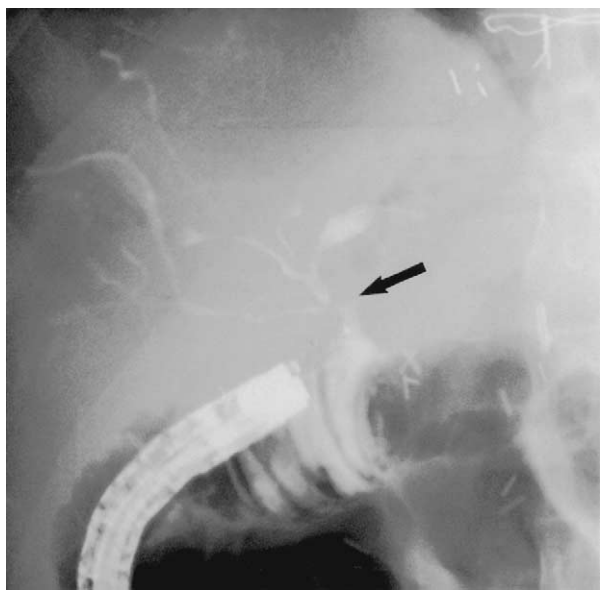

Fig. 4. Direct cholangiography in a patient with recurrent LP 7 months after pancreaticoduodenectomy demonstrates a single dominant biliary stricture in the common hepatic duct.

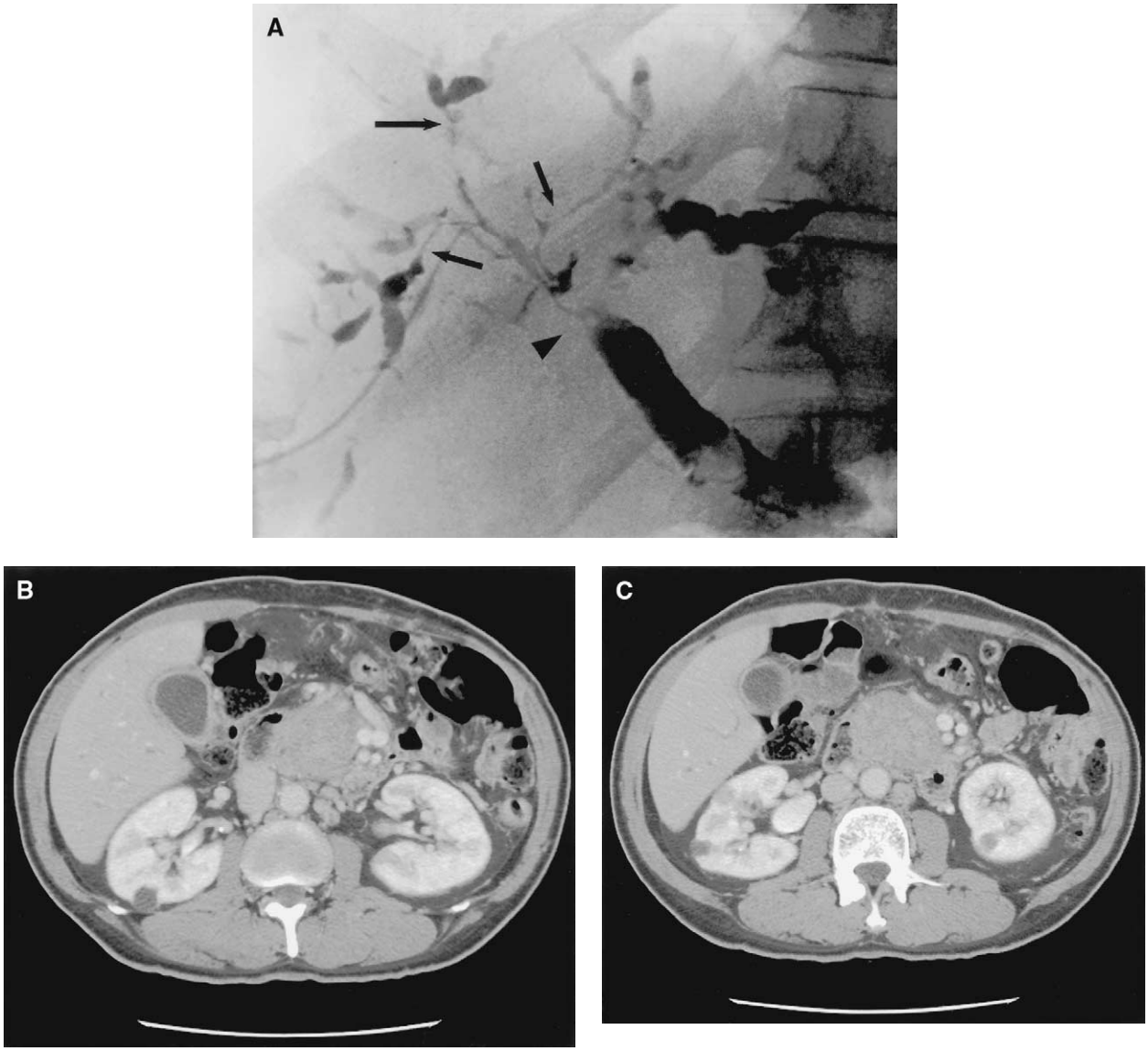

Fig. 5. Recurrent LP in a patient 11 months after distal pancreatectomy. **A,** Direct cholangiography demonstrates a stricture at the bifurcation of the hepatic ducts and multifocal intrahepatic strictures. **B** and **C,** Cross-sectional imaging reveals a diffuse pancreatic head mass that is displacing the superior mesenteric artery and superior mesenteric vein laterally and contacting the superior mesenteric vein for a portion of its length.

currence. All patients with recurrence were closely followed with serial CT scans; no patient had evidence of malignancy at the time of last follow-up. Of the two patients who were unresectable because of infiltration of the mesenteric vasculature, one died of disease at 4 months postoperatively and one was alive and well at 6 months postoperatively.

Because both the presentation and patterns of recurrence are different for patients with pancreatic body/tail lesions vs. patients with pancreatic head lesions, we analyzed patients subjected to pancreaticoduodenectomy as a separate group. In this select group of patients, recurrence of symptoms was associated with the absence of a preoperative mass on imaging ($P = 0.001$, Fig. 6). There was no association of recurrence with sex, type of LP (classic or intermediate), history of autoimmune disease, presentation, or presence of intraoperative mass.

DISCUSSION

Lymphoplasmacytic sclerosing pancreatitis is exceedingly difficult to diagnose preoperatively. In most

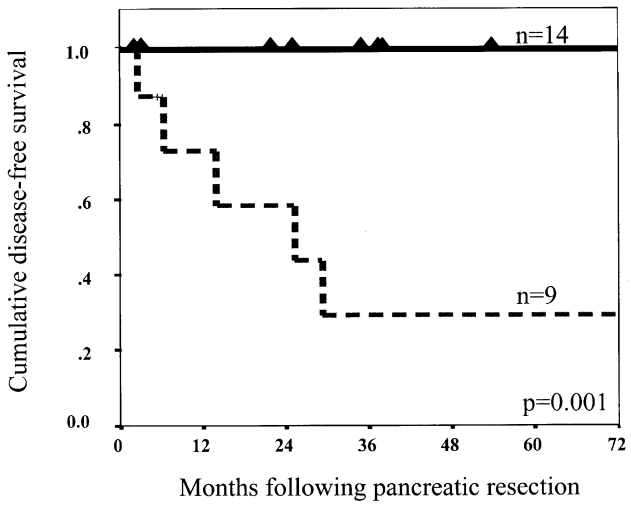

Fig. 6. Disease-free survival in patients undergoing pancreaticoduodenectomy: Preoperative pancreatic mass vs. no preoperative mass. _____ Preoperative mass; ---------- no preoperative mass.

patients, the presence of a pancreatic mass on cross-sectional imaging is thought to represent malignancy, and thus the majority are subjected to surgical exploration. By reexamining the clinical and pathologic features of these patients, we sought to improve our ability to identify these patients preoperatively, and to assess the risk of disease recurrence after resection.

There are no clear preoperative clinical signs of LP that help differentiate it from pancreatic adenocarcinoma, because most patients with both diagnoses present with jaundice, weight loss, and/or nonspecific abdominal pain. Intermittent jaundice, which may disappear spontaneously, has been reported in patients with LP.[2] In addition, the pancreatic mass may decrease in size after treatment with steroids,[7–9] although this is clearly not helpful in differentiating LP from pancreatic carcinoma preoperatively.

Several small studies have evaluated the imaging characteristics of LP. Cross-sectional imaging with enhanced CT or MRI scans may show delayed and prolonged enhancement of the involved area, with a diffusely enlarged pancreas.[10,11] This diffuse enlargement of the pancreas can also be seen on ultrasound images,[10,11] although other studies have found these features to be present in less than half of the patients with LP.[12] Images obtained on MRI may reveal a capsule-like rim that is hypointense on T1- and T2-weighted images; this is thought to correspond to an inflammatory process involving peripancreatic tissues, a characteristic finding of autoimmune pancreatitis.[11] Direct cholangiography, either ERCP or PTC, shows a classic pattern of obliteration or multifocal stenosis of the pancreatic duct.[9,10] Although it is often impossible to differentiate LP from pancreatic adenocarcinoma when the presentation is a pancreatic mass, clues to the diagnosis may include this diffuse enlargement of the gland on axial imaging and irregularity of the pancreatic duct. Only in rare cases will the patient have a history of autoimmune disease with all of the typical imaging findings in the pancreas. In most cases the diagnosis will not be suspected until after resection.

Interestingly, although many of these case series report that the majority of patients with LP have diffuse enlargement of the entire gland on preoperative imaging,[6,9–11] our series did not contain a single patient with this presentation. Most (18 [58%] of 31) of the patients in this series had a pancreatic mass on cross-sectional imaging. In addition, seven patients had biliary stricture alone, without any evidence of pancreatic abnormality. The fact that the majority of LP patients presented to our institution with a pancreatic mass is at least partially related to our referral pattern. Clearly, LP patients with a pancreatic mass, without diffuse involvement of the gland, are more difficult to diagnose without surgical resection.

Close follow-up of LP patients after resection is important because of the risk of recurrence of symptoms. We found that nearly one third of patients had recurrence of disease after resection (see Table 3). The majority of patients with recurrence (7 of 8) had jaundice at the time of recurrence, with one patient demonstrating recurrent pancreatitis resulting from multifocal pancreatic duct strictures. In attempting to evaluate risk factors for recurrence, we found that patients who had a lesion in the pancreatic body or tail were at higher risk for recurrence (see Fig. 2). Although there were only four patients undergoing distal pancreatectomy, this difference was statistically significant.

Because most of our patients had lesions in the pancreatic head, and because the pattern of disease progression is likely different for LP involving the pancreatic head compared to the body and tail, we analyzed the patients undergoing pancreaticoduodenectomy separately. These patients represented the majority of the patients (n = 23) in this study. Interestingly, we found that patients undergoing pancreaticoduodenectomy who present with a mass may have a *lower* risk of recurrence of disease than patients who present without a pancreatic mass. The risk of recurrence at three years was 71% for patients undergoing pancreaticoduodenectomy without a preoperative mass, whereas no patient with a preoperative pancreatic mass had a recurrence. These findings suggest that patients who present with a mass in the pancreatic head preoperatively may represent a more localized form of this disease.

The treatment of recurrent disease primarily includes palliation of symptoms, which can be accomplished with biliary stenting. In addition, as our understanding of LP as a true autoimmune disease has evolved, it has been suggested that patients with recurrence should be treated with bolus steroids. Because our understanding of LP as an autoimmune process is relatively recent, only two of our patients received steroids at the time of recurrence. Therefore the efficacy of steroids in treating patients with recurrence in this series is unclear, although clearly there are few other effective options in these patients.

Few patients in our study had a preoperative biopsy. Of the six patients who did undergo fine-needle aspiration, none had findings that were suggestive of LP. Clearly, an adequate sample of tissue is required to evaluate tissue for all of the pathologic features of LP. In most cases a core biopsy will not provide enough tissue to evaluate these features. This is demonstrated by the two patients in our study who were unresectable at the time of surgery, both of whom had larger volume intraoperative biopsies obtained, which were diagnostic for LP. In our series the diagnosis was established at the time of operation in all patients, which is the most common way of making the diagnosis. Upon review of our pathology database, we did not find any patients with unresectable disease in which a preoperative percutaneous or endoscopic biopsy was diagnostic of LP.

Although it is exceedingly difficult to obtain a definitive preoperative tissue diagnosis, the importance of attaining an intraoperative diagnosis in patients with unresectable tumors cannot be overemphasized. These tumors can closely mimic pancreatic adenocarcinoma, and have even been reported to result in gross peripancreatic adenopathy.[2] Because of this, if adequate tissue is not obtained intraoperatively, patients with unresectable disease may be inadvertently treated with chemotherapy and/or radiation.

We attempted to stratify patients based on pathologic criteria into two groups: classic and intermediate LP. Although our goal was to determine if there were certain pathologic findings that predicted recurrence of LP after resection, we found no difference in recurrence of symptoms between patients with classic and intermediate LP. In addition, there was no difference in presenting symptoms, history of autoimmune disease, and intraoperative findings between classic and intermediate LP patients. The only difference between these two groups was that a greater number of patients with classic LP presented with a true pancreatic mass compared to patients with intermediate LP. Clearly, the discovery that patients with either intermediate or classic LP follow a similar clinical course with an associated equal chance of recurrence not only expands our diagnostic criteria for this disease, but also reinforces the fact that close follow-up is important in all patients, regardless of the type of LP.

Many prior studies have found LP to be associated with autoimmune disease, including Sjögren's syndrome, systemic lupus erythematosus, and sclerosing cholangitis.[1,3–5,13–16] Elevated levels of several different autoantibodies have been associated with LP, including antinuclear antibody,[2,7] antimicrosomal antibody,[7] and anticarbonic anhydrase II.[9] However, we found only 19% (6/31) of the patients in this study had a history of an autoimmune disorder preoperatively. In addition, only one patient developed autoimmune disease after resection. Thus the majority of these patients appear to have an autoimmune disorder of the pancreas that manifests itself as a localized tumor.

An important article by Hamano et al.,[6] recently investigated the important issue of determining an early diagnosis in patients with LP to avoid surgical resection. Patients with LP were found to have markedly elevated levels of IgG4, a rare immunoglobulin, which can bind the C1q complement and activate the classic complement pathway, compared to patients with pancreatic cancer, chronic pancreatitis, primary sclerosing cholangitis, or Sjögren's syndrome. The diagnostic criteria for LP included irregular narrowing of the pancreatic duct and sonolucent swelling of the pancreas, which responded to bolus glucocorticoids. After 4 weeks of treatment with steroids, all patients had remission of symptoms and resolution of imaging abnormalities. In addition, the serum IgG4 level and the amount of IgG4 immune complexes found in the serum decreased after treatment with steroids. After initial treatment with bolus steroids, two patients had recurrence of pancreatitis. Other investigators have demonstrated a marked increase in the overall level of serum IgG.[2,7]

Clearly, these results need to be confirmed at other centers, because the repercussions of misdiagnosing a pancreatic malignancy as LP are profound. Because of this, our algorithm for treating patients with suspected LP consists of obtaining a serum IgG4 level. Because preoperative biopsy (fine-needle aspiration or core biopsy) is unlikely to be diagnostic and poses some risk to the patient, we do not recommend it. If the IgG4 level is elevated, the patient should be placed on a short trial of steroids followed by repeat imaging with CT in 4 to 6 weeks. If there is a decrease in the size of the mass, steroids should be continued. However, if there is no change or an increase in size, the patient should undergo definitive resection. The problem remains in defining the

patients with possible LP. Although patients who present with diffuse inflammation of the entire pancreas may be thought to have LP, the majority of patients with a pancreatic mass will continue to be appropriately treated with pancreatic resection.

CONCLUSION

Patients presenting with a pancreatic mass due to LP are extremely difficult to differentiate preoperatively from those with carcinoma. Although we attempted to predict the clinical outcome based on pathologic criteria, it appears that patients with classic and intermediate LP follow a similar clinical course. Because nearly one third of all patients have recurrence of disease after resection, close follow-up is mandatory for all patients.

We thank Diane Bassman for her thorough and diligent effort on this project.

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Discussion

Dr. A.L. Warshaw (Boston, MA): This is a very, very interesting experience and nicely presented. I have three questions for you. This entity is getting increasing attention, and one aspect of it is the presence of serologic abnormalities, including the elevation of IgG, which can be used as a preoperative diagnostic test. I recognize this is a retrospective study, but do you have any information on that? The second point is that your recurrences, if I understood your data, appear to be predominantly affecting the bile ducts rather than the pancreas. Is this the same lymphoplasmacytic response that involves the bile ducts or is it something else, such as a postoperative stricture? Third, there is some anecdotal experience with the treatment of this entity either preoperatively or postoperatively with steroids. Do you have any such experience? We have had a limited early experience in which there was a dramatic response.

Dr. S. Weber: These are all good questions. You refer to the issue of checking serology on these patients, which is in response to an article published in the *New England Journal of Medicine* in 2001. This is a Japanese series that correlated increased levels of IgG4 in patients with LP. This is very interesting because it may allow us to check a simple serologic test in patients with pancreatic masses who have a suspicious history of autoimmune disease or in those who do not fit the classic imaging pattern of pancreatic adenocarcinoma. In these cases, IgG4 can be an extremely helpful test, and we have evaluated it in three patients. Two of those patients have had elevated IgG4 levels, but we did have one patient who clearly had LP, with classic imaging findings and a great response to steroids, who also had a normal IgG4 level. We did not go back and evaluate the IgG4 level in patients retrospectively, because the level has been shown to correlate with disease activity, and so we would not expect an abnormal level in patients who have had a complete resection. As a corollary, the value of IgG4 in regard to screening patients for recurrence after resection has never been evaluated. Our recurrences did primarily affect the bile duct, although we have had two patients who had unusual sites of recurrence, including one patient with a retrobulbar mass that was biopsied and one patient with a small bowel mass that was resected last month by Dr. Conlon. Both of these patients' specimens were compared to the original pancreatic lesions, and similar pathologic features were identified in both. So the bottom line is that recurrences did primarily affect the bile ducts in our series. However, this may be because these are the patients who become symptomatic with jaundice, and so we detect the recurrence early in its course. Regarding the issue of steroids, if we can determine the diagnosis definitively preoperatively, which is clearly problematic, these patients should be treated with steroids because marked responses to steroids have been reported in numerous series. We have seen several patients with recurrences whom we have treated with steroids with good responses.

Dr. D. O'Toole (Clichy, France): In the patients with intrahepatic strictures, did you have a histology report to rule out primary sclerosing cholangitis? In these patients there is an association between sclerosing cholangitis and pancreatic autoimmune disease and various disorders. Second, did you retrospectively also exclude eosinophilic pancreatitis, which can also mimic this type of presentation?

Dr. Weber: We have not yet looked at eosinophils in this group. We are in the process of performing immunohistochemical staining of the pancreatic lesions to determine the type of inflammatory cells that are present, but this has not yet been completed. I agree that the pattern we see for patients with multiple intrahepatic strictures is similar to the pattern for sclerosing cholangitis. We performed brush biopsies in only a limited number of patients. Another interesting point in the *New England Journal of Medicine* article is that the IgG4 levels were specifically elevated in the patients who had LP but were not elevated in patients with pancreatic cancer, sclerosing cholangitis, or primary biliary cirrhosis. So I think this entity is different from sclerosing cholangitis, but I agree that the pattern of disease that we saw at the time of recurrence was very similar.

K.G. Billingsley (Seattle, WA): I am struck by the fact that in some of the CT scans you showed, the mass did not appear to be standard pancreatic adenocarcinoma. It looked like something clinically different. Did any of these patients undergo fine-needle aspiration or core biopsy prior to laparotomy, and can we take any lessons away from that? It seems as if this is a group of patients who may best be treated with medical therapy, steroids, or other anti-inflammatory treatment, rather than surgery; can you give us any insights or lessons on that?

Dr. Weber: The results of the preoperative imaging that we described were based on the imaging reports, which were obviously limited because many of these patients were resected before LP was well described. We are now in the process of obtaining the original films to review them retrospectively. I agree with you; when we look at several of these patients now, you can say that clearly this looks different from the imaging findings of typical pancreatic adenocarcinoma, but I think we did not really understand LP as an entity until recently. So we are trying to learn more about the preoperative imaging findings in these patients, and hopefully we will be able to report that data in the future. Regarding the issue of preoperative biopsies, we had very few patients who were subjected to biopsy. Of the six patients undergoing fine-needle aspiration, one was positive for acinar carcinoma and the other five were either benign or inconclusive. So clearly we have a great deal more to learn about this group of patients to help us determine the characteristics that differentiate LP from pancreatic cancer.

Dr. K.S. Kirkwood (San Francisco, CA): This group might actually be the appropriate group for endoscopic ultrasound (EUS)–guided fine-needle aspiration therapy.

Dr. Weber: Yes, EUS may be very helpful to evaluate patients for diffuse inflammation of the entire pancreas, which has been associated with LP in other series. Unfortunately, none of our patients had this finding, but perhaps EUS might be more sensitive for detecting it.

Invited Discussion—Expert Commentator

L. William Traverso, M.D. (Seattle, WA): This rare entity was found in 31 of 1287 (2.4%) pancreatic resections performed at the Memorial Sloan-Kettering Cancer Center. I have seen three cases in my own practice during the past 2 months. After adequate tissue sampling and surgical exploration, none of my patients required resection. They are being treated or considered for treatment with steroids.

What is this disease process and what does it mean to the patient? Consider the following clinical scenario: A 65-year-old man presents with jaundice, biliary stricture, and abdominal pain. The CT scan shows a pancreatic mass but does not show pancreatic duct dilation. ERCP was not done nor was it indicated. ERCP, however, would have been helpful in this case, because it might have shown a diffuse tightening of the pancreatic ductal system. If needle biopsies had been done and they were negative, the patient would still be a candidate for resection. After pancreaticoduodenectomy, the pathology is benign, and both the patient and the surgeon celebrate. The pathology report indicates chronic pancreatitis—a disease with the most common symptom being disabling abdominal pain. In addition, we know that when chronic pancreatitis is confined to the head, it responds well to head resection because the etiology of the pain appears to be driven by a smoldering fibrosis in the head of the gland. The head of the gland is known as the "pacemaker of chronic pancreatitis." Review of the specimen by the surgeon shows an infiltration with fibrosis and many more plasma cells and lymphocytes than would be expected with chronic inflammation. In fact, the pathologist says it looks like Hashimoto's thyroiditis of the pancreas. From today's presentation, what can we tell the patient about prognosis?

After a median follow-up of 38 months, this patient will have a 71% chance of recurrent symptoms if a mass was not seen on the preoperative CT scan—the most common symptom heralding this recurrence will be jaundice from continuing inflammation of the bile duct resulting in biliary stricture. The manuscript does not indicate what the histologic findings were in the bile duct among those who had the recurrent symptom of jaundice after pancreatic head resection.

Recent articles suggest that the process is due to immune complexes directed at the pancreas; in fact, a rare immunoglobulin may be at work here—IgG4. The surgeon should consider this entity and obtain more imaging studies than usual, particularly ERCP in patients with jaundice if on CT scan a mass is not seen or the main pancreatic duct is not dilated.

Close follow-up for the development of an occult cancer is always necessary. Even in the patients at Memorial Sloan-Kettering this is being done after resection, even when the specimen is benign.

Modulators of Ceramide Metabolism Sensitize Colorectal Cancer Cells to Chemotherapy: A Novel Treatment Strategy

David A. Litvak, M.D., Anton J. Bilchik, M.D., PhD., Myles C. Cabot, Ph.D.

Irinotecan is a first-line chemotherapeutic agent for patients with metastatic colorectal cancer (CRC). Response rates of less than 40% underscore the problem of treating CRC with irinotecan. Our studies have shown that chemosensitization correlates with high levels of ceramide, whereas resistance correlates with high levels of glucosylceramide (GlcCer). The purpose of this study was to characterize the role of ceramide in irinotecan-mediated CRC cell death. We used four human CRC cell lines to assess ceramide metabolism, cell viability, and apoptosis after treatment with irinotecan. Fumonisin B_1 (FB_1) and 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP) were used to inhibit de novo ceramide synthesis and GlcCer production, respectively. L-*threo*-dihydrosphingosine (safingol) was used to inhibit secondary proliferative pathways mediated by an atypical protein kinase C that is activated by ceramide. Irinotecan elicited dose- and time-dependent increases in ceramide, which preceded apoptosis. When FB_1 was added to irinotecan, CRC cell death was significantly decreased. A significant increase in intracellular levels of GlcCer also was noted after treatment with irinotecan. When GlcCer production was blocked by treating cells with PPMP in addition to irinotecan, ceramide levels increased to 228% of control values and cell death increased by 88%, compared to irinotecan alone. When irinotecan was combined with both PPMP and safingol, cell death was increased by 225% to 325%, compared to irinotecan lone. CRC cell death due to irinotecan is mediated, at least in part, by the de novo synthesis of ceramide. Blocking further metabolism of ceramide can enhance this cytotoxicity. Targeting ceramide pathways is a novel strategy for the treatment of patients with CRC. (J GASTROINTEST SURG 2003;7:140–148.)

KEY WORDS: Irinotecan, ceramide, glucosylceramide, drug resistance, colorectal cancer, L-*threo*-dihydrosphingosine (safingol), 1-phenyl-2-palmitoylamino-3-morpholino-1-propanol (PPMP)

Colorectal cancer (CRC) is the second leading cause of cancer-related mortality in the United States with 56,700 deaths annually.[1] The topoisomerase (topo) I inhibitor irinotecan (CPT-11; Camptosar [Pharmacia-Upjohn, Peapack, NJ]) is effective against a number of solid tumors including untreated and fluoropyrimidine-resistant CRC.[2–5] Currently, irinotecan is regarded as a first-line agent for metastatic CRC.[2] Studies may prove it clinically effective in the adjuvant setting as well. Nevertheless, response rates of less than 40% are typical of treatment with irinotecan in CRC. Most treated patients eventually become resistant, leaving them with few therapeutic options. This underscores the problem with drug resistance in this disease.

Irinotecan is a semisynthetic derivative of camptothecin (CPT),[6] which was originally isolated from the *Camptotheca acuminata* tree more than 30 years ago.[7] Topo I introduces reversible single-strand breaks in cellular DNA to relax supercoiling. This forms a transient DNA-enzyme intermediate, referred to as the cleavable complex.[8] Irinotecan inhibits the separation of the cleavable complex into its components. The cleavable complex that is stabilized by irinotecan may then collide with other molecular machinery (e.g., rep-

Presented in part at the Surgical Forum of the American College of Surgeons, New Orleans, Louisiana, October 7–12, 2001; and at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (oral presentation).
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Supported in part by a grant from the National Cancer Institute (CA 77632); the Rogovin-Davidow Foundation, Los Angeles, California; and the Rod Fasone Memorial Cancer Fund, Indianapolis, Indiana.
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 Published by Elsevier Science Inc.
1091-255X/03/$—see front matter
PII: S1091-255X(02)00126-9

lication forks) involved in DNA replication; this may initiate a series of events that lead to cell death.[9] A number of cellular features may modulate sensitivity to irinotecan. These include levels and activity of topo I[10] and the related enzyme topo II.[11] Exposure of cancer cells to CPT or its derivatives has been shown to activate a number of cellular pathways leading to cell cycle arrest or cell death. The study by Suzuki et al.[12] has suggested that irinotecan-induced death of murine fibroblasts may involve ceramide, a ubiquitous sphingolipid second messenger.

Ceramide initiates a cell death signal in response to various toxic stimuli. These stimuli include Fas ligand, tumor necrosis factor-alpha (TNF-α), ionizing radiation, and several chemotherapeutic agents.[13,14] Ceramide can be formed from either cleavage of the phosphodiester bond of sphingomyelin by sphingomyelinase or by *N*-acylation of dihydrosphingosine by ceramide synthase as part of the de novo pathway. An increase in levels of ceramide induces cell death in response to anthracyclines,[15,16] etoposide,[17] paclitaxel,[18] and *Vinca* alkaloids[19] in breast cancer, squamous cell carcinoma, and leukemia cell lines. However, the signal for cell death initiated by an increase in ceramide may be muted by two intrinsic processes. First, cancer cells may convert ceramide to the noncytotoxic glucosylceramide (GlcCer) that confers a drug-resistant phenotype.[20,21] Second, the activation of proliferative pathways by the ceramide-mediated atypical protein kinase C–ζ (PKC-ζ) may short-circuit the signal for cell death.[22]

A better understanding of the mechanisms contributing to CRC cell resistance may improve existing chemotherapy and provide targets for novel therapies. Herein, using four human CRC cell lines, we characterize the role of ceramide in CRC cell death due to irinotecan. We found that exposure of CRC cells to irinotecan causes a significant increase in both ceramide and GlcCer levels. The cytotoxicity of irinotecan is mediated, at least in part, by the de novo synthesis of ceramide. Moreover, we noted that blocking both the metabolism of ceramide to GlcCer and the activation of secondary PKC-ζ–mediated proliferative pathways significantly enhances the cytotoxicity of irinotecan.

MATERIAL AND METHODS

Irinotecan was purchased from Pharmacia-Upjohn. RPMI-1640 and HAMS F-12 culture media were obtained from Life Technologies, Inc. (Grand Island, NY). Fetal bovine serum (FBS) was from HyClone (Logan, UT). L-*threo*-dihydrosphingosine (safingol) and1-phenyl-2-palmitoylamino-3-morphilino-1-propanol (PPMP) were obtained from Matreya, Inc. (Pleasant Gap, PA). Fumonisin B_1 (FB_1) was purchased from Biomol (Plymouth Meeting, PA); C_6-ceramide and sphingomyelin (brain-derived) were from Avanti Polar Lipids (Alabaster, AL). [9,10-^{3}H]Palmitic acid (50 Ci/mmol) was from Dupont/NEN (Boston, MA), and water-compatible liquid scintillation fluid (Eco-Lume) was from ICN Biomedicals (Costa Mesa, CA). Thin-layer chromatography plates (Silica Gel G) were purchased from Analtech (Newark, DE). Plastic tissue cultureware was obtained from Costar (Cambridge, MA) and Corning (Corning, NY).

Cell Culture

The human CRC cell lines HT-29, SW 403, and SW 1417 were obtained from American Type Culture Collection (Rockville, MD). The CeNU cell line was established in our laboratory from a lymph node of a patient with metastatic CRC. Cells were maintained in either RPMI-1640 with 10% (HT-29, CeNU) or 20% FBS (SW 1417, SW 403). All media were supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 584 mg/liter L-glutamine. Cells were cultured in a humidified, 5% CO_2 tissue incubator at 37° C and subcultured weekly using 0.05%/0.53mmol/L trypsin/EDTA solution. For all experiments, cells were trypsinized and counted with a hemacytometer. The appropriate numbers of cells were then suspended in media with 10% to 20% FBS and placed in six-well or 96-well plates. After 24 hours, treatment (irinotecan, FB_1, safingol, C_6-ceramide, or PPMP, alone or in combination) or vehicle (ethanol and/or media) was added to the cells. The serum content of the medium was lowered to 5% for HT-29 and CeNU cells or 10% for SW 403 and SW 1417 cells after 24 hours.

Cell Radiolabeling and Lipid Analysis

As described previously, [20], [^{3}H]palmitic acid (1.0 to 2.5 μCi/ml culture medium) was used as a lipid precursor to trace cellular metabolism of ceramide, GlcCer, and sphingomyelin. After radiolabeling cells for specified times, 100 μl aliquots of media were removed and analyzed by liquid scintillation counting to determine cellular uptake of the [^{3}H]palmitic acid. The radiolabeled medium was then aspirated, and cell monolayers were rinsed twice with ice-cold phosphate-buffered saline solution. Ice-cold methanol containing 2% acetic acid was added, and cells were scraped free of the substratum (plastic scraper) for lipid extraction using chloroform and water in 1-dram glass vials. After centrifugation, the resulting organic lower phase was withdrawn, transferred to a glass vial, and evaporated to dryness under a stream of nitrogen. Lipids were dispersed in 50 to 100 μl aliquots of chloro-

form/methanol (2:1) for analysis by thin-layer chromatography. [^{3}H]Ceramide was resolved from other radiolabeled lipids by thin-layer chromatography in a solvent system containing chloroform/acetic acid (90:10 v/v), and [^{3}H]sphingomyelin was resolved by thin-layer chromatography in chloroform/methanol/acetic acid/water (60:30:7:3 v/v/v/v). Commercial standards were run with the lipid samples. After drying the thin-layer chromatography plates, lipids were visualized by iodine vapor staining. Spots of interest were scraped into 0.5 ml of water in plastic vials, vortexed, and 4.5 ml EcoLume was added for analysis of tritium by liquid scintillation counting.

Cell Viability Assay

CRC cells were seeded in 96-well plates (3000 to 5000 cells/well) in 0.1 ml of medium containing 10–20% FBS. Perimeter wells contained 0.2 ml of water. Cells were cultured for 24 hours before addition of the treatment or vehicle in 0.1 ml media. Cells were incubated at 37° C for an additional 48 to 72 hours. Cell viability was determined using the Promega Cell Titer 96 aqueous cell proliferation kit (Promega Corp., Madison, WI), according to the manufacturer's directions. Absorbance was recorded using an FL600 microplate reader (Biotek, Winooski, VT).

Apoptosis Assays

Apoptosis was quantitatively measured using the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics, Indianapolis, IN). This is a photometric enzyme immunoassay that uses mouse monoclonal antibody directed against DNA and histone. The assays were conducted as detailed in the manufacturer's instructions. Apoptosis was confirmed as the mode of cell death by DNA gel electrophoresis. Briefly, after treatment, cells were collected and lysed with hypotonic buffer (0.5% Triton X-100, 20 mmol/L EDTA, 25 mmol/L Tris-HCl, pH 8.0). Disrupted cells were then microfuged at 10,000 RPM for 5 minutes and the low-molecular-weight DNA-enriched supernate was collected. RNase (100 units/ml) was added to the supernate and then 7.5 mol/L ammonium acetate and 100% ethanol (2:1 v/v) were added to precipitate DNA at −20° C. After pelleting the DNA precipitate by centrifugation, the pellet was resuspended in TAE buffer (40 mmol/L Tris-acetate, 1.0 mmol/L EDTA, pH 8.3) and run on a 1.5% agarose gel stained with ethidium bromide. The gel was inspected for DNA laddering in multiples of 200 base-pairs, which is characteristic of apoptosis.

Statistical Analysis

All data are expressed as mean ± standard deviation. Comparisons between groups were made using either Student's unpaired t test or analysis of variance with Fisher's least significant difference test (for comparisons between multiple groups). $P < 0.05$ was considered significant.

RESULTS

Cytotoxicity of Irinotecan and Ceramide Formation

A dose-dependent increase in levels of ceramide preceded the onset of CRC cell death following exposure to irinotecan (Fig. 1). No significant cell death was detected within 24 hours of treatment with irinotecan (0.1 to 20 μmol/L; see Fig. 1, *A*). The absence of cell death 24 hours after treatment was confirmed by the ability of cells to exclude the vital dye Trypan blue (data not shown). A significant 142% to 221% increase in ceramide over control (untreated) cells was noted with irinotecan (10 to 20 μmol/L) within 12 hours after treatment (see Fig. 1, *B*), thus preceding cell death by at least 24 hours. To better define the relationship between ceramide formation and cell death, we assessed levels of ceramide and apoptosis over a time-course after treatment with irinotecan (10 μmol/L) (Fig. 2). We noted that ceramide levels increased to 144% of the control value after 12 hours and 394% of the control value after 36 hours. In contrast to the early increase in ceramide, apoptosis was not significantly increased until 36 hours after treatment (160% of control). Apoptosis was confirmed as the mode of cell death by DNA gel electrophoresis, demonstrating characteristic laddering (data not shown). Collectively these data suggest a causal association between increases in intracellular ceramide levels and cell death in response to exposure to irinotecan.

We determined whether blocking the formation of ceramide would abrogate the cytotoxic effects of irinotecan. First, we confirmed previous results that ceramide was cytotoxic to CRC cells[23,24] by treating them with C_6-ceramide, a cell-permeable analog (Fig. 3). The EC_{50} (concentration of drug causing a 50% cell death) for C_6-ceramide was 0.75 ± 0.03 μmol/L, and all cells were killed at a dose of 2.5 μmol/L (confirmed by failure of cells to exclude Trypan blue [data not shown]). Next we demonstrated that irinotecan enhanced the formation of ceramide through activation of the de novo pathway. For this we blocked a key enzyme involved in this pathway, ceramide synthase, with FB_1 and assessed ceramide levels and cell viability (Fig. 4). When FB_1 (10 μmol/L) was added to cells before exposure to irinotecan, as expected, ceramide

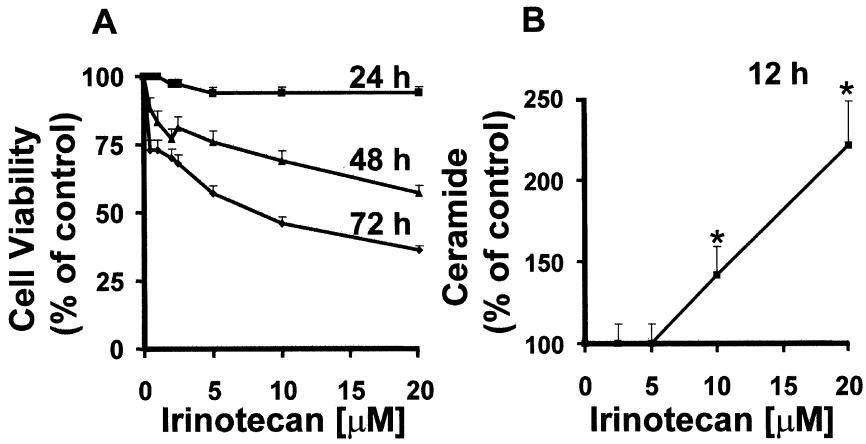

Fig. 1. Cell viability (**A**) and ceramide levels (**B**) in CeNU CRC cells after treatment with irinotecan. To determine ceramide levels, subconfluent cells were exposed to irinotecan for 12 hours in medium containing [^{3}H]palmitic acid (1.0 to 2.5 μCi/ml). Total cellular lipids were extracted and [^{3}H]ceramide was analyzed. To measure viability, cells were seeded in 96-well plates and treated the following day with either vehicle (control) or varying doses of irinotecan. Viability was determined 24 to 72 hours later by means of a colorimetric assay. Data are expressed as mean ± SD (n = 3 for ceramide assays; n = 6 for viability assays). *$P < 0.05$ vs. untreated cells (control).

levels were reduced to 15% of control (untreated cells) values (see Fig. 4, *A*). Cell viability was restored to 90% of the control value when FB_1 was present with irinotecan, a 95% increase compared to treatment with irinotecan alone (see Fig. 4, *B*). Consistent with previous results,[25] the addition of FB_1 alone resulted in a slight decrease in cell viability to 90% of the control value; this appears to be due to the accumulation of sphinganine, a cytotoxic precursor of ceramide.[25] Experiments with L-cycloserine, a competitive inhibitor of serine palmitoyl-transferase, a second key enzyme in the de novo synthesis of ceramide, were also performed; however, this agent was too toxic to CRC cells to be useful (data not shown). We determined that sphingomyelin levels remained constant up to 36 hours after treatment with irinotecan (data not shown). The degradation of sphingomyelin does not appear to contribute to the accumulation of ceramide in response to irinotecan. Collectively these results show that ceramide generated via the de novo pathway is critical to inducing CRC cell death after exposure to irinotecan; when ceramide formation is blocked, cytotoxicity is significantly diminished.

Ceramide Metabolism and Sensitization to Irinotecan

Ceramide can be metabolized by any of several cellular pathways. The conversion of ceramide to GlcCer by the enzyme GlcCer synthase (GCS) has been shown to correlate with resistance of breast cancer cells to chemotherapeutic agents in vitro.[20,21] From our cell viability assays (see Fig. 1), we noted that up to 40% of CRC cells remained viable after prolonged treatment with high concentrations of irinotecan (20 μmol/L). We speculated that this resistance to irinotecan may be caused by the shunting of ceramide to a noncytotoxic metabolite such as GlcCer. We assessed GlcCer levels in each of the CRC cell lines after treatment with irinotecan (Fig. 5). Ceramide levels were significantly elevated after treatment with irinotecan in all four cell lines (range 144% to 184% of control values). We also noted that levels of GlcCer were significantly increased in HT-29, CeNU, and SW 403 cells (range 140% to 156% of control values); GlcCer was only slightly increased to 115% of control in SW 1417 cells. We then treated CRC cells with the GCS inhibitor PPMP to determine whether blocking the production of GlcCer would enhance the cytotoxicity of irinotecan (Fig. 6). When CRC cells were treated with PPMP (10 μmol/L) in addition to irinotecan (10 μmol/L), GlcCer levels were reduced to 43% of control values (untreated cells) and ceramide levels were increased 46%, compared to treatment with irinotecan alone (see Fig. 6, *A*). Cell viability was decreased by 52% when PPMP was added to irinotecan, compared to irinotecan alone (see Fig. 6, *B*). These results suggest that blocking the formation of GlcCer can sensitize CRC cells to irinotecan.

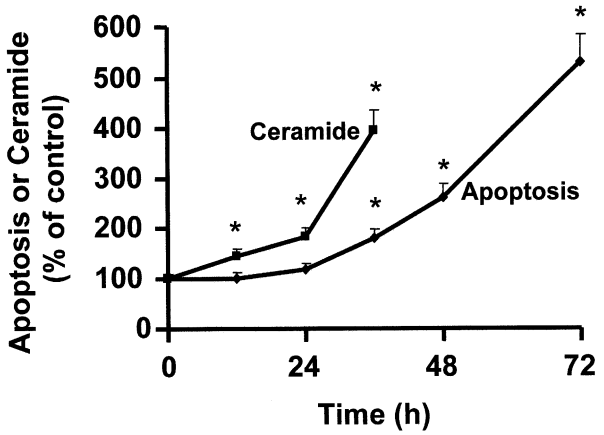

Fig. 2. Relationship between ceramide formation and apoptosis in CeNU CRC cells after exposure to irinotecan (10 μmol/L). To determine ceramide levels, subconfluent cells were exposed to either irinotecan or vehicle (control) for the times indicated in medium containing [^{3}H]palmitic acid (1.0 to 2.5 μCi/ml). Total cellular lipids were extracted, and [^{3}H]ceramide was analyzed. Data expressed as mean ± SD (n = 3). *$P < 0.05$, compared with untreated (control) cells. To determine apoptosis, cells were cultured in the absence (vehicle only) or presence of irinotecan (10 μmol/L) and incubated for the time periods indicated. Apoptosis was quantitatively measured by colorimetric assay. Data are expressed as mean ± SD (n = 6). *$P < 0.05$, compared with control (untreated) cells.

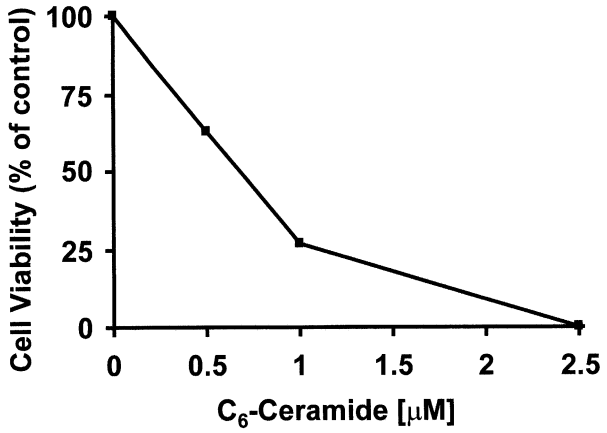

Fig. 3. Influence of C_6-ceramide on the viability of CeNU CRC cells. CeNU cells were seeded in 96-well plates and treated the following day with either vehicle (control) or C_6-ceramide. Cell viability was determined after 72 hours by colorimetric assay. Data expressed as mean ± SD (n = 6). *$P <$ 0.05 vs. untreated (control) cells.

Modulating Ceramide Metabolism and Enhancing Sensitivity of Cells to Irinotecan

The multidrug resistance modulator safingol blocks ceramide-activated PKC-mediated proliferative pathways.[26] We evaluated the effects of the combination of irinotecan, PPMP, and safingol in the four CRC cell lines (Fig. 7). Preliminary experiments (data not shown) were conducted to ascertain the most effective doses for the three-agent regimen. This avoided significant single-agent cytotoxicity. We used a reduced dose of irinotecan (8 μmol/L) over a 48-hour treatment period and then assessed cell viability. When combined with PPMP (8 μmol/L), this reduced dose of irinotecan still caused an 18% to 40% decrease in cell viability compared to irinotecan alone. SW 1417 cells were least affected by the combination of irinotecan and PPMP. We speculate that this may reflect that SW 1417 cells produce very little GlcCer after exposure to irinotecan; we detected only a 15% increase in GlcCer levels after treatment with irinotecan alone in these cells (see Fig. 3). Irinotecan (8 μmol/L) combined with safingol (2.4 μmol/L) caused a 27% to 56% decrease in cell viability, compared to irinotecan in each cell line. More important, the combination of irinotecan, PPMP, and safingol significantly reduced cell viability by 71% to 90% in each of the CRC cell lines compared to irinotecan alone. The efficacy of the three-drug combination was greatest when the cell lines were in log-phase growth. This is likely because irinotecan works best when cells are progressing through the cell cycle.

DISCUSSION

The cytotoxic effects of irinotecan may not be due solely to its ability to interfere with DNA replication at the level of the topo I-DNA cleavable complex.[8,9] Prior studies have shown that the development of CPT-resistant cancer cells in vitro frequently correlates with decreased mRNA or protein levels of topo I.[10,27] However, the results from other studies evaluating both the sensitivity of chemotherapy-naïve cancer cells to CPT in vitro[28] and the expression of topo I in patients with CRC who are resistant to irinotecan[29] do not support this hypothesis. It appears, instead, that sensitivity to irinotecan may be due to a number of different, and perhaps cell-type specific, molecules. The lipid second-messenger ceramide initiates a death signal in response to a number of different toxic stimuli including several chemotherapeutic agents.[14–18,30] Our present study demonstrates that irinotecan-governed CRC cell death is in part mediated by the de novo formation of ceramide, as cell viability was maintained when cera-

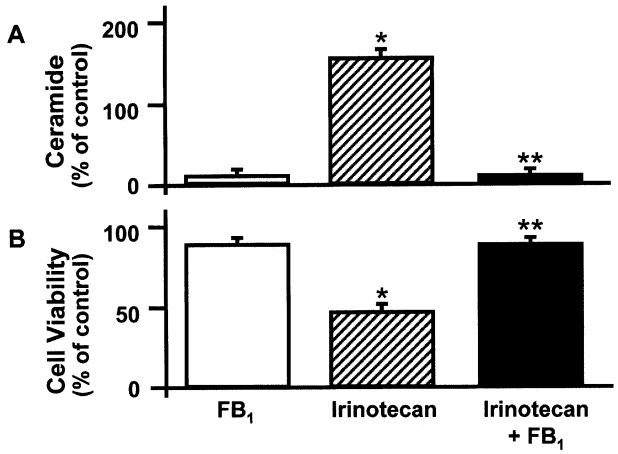

Fig. 4. The effect of FB_1 on ceramide levels in (**A**) and viability of (**B**) CeNU cells exposed to irinotecan. To assess ceramide levels, subconfluent CeNU cells were treated with vehicle (control), irinotecan (10 μmol/L), FB_1 (10 μmol/L), or a combination of irinotecan and FB_1 in medium containing [^{3}H]palmitic acid for 12 hours. Cellular lipids were analyzed for [^{3}H]ceramide. Data are expressed as mean ± SD (n = 3). *$P < 0.05$ vs. untreated (control) cells; **$P < 0.05$ vs. cells treated with irinotecan. Cell viability was determined by colorimetric assay after 72 hours. Data are expressed as mean ± SD (n = 6). *$P < 0.05$ vs. untreated cells (control); **$P < 0.05$ vs. cells treated with irinotecan.

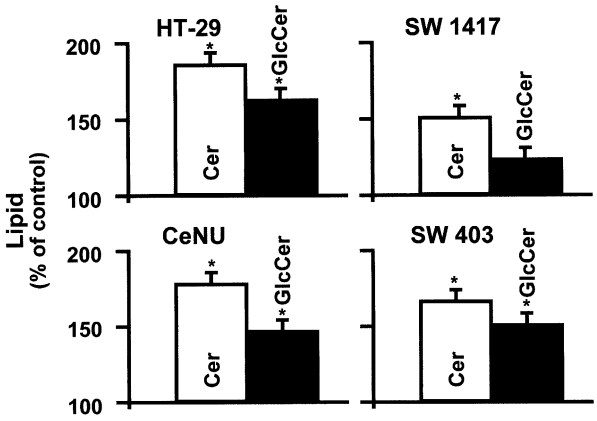

Fig. 5. Ceramide and glucosylceramide levels of four CRC cell lines treated with irinotecan. Cellular lipids were isolated and measured from cells incubated in the absence or presence of irinotecan (10 μmol/L) in medium containing [^{3}H]palmitic acid for 12 hours, and quantitated by thin-layer chromatography. Data are expressed as mean ± SD (n = 3), *$P < 0.05$ vs. untreated (control) cells. Cer = ceramide; GlcCer = glucosylceramide.

mide synthesis was inhibited. In addition, we have shown that the cytotoxicity of irinotecan may be augmented several-fold by the addition of modulators of ceramide metabolism, such as PPMP and safingol.

Our results suggest that the de novo synthesis of ceramide plays a critical role in signaling CRC cell death after treatment with irinotecan. We noted that levels of ceramide were significantly elevated in all four CRC cell lines (see Fig. 1, *B*). When we looked at ceramide formation over a time course in CeNU CRC cells, we noted that levels of ceramide continued to increase up to 394% of control values (untreated cells) by 36 hours (see Fig. 2). We determined that this increase in ceramide was due to its synthesis via the de novo pathway and not by the degradation of sphingomyelin. However, we acknowledge that our assay may lack the sensitivity to detect small changes in intracellular concentrations of sphingomyelin. Previous studies have shown that irinotecan elicits an increase in ceramide levels through the de novo pathway and by the metabolism of sphingomyelin.[12,31] Similar to our findings, the preliminary results of Bennouna et al.[31] showed that the formation of ceramide in CRC cells after their exposure to irinotecan was abrogated by the addition of FB_1. However, the study by Suzuki et al.[12] showed an increase in cell death when exogenous sphingomyelin was added to cultures of mouse fibroblasts treated with irinotecan, suggesting the involvement of this pathway. More important, we noted that increases in ceramide preceded detectable increases in cell death (see Figs 1 and 2) and that the addition of FB_1 to irinotecan resulted in a 95% increase in CRC cell viability (see Fig 4, *B*). Suzuki et al.[12] previously demonstrated that FB_1 could prevent cell death after exposure of 4B1 mouse fibroblasts to irinotecan. Our laboratory has obtained similar results for paclitaxel, an antimicrotubule agent, in breast cancer cells; when the de novo synthesis of ceramide was blocked, cell death was prevented.[18] Collectively these data suggest that ceramide formation via the de novo pathway is a critical signal for cell death after exposure to irinotecan.

The sensitivity of CRC cells to irinotecan is enhanced by blocking the formation of the noncytotoxic metabolite of ceramide, GlcCer; this effect appears to be due to the enhancement of intracellular levels of ceramide. We noted that levels of GlcCer were significantly increased in three of four of the CRC cell lines after treatment with irinotecan (see Fig. 5). When we inhibited the GCS enzyme with PPMP, levels of ceramide and cell death were increased (see Fig. 6). We speculate that the metabolism of ceramide to GlcCer in CRC cells might limit the cytotoxicity of irinotecan. The ability of cells to metabolize ceramide to GlcCer has been shown to correlate with the development of chemotherapy-resistant cancer cells in vitro.[20,21,32] In addition, in-

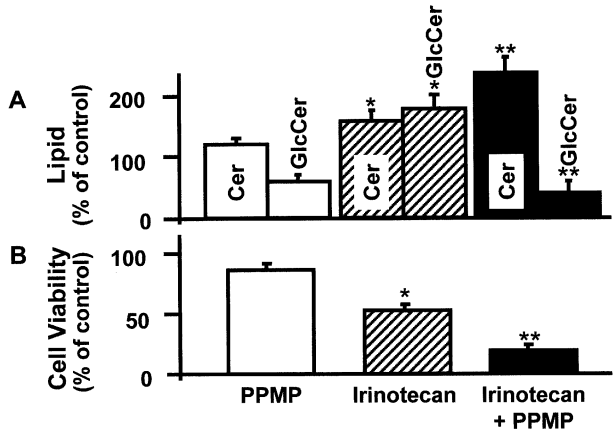

Fig. 6. Effect of PPMP on levels of ceramide and glucosylceramide in (**A**) and viability of (**B**) CeNU cells treated with irinotecan. Subconfluent cultures were treated with either vehicle (control), irinotecan (10 μmol/L), PPMP (10 μmol/L), or a combination of irinotecan and PPMP in medium containing [^{3}H]palmitic acid for 12 hours. Cellular lipids were analyzed for [^{3}H]ceramide and [^{3}H]glucosylceramide. Data are expressed as mean ± SD (n = 3). *$P < 0.05$ vs. untreated (control) cells; **$P < 0.05$ vs. cells treated with irinotecan. Cell viability was determined by colorimetric assay after 72 hours. Data are expressed as mean ± SD (n = 6). *$P < 0.05$ vs. untreated (control) cells; **$P < 0.05$ vs. cells treated with irinotecan. Cer = ceramide; GlcCer = glucosylceramide.

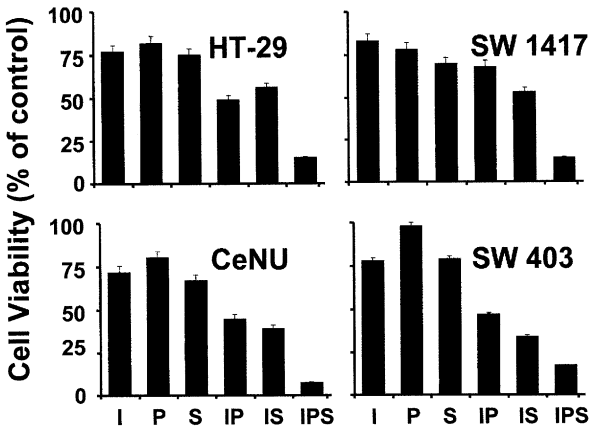

Fig. 7. Effects of the combination of irinotecan, PPMP, and safingol on viability of four CRC cell lines. Cells were treated with either irinotecan (*I*; 8 μmol/L), PPMP (*P*; 8 μmol/L), or safingol (*S*; 2.4 μmol/L), separately or in combinations of two (*IP*, *IS*) or three (*IPS*) agents. Cell viability was determined 48 hours after treatment. Data are expressed as mean ± SD (n = 6).

creased tumor levels of GlcCer correlate with resistance to chemotherapy in patients with breast cancer and melanoma.[21] Our previous studies have shown that the sensitivity of breast cancer and leukemia cells to chemotherapy may be enhanced by blocking GCS activity.[33–36] We have accomplished this pharmacologically by using agents such as PPMP or tamoxifen (a somewhat nonspecific inhibitor)[26,34–36] or by introducing antisense GCS cDNA into drug-resistant cancer cells.[33]

The combination of modulators of ceramide metabolism (e.g., PPMP and safingol) further enhances the cytotoxic effects of chemotherapeutic agents. The atypical PKC inhibitor safingol appears to inhibit the regulatory subunit of PKC-ζ and partially reverses drug resistance in selected in vitro cancer models.[37] In our study we noted a significant decrease in cell viability with the combination of irinotecan, PPMP, and safingol, compared to treatment with irinotecan alone in each of the CRC cell lines. Other studies have similarly showed the usefulness of combining a cytotoxic agent that generates ceramide with modulators of ceramide metabolism. In the study by Maurer et al.,[26] the combination of the novel retinoid N-(4-hydroxyphenyl)-retinamide (4-HPR; fenretinide), safingol, and tamoxifen or PPMP caused a multifold increase in cell death in several different types of cancer cell lines[26] compared to treatment with 4-HPR alone. Investigators from our laboratory have previously noted a synergistic cytotoxic effect by combining doxorubicin (which generates ceramide) and tamoxifen in breast cancer cells.[36] In our studies, combined treatment with irinotecan, PPMP, and safingol enabled a 20% reduction in the dose of irinotecan while augmenting its cytotoxicity. Reducing the dose of irinotecan (without sacrificing efficacy) may be of additional clinical interest because of the potential life-threatening toxicity of irinotecan to patients (e.g., severe diarrhea or neutropenia).

Several studies are in progress to evaluate the toxicity of agents that modify ceramide metabolism. Oral and intravenous formulations of 4-HPR are being evaluated in nude mouse xenograft models,[38] and preliminary preclinical testing is ongoing to evaluate a paclitaxel/ceramide regimen in squamous cell carcinoma xenografts (Wanebo HJ, personal communication). Drugs that slow the rate of formation of glycolipids, such as PPMP and OGT-918 (N-butyldeoxynojirimycin), are being tested in patients with Gaucher disease,[39,40] and a Rapid Access to Preventive Intervention Development grant has been issued by the Developmental Therapeutics Program of the National Cancer Institute to support toxicology studies of agents such as safingol.[26] In in vitro studies, safingol was found to be minimally toxic to normal fibroblasts and bone marrow myeloid progenitor cells.[26]

CONCLUSION

The results of our study indicate that the cytotoxic effects of irinotecan are mediated, at least in part, by the de novo formation of ceramide. CRC cells also accumulate the noncytotoxic metabolite GlcCer. We have shown that the addition of modulators of ceramide metabolism blocks the formation of GlcCer and augments CRC cell death in response to irinotecan. Our study and others have shown that CRC cells are sensitive to increases in endogenous levels of ceramide stimulated by certain cytotoxic agents and to exogenous cell-permeable analogs of ceramide.[23,24] Based on these results, we suggest that targeting ceramide pathways may be an attractive and potentially effective treatment strategy for killing CRC cells. Future studies may better delineate the cellular mechanisms involved in irinotecan-dependent and ceramide-mediated cell death and whether this treatment strategy may be effective clinically for patients with CRC.

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Discussion

Dr. Z. Cohen (Toronto, Ontario, Canada): The chemotherapeutic agents are not selective for cancer cells. They kill normal cells, as well. What happens to the normal cells as you increase the activity against the cancer cells?

Dr. D. Litvak: We did not look at that specifically, but that has been studied previously. A recently published study,[26] a collaborative project between the group from Childrens' Hospital in Los Angeles and investigators from our laboratory, looked at the effects of ceramide on normal cells. These investigators used a different ceramide-generating chemotherapeutic agent, the novel retinoid N-(4-hydroxyphenyl) retinamide (fenretinide; 4-HPR). When they combined 4-HPR with safingol and either PPMP or tamoxifen, they saw very little in the way of cytotoxicity to normal cells. We are not really sure why there was so little toxicity. It is possible that the ceramide pathways in cancer cells are different from the ceramide pathways in normal cells. There is a hint that this may be the case. A recent study that looked at biopsy specimens of normal colons and cancerous colons showed that the levels of ceramide in these samples are significantly different. Perhaps there are some mechanistic differences as well in how these tissues respond to an increase in intracellular ceramide.

Dr. K.E. Behrns (Chapel Hill, NC): There is a lot of redundancy in these survival or proliferation pathways, and ceramide is one of many signal transducers that could be involved. Have you looked at others, such as Akt or the NF-κB

Dr. Litvak: We are really in the preliminary stages, that is, characterizing the effects of combining these compounds, irinotecan, PPMP, and safingol, in regard to levels of ceramide and glucosylceramide. Additionally, we are manipulating these pathways and seeing what happens to the cytotoxic effects of irinotecan. The next step, obviously, is to look more mechanistically at potential upstream and downstream factors. In general, it is not yet known where exactly ceramide fits into the whole picture of the cell-signaling pathway that leads to cell death. It is somewhat unclear what is upstream to or downstream from ceramide. Although there is evidence that caspases are downstream effectors in response to increases in ceramide, at the present time there is a "black box" as to what is going on in between.

Epidermal Growth Factor Activation of Intestinal Glutamine Transport Is Mediated by Mitogen-Activated Protein Kinases

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Glutamine is an essential nutrient for gut functions, but the regulation of its uptake by intestinal mucosal cells is poorly understood. Given the pivotal role of epidermal growth factor (EGF) in regulating gut metabolism, growth, and differentiation, this in vitro study was designed to investigate the intracellular signaling pathways involved in the regulation of EGF-mediated intestinal glutamine transport in intestinal epithelia. Continuous incubation with EGF (>30 hours, 100 ng/ml) stimulated glutamine transport activity across intestinal epithelial Caco-2 cell apical membrane. Exposure to EGF for 48 hours resulted in an increase in transport activity (50%) and glutamine transport system B gene ATB^0 mRNA levels (ninefold). EGF stimulated glutamine transport activity by increasing the glutamine transporter maximal velocity (V_{max}) without altering the transporter apparent affinity (K_m). Furthermore, EGF stimulated both intracellular protein kinase C and mitogen-activated protein kinase MEK1/2 activities. The EGF-stimulated glutamine transport activity was attenuated individually by the specific protein kinase C inhibitor chelerythrine chloride and the mitogen-activated protein kinase MEK1 inhibitor PD 98059. These data suggest that EGF activates glutamine transport activity across intestinal epithelial membrane via a signaling mechanism that involves activation of protein kinase C and the mitogen-activated protein kinase MEK1/2 cascade. EGF activates glutamine transport via alterations in transporter mRNA levels and the number of functional copies of transporter units. (J GASTROINTEST SURG 2003;7:149–156.)

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

Amino acid glutamine is vital for maintaining intestinal and systemic nutritional and immunologic functions, especially during catabolic states in which the requirement for glutamine is increased.[1–4] Movement of luminal glutamine by discrete membrane transport systems across the intestinal epithelial brush-border membrane into the enterocyte is a critical initial step for delivering exogenous glutamine to the systemic circulation. Transport of glutamine is regulated by various local and systemic factors.[5,6] Intestinal luminal paracrine factors such as epidermal growth factor (EGF) affect the luminal epithelium and regulate many biological functions in the small intestine.[7,8] Endogenous sources of EGF include secretions from submaxillary glands and jejunal/ileal mucosa,[9] whereas exogenous sources include milk.[7,8,10] EGF stimulates cell growth, proliferation, and differentiation in epithelial cells, and is the main stimulator in promoting intestinal mucosal wound healing in mucosal injury.[11] Previously we characterized the apical membrane glutamine sodium–dependent transport system B (90%) and the sodium-independent system L (10%) in a cultured Caco-2 cell line,[12] an in vitro model commonly used for intestinal epithelial nutrient and drug transport studies.[13,14] In this study we explored the intracellular signaling pathways involved in the activation of intestinal glutamine transport by EGF in Caco-2 cells.

Presented at the Forty-Third Annual Meeting of The Society for Surgery of the Alimentary Tract, San Francisco, California, May 19–22, 2002 (poster presentation).

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1091-255X/03/$—see front matter
PII: S1091-255X(02)00130-0

MATERIAL AND METHODS

Caco-2 Cell Cultures

The human intestinal epithelial Caco-2 cell line was obtained from American Type Culture Collection (Rockville, MD) at passage 16. Cells were grown in a humidified incubator at 37° C in 10% CO_2/90% O_2. Cells were routinely grown in Dulbecco's modified Eagle medium (DMEM) containing 25 mmol/L glucose, 4 mmol/L glutamine, and 0.4 mmol/L sodium bicarbonate, supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 1% nonessential amino acids. Caco-2 cells were passaged weekly after treatment with 0.05% trypsin and 0.02% EDTA. Cells were reseeded at a density of 4.5×10^6 cells/100 mm dish for future subculturing, seeded in six-well cluster Costar tissue culture plates at a density of 10^5 cells per well for Northern blot or Western blot analysis, or seeded in the 24-well cluster Costar tissue culture plates at a density of 10^4 cells per well for transport experiments. Confluent cells (day 7, passages 20 to 40) were used for experiments. The day of seeding was designated as day 0. The growth medium was changed daily, and cultures were inspected daily using a phase-contrast microscope.

Cell Treatments

To treat cells, growth medium was first replaced with serum-free medium (i.e., DMEM containing amino acids, penicillin, and streptomycin but lacking fetal bovine serum) for 2 hours at 37° C. The cell monolayer was then washed three times in serum-free medium. The cells were then exposed to each agent for various times and concentrations described below in a 37° C 10% CO_2/90% air-humidified incubator. Treatment media were replenished every 6 hours to ensure consistent agent concentrations. Cells were treated individually with EGF (0 to 100 ng/ml) for various periods of time (minutes to 72 hours). Cells were also treated with individual inhibitors: PD 98059 (0 to 100 μmol/L, dimethylsulfoxide [DMSO] as control) for mitogen-activated protein kinase (MAPK), MEK 1, and chelerythrine chloride (CHE; 0 to 6.6 μmol/L, DMSO as control) for protein kinase C (PKC), as well as actinomycin (Act-D; 0 to 0.1 μmol/L), and cycloheximide (CHX; 0 to 10 μmol/L). Caco-2 cells remained viable (viability >99% by dye exclusion) during at least 72 hours of exposure to serum-free media.

L-Glutamine Uptake Measurements

L-Glutamine transport activity was measured at 37 ± 1.0° C.[15] After cells were pretreated with various agents (described previously), cells were rinsed with "uptake buffer" (37° C) comprised of 137 mmol/L NaCl (or 137 mmol/L choline Cl), 10 mmol/L HEPES/Tris buffer (pH 7.4), 4.7 mmol/L KCl, 1.2 mmol/L $MgSO_4$, 1.2 mmol/L KH_2PO_4, and 2.5 mmol/L $CaCl_2$. Transport was initiated by adding 1 ml of uptake buffer that also contained L-[^{3}H] glutamine (2 μCi/ml, 1 μmol/L to 10 mmol/L). Transport cell culture plates were continuously shaken by an orbital shaker (1 Hz) during the uptake period. Uptake was arrested by discarding the uptake buffer and washing cells three times with ice-cold uptake buffer lacking [^{3}H]-labeled substrate. Radioactivity of isotope extracted from the cells with 1 ml 1N NaOH was neutralized with acetic acid and assayed by liquid scintillation spectrometry. Protein in the NaOH extract was measured by means of the Bio-Rad protein assay.[16] Initial rates of transport activity were determined during the linear uptake period (2 minutes), with zero time points serving as blanks.[16,17] Uptake rates are expressed as nanomoles of glutamine per minute per milligram of cell protein. Sodium-dependent system B glutamine transport was obtained by subtracting total glutamine transport measured in choline Cl buffer from that in NaCl buffer.

Northern Blot Analysis of System B ATB^0 mRNA

After cells were pretreated with various agents (described previously), cells were rinsed three times with phosphate-buffered saline solution. All procedures were performed under RNase-free conditions. Total RNA was isolated from control and treated Caco-2 cells using the "Totally RNA" isolation kit (Ambion, Austin, TX). Total RNA (10 μg) was separated on a 1% formaldehyde gel and transferred to GeneScreen membrane (PerkinElmer LifeSciences, Boston, MA) in 20× standard sodium citrate. The membrane was hybridized with an antisense oligonucleotide probe specific to human ATB^0 (5′-TTACATGACTGATTCCTTCTCAGAG-3′),[18] and then stripped and rehybridized with an oligonucleotide probe specific for 18S ribosomal RNA (5′-GTTATTGCTCAATCTCGGGTG-3′). Autoradiographs were scanned with a laser densitometer and the ATB^0 signal was normalized to 18S RNA.[15] The ATB^0 probe was 3′ end-labeled using terminal transferase and ^{32}P-dATP, and the 18S probe was 5′ end-labeled using T_4 polynucleotide kinase and ^{32}P-ATP.

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

After cells were pretreated with various agents (described previously), cells were rinsed three times

with phosphate-buffered saline. Total Caco-2 cell lysate was obtained by incubating cells in lysis solution (50 mmol/L HEPES, 150 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 1.0 mmol/L EGTA, 100 mmol/L NaF, 0.2 mmol/L Na_3VO_4, 1 mmol/L PMSF, and 10 μg/ml aprotinin) for 30 minutes on ice, and supernate was collected.[15] Equal amounts of protein from control and treated cells were separated on sodium dodecylsylfate–polyacrylamide gel electrophoresis and transferred to PVDF membrane (Millipore, Bedford, MA). The membrane was then incubated with phospho–protein kinase C (PKC) (pan) antibody, phospho-MEK1/2, or mitogen-activated protein kinase (MAPK) p44/42 antibodies (1:1000, Cell Signaling Technology, Beverly, MA) overnight at 4° C and then incubated with horseradish peroxide–conjugated secondary antibody (1:50,000). Phospho-PKC (pan), phospho-MEK1/2, and p44/42 proteins were detected using the ECL system (Amersham, Piscataway, NJ). Autoradiographs were scanned with a laser densitometer.

Statistical Analysis

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

RESULTS

Effect of Epidermal Growth Factor on L-Glutamine Uptake Activity

Uptake of glutamine (50 μmol/L) was measured in the Caco-2 cells after the cells had been incubated in EGF (0 to 100 ng/ml) for various times (minutes to 72 hours) (Fig. 1). At least 30 hours of continuous incubation was required for EGF to stimulate glutamine transport activity. Continuous incubation (48 hours) of EGF (100 ng/ml) resulted in a 50% increase in glutamine uptake activity. Pulse EGF stimulation, where cells were exposed to EGF for up to 6 hours and reincubated in EGF-free medium for the remaining incubation period (42 hours), did not affect the glutamine transport activity. EGF stimulated glutamine transport activity in a dose-dependent manner. Significant stimulation was observed at [EGF] > 50 ng (see Fig. 1). Therefore a 48-hour EGF (100 ng/ml) treatment point was selected for the subsequent experiments in this study. The EGF analog transforming growth factor-alpha (TGF-α, 0 to 20 ng/ml) exhibited a similar stimulatory effect on the glutamine transport activity.

Uptake of glutamine of various concentrations (1 μmol/L to 10 mmol/L) was measured in control and EGF-treated (100 ng/ml, 48 hours) cells. EGF stimulated the system B glutamine transport maximal velocity (V_{max}, 1.41 ± 0.25 nmole/mg/min control vs. 2.35 ± 0.19 nmole/mg/min EGF treatment; $P < 0.01$). However, the transporter apparent affinity (K_m) was not affected by EGF incubation (K_m, 207 ± 22 μmol/L glutamine control vs. 224 ± 26 μmol/L glutamine EGF treatment; $P > 0.05$) (Fig. 2)

Involvement of De Novo Transcription and Translation Processes in the Epidermal Growth Factor Stimulation of System B Glutamine Transport Activity

Glutamine transport activity was measured in control and EGF-treated cells with Act-D (0 to 0.1 μmol/L) or CHX (0 to 1 μmol/L) in the incubation medium. Act-D and CHX individually blocked the EGF-induced system B glutamine uptake (Fig. 3). The concentration of actinomycin and cycloheximide was selected so that baseline control cell transport activity was not affected to minimize the nonspecific inhibition effect of Act-D and CHX. The protein content and cell numbers of the 48-hour Act-D– or cycloheximide-treated cells was comparable to the pretreatment levels. The viability (by dye exclusion) of both control and Act-D/CHX–treated cells was greater than 99%. Compared to the control group (with only DMEM treatment), the Act-D/CHX–treated cells had 20% less protein and 40% less cells. The inhibitory effect of Act-D or CHX on the system B glutamine uptake was likely due to inhibition of protein synthesis rather than a cytotoxic effect.

To assess the effect of EGF on system B transporter gene ATB^0 expression, ATB^0 mRNA levels were measured in control and EGF-treated cells. The ATB^0 mRNA level was increased eightfold after 48 hours of continuous EGF incubation (relative levels: 1.0 control vs. 8 ± 2 EGF group; $P < 0.001$) (Fig. 4).

Involvement of Protein Kinase C Activation in the Epidermal Growth Factor Stimulation of System B Glutamine Transport Activity

To assess the effect of EGF on cellular PKC activity, phospho-PKC (pan) activity was measured by Western blot analysis using commercially available phospho-PKC (pan) antibody in control and EGF-treated cells. Phospho-PKC (pan) levels were elevated in EGF-

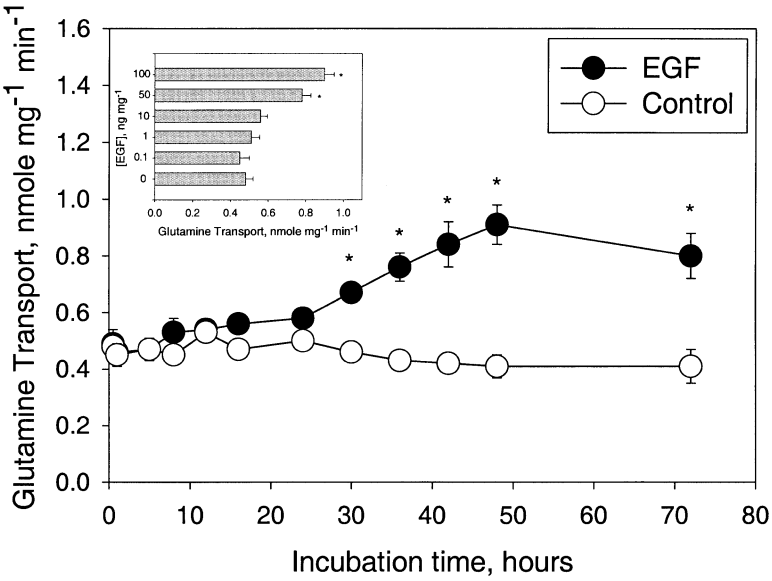

Fig. 1. Effect of EFG on system B glutamine transport activity. Uptake of glutamine (50 μmol/L) was measured in cells incubated with EGF (0 to 100 ng/ml) for various periods of time (minutes to 72 hours). Transport values are means ± SEM (n = 6, $^*P < 0.05$).

treated cells (Fig. 5), suggesting EGF-stimulated activation of PKC.

To further define the involvement of PKC activation in EGF stimulation of system B glutamine transport, glutamine transport activity was measured in both control and EGF-treated Caco-2 cells in the presence and absence of the specific PKC inhibitor CHE (0 to 6.6 μmol/L, DMSO as control). CHE (0 to 6.6 μmol/L) attenuated EGF-induced glutamine transport in a dose-dependent manner without affecting baseline transport activity. CHE (6.6 μmol/L) abolished the EGF-stimulated system B glutamine transport activity (Fig. 6) and EGF-induced system B transporter ATB^0mRNA level (Fig. 7).

Involvement of Mitogen-Activated Protein Kinases in the Epidermal Growth Factor Stimulation of System B Glutamine Transport Activity

To assess the effect of EGF on MAPK activities in Caco-2 cells, MAPK p44/42, MAPK phospho-p44/42, and MAPK phospho-MEK1/2 activity was measured by Western blot analysis using commercially available MAPK p44/42, MAPK phospho-p44/42, and MAPK phospho-MEK1/2 antibodies in cells treated with EGF (0 to 100 ng/ml) for 48 hours. EGF stimulated the active form MAPK phospho-p44/42 activity but not the total MAPK p44/42 levels, and MAPK phospho-MEK1/2 (Fig. 8), suggesting that EGF activated the MAPK MEK1/2 cascade.

To further define the role of MAPKs in the EGF activation of system B glutamine transport activity, Caco-2 cells were incubated in EGF with or without coincubation of the MAPK MEK1 inhibitor PD 98059 (0 to 50 μmol/L; DMSO as control). PD 98059 blocked the EGF-induced activation of glutamine transport activity and transporter ATB^0 mRNA levels without affecting the control cells (see Figs. 6 and 7).

DISCUSSION

In the present study we investigated in vitro regulation of intestinal apical membrane glutamine transport by the peptide growth factor EGF and associated intracellular signaling pathways, such as PKC activation and MAPK activation.

Glutamine is the most abundant amino acid in the body, accounting for 60% of the free amino acid pool. It plays a central role in interorgan nitrogen transfer and is considered a conditionally essential amino acid.[1,2] Glutamine has profound effects on gut-related immune functions and an anabolic effect on host protein synthesis.[1,2] In the enterocyte, glutamine is the preferred metabolic fuel, as well as a major precursor for biosynthesis of biological compounds.[1,2] Intestinal glutamine absorption is mediated by discrete amino acid transport systems.[5] In our previous studies we characterized L-glutamine transport systems in the human intestinal epithelial

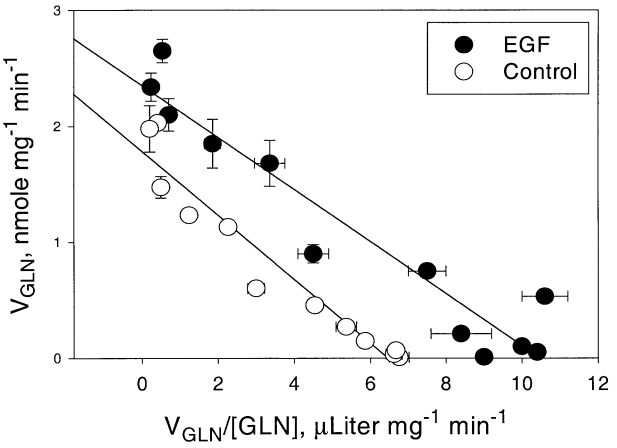

Fig. 2. Eadie-Hofstee transformation of system B glutamine uptake kinetics. Uptake of glutamine (1 μmol/L to 5 mmol/L) measured in cells incubated with EGF (0 and 100 ng/ml) for 48 hours. Transport values are means ± SEM (n = 9).

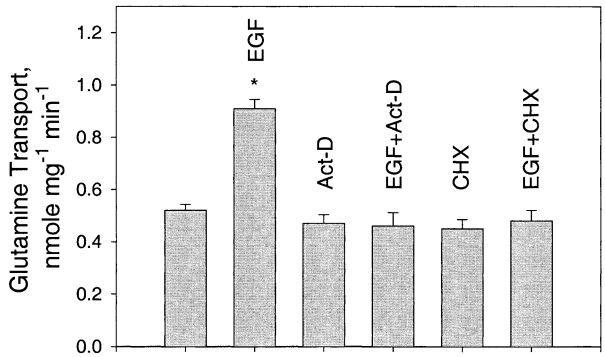

Fig. 3. Effect of actinomycin-D (*Act-D*) and cycloheximide (*CHX*) on EGF-stimulated system B glutamine transport activity. Uptake of glutamine (50 μmol/L) was measured in cells incubated with EGF (100 ng/ml) ± Act-D (0.5 μmol/L) and cycloheximide (*CHX*; 10 μmol/L. Transport values are means ± SEM (n = 6).

Caco-2 cell brush-border membrane. Glutamine is predominantly transported by the sodium-dependent transport system B (90%) with minimal contribution from the sodium-independent transport system L and passive diffusion.[12] Cultured Caco-2 cells undergo spontaneous differentiation in cell culture environment, and the differentiated cells display small intestinal epithelial characteristics such as polarized cell membrane with specific membrane marker enzymes such as alkaline phosphatase, sucrase, and sodium-potassium ATPase.[13,19,20] Caco-2 cells have been widely used as the in vitro small intestinal epithelia model for nutrient transport and drug transport studies.[13,19,20]

The intestinal epithelium is continuously exposed to various stimuli including luminal growth factors such as epidermal growth factor. EGF, which is normally present in the intestinal lumen and in circulation, regulates epithelial cell growth, proliferation, and differentiation.[7,8] EGF also has been shown to promote intestinal protein synthesis and mucosal repair.[11] Intestinal luminal EGF can come from endogenous sources such as the submandibular glands and intestinal jejunal mucosa[7,8,10] or exogenous sources such as milk.[10] EGF elicits its functions through binding to the same EGF receptor, a tyrosine kinase in the plasma membrane, which regulates many biological activities.[11] The EGF receptor then activates phospholipase, MAPK, lipoprotein I, c-erbB-2, and phosphoinositol-3 kinase.[21–24] These mechanisms are designed to allow the enterocyte to maintain intestinal homeostasis. One characteristic response is upregulation of amino acid uptake. In our previous studies we have shown that EGF stimulates sodium-independent arginine uptake via intracellular PKC activation.[25] Information on cellular regulation of intestinal glutamine transport remains limited.

EGF elicits its biological activities through two classes of mechanisms: an acute phase mechanism (minutes) and a chronic phase (hours). The acute phase involves intracellular phosphorylation, triggering rapid responses. On the other hand, the chronic phase normally involves intracellular cascades and protein synthesis to provide slow but sustained responses.[26] Many EGF-induced changes in intestinal biological activity occur in the chronic phase (hours to days).[27–30] As shown in Fig.1, prolonged exposure to EGF stimulated glutamine transport activity in a time- and dose-dependent manner. More than 30 hours of continuous incubation was required for EGF to elicit the stimulatory effect, suggesting that EGF-induced glutamine transport stimulation participates in the chronic phase of EGF activity rather than triggering an acute effect. Pulsed EGF stimulation, where cells were exposed to EGF for up to 6 hours and reincubated in EGF-free medium for the remaining incubation period (42 hours), did not affect the glutamine transport activity. These data suggest that continuous exposure of EGF is required for this stimulation. During the EGF continuous incubation, the incubation medium was changed every 6 hours to ensure a consistent EGF exposure and minimize the possible involvement of a paracrine effect and EGF degradation that may be associated with the prolonged EGF exposure.

Act-D or CHX in the incubation medium each blocked the EGF-induced glutamine uptake (see Fig. 3), indicating the possible involvement of transcription and de novo protein synthesis. Low concentra-

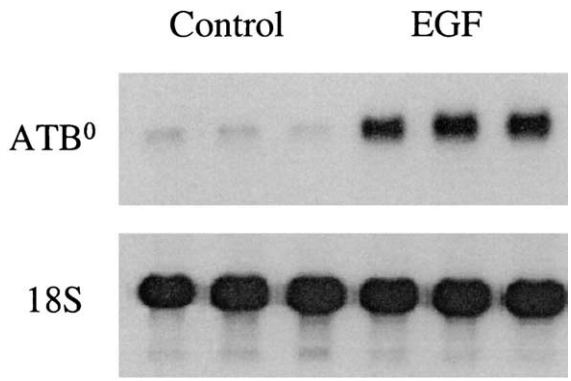

Fig. 4. Northern blot of system B mRNA (ATB^0). Glutamine transporter ATB^0 levels were measured in cells incubated with EGF (0 and 100 ng/ml) for 48 hours.

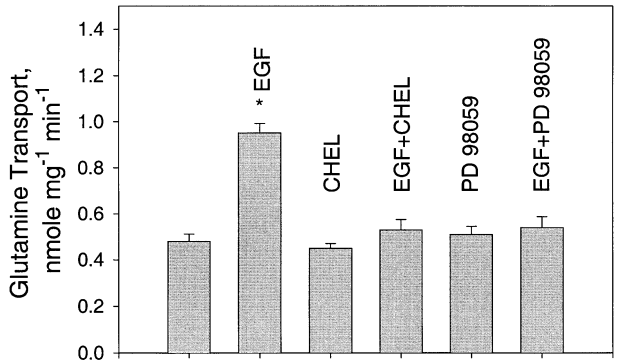

Fig. 6. Effect of inhibitors of PKC and MAPK MEK1 on EGF stimulation of system B glutamine transport. Uptake of glutamine (50 μmol/L) was measured in cells incubated with EGF (100 ng/ml) ± PKC inhibitor chelerythrine chloride (*CHEL*; 6.6 μmol/L), and MAPK MEK 1 inhibitor PD 98059 (50 μmol/L). Transport values are means ± SEM (n = 9, $^*P < 0.01$).

tions of Act-D and CHX were selected to minimize the nonspecific inhibitory effect Act-D and CHX might have on cells. The concentration of Act-D and CHX was selected so that baseline control cell transport activity was not affected to minimize the nonspecific inhibition effect. The viability (by dye exclusion) of both control and Act-D/CHX–treated cells was greater than 99%. The in vitro Caco-2 cells are a growing cell line in a culture environment and continue to grow even under experimental conditions. Compared to the control group (with only DMEM treatment), the 48-hour Act-D–treated or CHX-treated cells had 20% less protein and 40% fewer cells but these were comparable to the pretreatment levels. These findings suggest that Act-D or CHX blocks overall new transcription and new protein synthesis without affecting the existing cells. The inhibitory effect of Act-D or CHX on the system B glutamine uptake was likely due to inhibiting new protein synthesis rather than cytotoxic effect.

The elevation of transporter ATB^0 mRNA after EGF treatment (see Fig. 4) indicates that EGF stimulates system B glutamine transport activity by either specifically enhancing the system B transporter ATB^0 transcription or stabilizing the transcribed mRNA. Further studies such as nuclear runoff assays measuring changes in transcription of ATB^0 genes and ATB^0 mRNA stability assays measuring mRNA half-life, currently being conducted in our laboratory, will determine whether the induction of the transporter mRNA level results from increased ATB^0 gene transcription and/or increased ATB^0 mRNA stability. Kinetic analyses of system B activity showed that EGF stimulated the transport maximal capacity V_{max} without affecting the apparent K_m (see Fig. 2). These data indicated that EGF stimulates glutamine uptake by increasing functional copies of system B transport units rather than by modifying transport affinity. Because a system B antibody is currently not available, it is unclear whether the observed increase in transport activity V_{max} reflects de novo protein synthesis of the transporter protein itself or another regulatory protein.

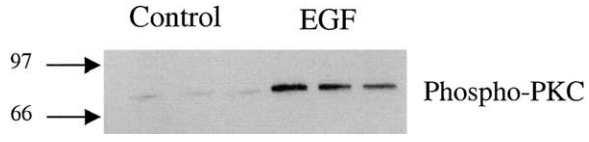

Fig. 5. Western blot of Phospho-PKC (pan). Whole-cell phospho-PKC (pan) levels were measured using monoclonal phospho-PKC antibody in cells incubated with EGF (0 and 100 ng/ml) for 48 hours.

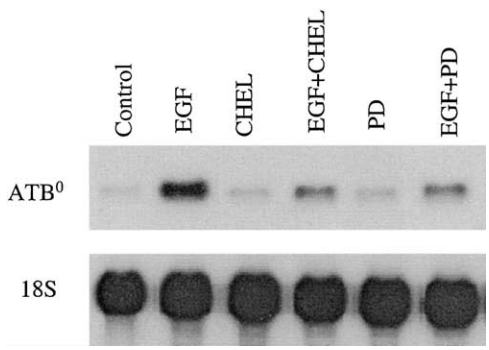

Fig. 7. Northern blot of system B mRNA (ATB^0). Glutamine transporter ATB^0 levels were measured in cells incubated with EGF (0 and 100 ng/ml) for 48 hours ± PKC inhibitor CHEL (6.6 μmol/L), or MAPK MEK 1 inhibitor PD 98059 (50 μmol/L).

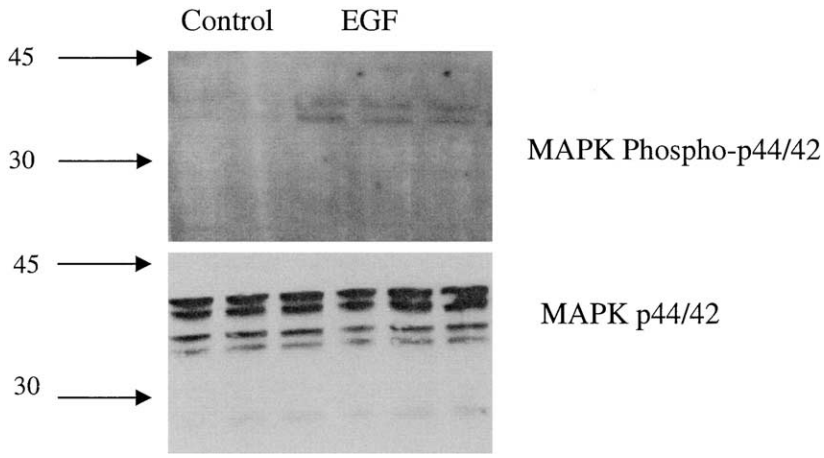

Fig. 8. Western blot of MAPK p44/42 and phospho-p44/42. Whole-cell p44/42 and phospho-p44/42 levels were measured using monoclonal MAPK p44/42 and MAPK p44/42 antibodies in cells incubated with EGF (0 and 100 ng/ml) for 48 hours.

As shown in Fig. 5, EGF incubation increases the phospho-PKC level indicating increased PKC activity. By itself, increased phospho-PKC only indicates that EGF activates PKC; it does not establish a linkage between EGF and glutamine transport. The blockade of the EGF-induced glutamine transport activity and transporter ATB^0 mRNA level by the specific PKC inhibitor CHE,[27] in addition to the direct activation of PKC by EGF, demonstrates the involvement of PKC in signaling events associated with EGF system B induction in Caco-2 cells.

MAPKs are a family of kinases that mediate various biological activities and regulation of gene expression in response to various stimuli.[31–33] There are at least four distinctly regulated groups of MAPKs: extracellular signal-related kinases (ERK)-1/2, Jun amino–terminal kinases (JNK1/2/3), p38 protein (p38α/β/γ/δ), and ERK5. Each group is activated by specific MAPK kinases such as MEK1/2 for ERK1/2 and MKK3/6 for p38.[28–30] EGF initiates many of its biological activities via activation of intracellular MAPK pathways.[31–34] The intracellular signaling cascade for EGF-induced system B glutamine transport in Caco-2 cells is unknown.

To assess the role of MAPKs in EGF-induced system B glutamine transport, we first demonstrated that EGF activates the MAPK kinase cascade. EGF stimulated the MAPK phospho-p44/42, the active form of MAPK P44/42, without altering total p42/p44, indicating that EGF activates MAPK p44/42 (Fig. 8). Furthermore, MAPK MEK1/2 activity was elevated by EGF-exposure (see Fig. 8), suggesting that the EGF in our experimental conditions activates MAPK MEK1/2 cascade. These data demonstrate that EGF is an activator of MAPK MEK cascade but do not establish a linkage between MAPK and glutamine transport. To delineate the relationship among EGF, MAPK, and system B glutamine transport, we measured Caco-2 system B glutamine transport activity in the presence of individual MAPK inhibitors. 2′-amino-3′-methoxyflavone (PD 98059) is a potent cell-permeable and selective inhibitor of MAPK/ERK kinase 1 (MEK1).[35] This inhibitor blocks the activation of MEK1, therefore inhibiting the subsequent phosphorylation and activation of MAPKs such as ERK and biological responses. As shown in Figs. 6 and 7, PD 98059 blocked EGF-stimulated glutamine transport activity and transporter ATB^0 mRNA level without affecting the baseline activity, suggesting that EGF-induced upregulation of system B glutamine transport activity involved MAPK ERK cascade. These data demonstrate that EGF stimulates the MAPK MEK1/2 cascade, which mediates the EGF stimulation of system B glutamine transport activity in Caco-2 cells.

In summary, EGF stimulates intestinal system B glutamine transport activity and transporter ATB^0 mRNA expression. This stimulation is the result of an increase in transporter units rather than modifying transporter affinity, most likely because of a de novo synthesis of new transporters. Furthermore, this EGF-activated system B glutamine transport is mediated by intracellular PKC and MAPK MEK1/2 pathways.

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Southwestern Center for Minimally Invasive Surgery (SCMIS): Laparoscopic Bariatric Surgery, January 24–25, 2003; May 30–31, 2003; September 26–27, 2003; The University of Texas Southwestern Medical Center at Dallas. Cost: physicians ($300, lecture only; $1050, lecture and lab); UTSW and SCMIS Alumni ($250, lecture only; $950, lecture and lab); nurse ($175, lecture only; $375, lecture and lab). For further information contact: Jennifer Leedy, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9059. Phone: 214-648-3792; fax: 214-648-2317; e-mail: jennifer.leedy@utsouthwestern.edu

14th Annual Colorectal Disease Symposium, An International Exchange of Medicinal and Surgical Concepts, February 13–15, 2003, Marriott's Harbor Beach Resort, Fort Lauderdale, Florida. Sponsor: Cleveland Clinic Florida. Symposium director: Steven D. Wexner, M.D. CME credit: 26 Category 1. Contact information: Cleveland Clinic Florida, Department of Continuing Education, 2950 Cleveland Clinic Boulevard, Weston, FL 33331. Phone: 954-659-5490; toll free: 866-293-7866, ext. 55490; fax: 954-659-5491; e-mail: cme@ccf.org

Surgery of the Foregut, February 17–18 2003, Biltmore Hotel, Coral Gables, Florida. Meeting sponsor: Cleveland Clinic Florida. For further information contact: Cleveland Clinic Florida, Office of CME, 2950 Cleveland Clinic Boulevard, Weston, FL 33331. Phone: 954-659-5490; toll free: 866-293-7866 ext. 55490; fax: 954-659-5491; e-mail: cme@ccf.org

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Female Pelvic Floor Disorders, March 14–16, 2003, Sheraton Yankee Trader Beach Hotel, Fort Lauderdale, Florida. Meeting sponsor: Cleveland Clinic Florida. For further information contact: Cleveland Clinic Florida, Office of CME, 2950 Cleveland Clinic Boulevard, Weston, FL 33331. Phone: 954-659-5490; toll free: 866-293-7866 ext. 55490; fax: 954-659-5491; e-mail: cme@ccf.org

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Southwestern Center for Minimally Invasive Surgery (SCMIS): Laparoscopic Management & Percutaneous Ablation of Small Retinal Tumors, July 25–26, 2003; The University of Texas Southwestern Medical Center at Dallas. For further information contact: Jennifer Leedy, UT Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390-9059. Phone: 214-648-3792; fax: 214-648-2317; e-mail: jennifer.leedy@utsouthwestern.edu

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