Randomized Prospective Trial of Pylorus-Preserving vs. Classic Duodenopancreatectomy (Whipple Procedure): Initial Clinical Results

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During the past decades, the classic Whipple resection (cWhipple) and the pylorus-preserving Whipple (ppWhipple) operation have been advanced for the resection of cancer of the pancreatic head. However, no definitive answer exists as to whether the more conservative ppWhipple operation indeed equalizes the short- and long-term results of the cWhipple procedure. Therefore we conducted a randomized prospective trial in a nonselected series of consecutive patients. Demographics, diagnostic, intraoperative, and histologic findings (tumor type and tumor stage of these patients) as well as postoperative mortality, morbidity, and follow-up after discharge were analyzed. For statistical evaluation Kruskal-Wallis and chi-square tests were used where appropriate. Survival was analyzed according to Kaplan-Meier curves, and differences were examined using the log-rank test. From June 1996 to April 1999, a total of 114 patients with suspected pancreatic or periampullary tumors were prospectively randomized to undergo either a cWhipple or a ppWhipple (intention to treat) operation. Based on the inclusion and exclusion criteria, 77 of these patients were included in the final analysis. Forty had a cWhipple and 37 had a ppWhipple resection. There were no differences with regard to age, sex distribution, ASA classification, histologic classification, UICC stage, length of stay in the intensive care unit, and length of hospital stay. The ppWhipple group had a significantly shorter operative time, reduced blood loss, and fewer blood transfusions. There was no difference in mortality, but the cWhipple group showed a significantly higher total morbidity. The incidence of delayed gastric emptying was identical in both groups. For long-term follow-up, a total of 61 patients with histologically proven pancreatic or periampullary carcinoma were analyzed. There were no differences in tumor recurrence or in long-term survival at a median follow-up of 1.1 years (range 0.1 to 2.9 years). Our initial results demonstrate that the cWhipple and ppWhipple operations are equally radical. However, ppWhipple may be the procedure of choice for the treatment of pancreatic and periampullary cancer. (J GASTROINTEST SURG 2000;4:443-452.)

KEY WORDS: Randomized trial, classic Whipple, pylorus-preserving Whipple, pancreatic neoplasm, periampullary neoplasm, morbidity and mortality, survival

Pancreatic cancer is a devastating disease that is currently the fifth leading cause of cancer-related death in the Western world.[1] During the past two decades, advances in surgical technique have drastically reduced the mortality for pancreatic resections, and a mortality rate of less than 5% has been achieved at some referral centers.[2-4] Because of the aggressive nature of these tumors and their resistance to oncologic treatment modalities, most patients suffer from early local tumor recurrence or metastatic disease.[1] Therefore 5-year survival after pancreatic resection with curative intention averaged approximately 10% to 15% in recently reported large series.[5-7]

For many years the surgical procedure of choice was the duodenopancreatectomy or classic Whipple operation inaugurated by Drs. Walter Kausch[8] and Allen O. Whipple.[9] The resection encompasses an en bloc removal of the pancreatic head, the duodenum, the common bile duct, and the gallbladder as well as the distal portion of the stomach together with the adjacent lymph nodes.[2] However, this operation is associated with the well-known side effects of a partial

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stomach resection such as postoperative weight loss and early and late dumping. Therefore a more conservative procedure that preserves the antral and pyloric region was developed. The first pylorus-preserving operation in combination with a pancreatic resection was performed in 1942 by Dr. Kenneth Watson,[10] a surgeon from Great Britain. The procedure was then popularized by Traverso and Longmire.[11] They like Watson reasoned that preservation of the stomach would eliminate the side effects of partial gastrectomy and therefore benefit their patients.[12]

Although this method was originally described for the treatment of periampullary tumors, many surgeons began using the pylorus-preserving Whipple procedure to treat cancer of the pancreatic head as well. However, controversy still exists among surgeons concerning the radicality of this surgical procedure in pancreatic cancer. According to Roder et al.,[13] patients with UICC stage III pancreatic cancer showed a reduced survival after the pylorus-preserving procedure, but theirs was a retrospective analysis.[13] Several other nonrandomized studies did not show any significant difference in survival between the two procedures in patients at comparable tumor stages.[14-19] The occurrence of postoperative gastric ulcers was claimed to be a typical complication of the pylorus-preserving procedure. However, recent data have shown a similar or even lower incidence of anastomotic ulceration after the pylorus-preserving Whipple technique.[14,16,20] There is also still some concern about disturbed postoperative gastric function resulting in significant delayed gastric emptying. However, findings in recent studies can no longer support this argument against pylorus-preserving resection.[13,20-23]

The main advantages of the pylorus-preserving technique seem to be the decrease in operative time and the reduced blood loss because the gastric resection has been omitted. Furthermore, access to the biliary anastomosis may be more easily accomplished for postoperative endoscopic investigations than is possible after classic Whipple resection in patients with recurrent biliary obstruction.[24,25] The perioperative morbidity and mortality of the pylorus-preserving Whipple procedure seems to be lower or at least similar to that of the classic pancreatoduodenectomy.[3,19] In addition, preservation of the pylorus seems to result in an improved postoperative weight gain and a superior quality of life compared to the standard Whipple procedure.[14,15] Until now, no randomized controlled trials existed that could provide unbiased answers to these important questions. We therefore conducted a randomized prospective and consecutive trial in a nonselected group of patients referred to our surgical department with suspected pancreatic or periampullary cancer.

PATIENTS AND METHODS

This report was prepared in accordance with the Consolidated Standards of Reporting Trials (the "CONSORT" statement).[26]

Study Design and Protocol

The study protocol was approved by the Ethics Committee of the University of Bern, Switzerland. The design of the trial consisted of a pretreatment evaluation, randomized treatment with either a classic (cWhipple) or a pylorus-preserving Whipple (pp-Whipple) resection, in-hospital postoperative follow-up, and long-term follow-up every 3 months until postoperative month 60 (the trial remains ongoing).

Inclusion Criteria. All patients considered suitable for surgery, with suspected pancreatic or periampullary cancer assumed resectable according to preoperative diagnostic imaging (CT and/or MRI) and with no history of previous gastric resection, were included.

Exclusion Criteria. Patients with direct invasion of the proximal duodenum, pylorus, or stomach as well as patients with positive peripyloric lymph nodes were excluded. Also excluded were patients who had distant metastases or tumors that were locally unresectable because of major retroperitoneal infiltration according to intraoperative findings. Patients requiring emergency resections were also excluded. All of the remaining patients were included in the analysis for efficacy. However, for follow-up evaluation and analysis of survival, patients with lesions other than pancreatic or periampullary adenocarcinomas were excluded.

Preoperative Evaluation. All patients had a complete physical examination. Routine laboratory tests consisted of hematologic, liver, and renal function tests and determination of the tumor markers CEA and CA19-9. Each patient underwent lung spirometry and ergometry. The perioperative risk was assessed according to the ASA classification.[27] Basic diagnostic imaging included a chest x-ray examination and CT scan, and/or MRI was performed in all patients. Patients with jaundice underwent endoscopic retrograde cholangiopancreatography with stent placement in the common bile duct if technically feasible.

Blinding and Randomization. An equal number of envelopes with detailed protocols for the cWhipple procedure or ppWhipple resection were prepared in a blinded manner. The envelopes were used sequentially as patients were enrolled in the study. There was thus a strict randomization in two arms. Envelopes from patients who were excluded were not used for the study and were discarded. The randomization was carried out the evening before the operation.

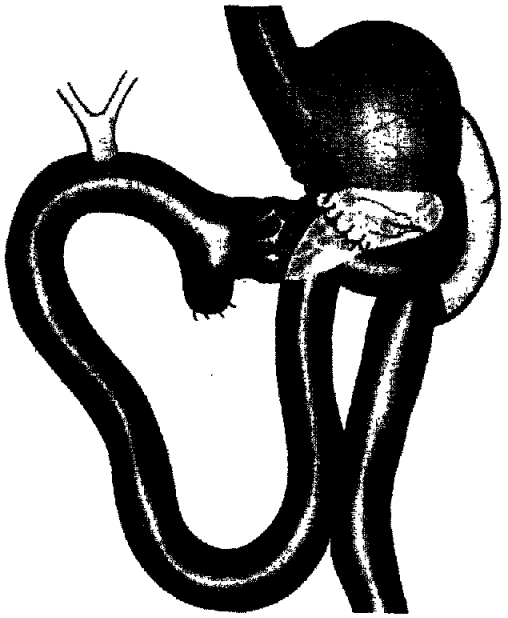

Fig. 1. Classic Whipple resection. For this reconstruction, an end-to-side pancreatojejunostomy is performed in an interrupted two-layer fashion. Six to eight stitches are used to suture the duct to ensure direct anastomosis of the pancreatic duct to the jejunal mucosa. Then an end-to-side hepaticojejunostomy, 10 to 15 cm distal to the pancreatic anastomosis, is created and the reconstruction is completed with an end-to-side gastrojejunostomy approximately 40 cm distal to the biliodigestive anastomosis fashioned with a retrocolic omega loop technique followed by a Braun jejunojejunostomy.

Surgery. All patients were placed on a regimen of prophylactic antibiotics consisting of 4 g piperacillin (Pipril, Lederle, Zug, Switzerland) and 1 g ornidazol (Tiberal, Roche Pharma AG, Reinach, Switzerland). In addition, octreotide (Sandostatin, Novartis, Bern, Switzerland) was administered to all patients undergoing a pancreatic resection, starting perioperatively and continuing for 7 days at dosages ranging from 100 to 200 μg given subcutaneously three times a day.[28]

The abdominal cavity was accessed through a midline incision. If there was no evidence of distant metastases or major infiltration of the retroperitoneum, further preparation for resection was undertaken. For a standard Whipple resection, dissection of the gastrocolic ligament was followed, the lesser sac was opened widely, and a Kocher maneuver was performed to fully expose the pancreatic head. The neck of the pancreas was mobilized from the superior mesenteric vein. Resection encompassed removal of the gallbladder and the common hepatic duct together with all adjacent lymphatic tissue. The pancreatic head was removed en bloc with the duodenum and the distal stomach together with the peripancreatic lymph nodes. Reconstruction was performed by means of an interrupted two-layer end-to-side pancreatojejunostomy with the addition of six to eight stitches for suturing the duct to ensure direct anastomosis of the pancreatic duct to the jejunal mucosa (Fig. 1). Reconstruction was completed by an end-to-side hepaticojejunostomy 10 to 15 cm distal to the pancreatic anastomosis and an end-to-side gastrojejunostomy approximately 40 cm distal to the biliodigestive anastomosis followed by a Braun jejunojejunostomy (see Fig. 1). Two soft drains were placed, one close to the pancreatic anastomosis and the other near the hepaticojejunostomy. Then the abdomen was closed with a one- or two-layer running suture.

The pylorus-preserving technique followed basically the same steps as those used for a Whipple resection with a few important exceptions. The pyloric branches of the right gastric artery were dissected in most of the ppWhipple operations to ensure oncologic radicality. The gastroepiploic arteries along the greater curvature of the stomach as well as the vagal branches to the pylorus were preserved. The gastroduodenal artery was dissected near its origin, and the duodenum was divided approximately 2 to 3 cm distal to the pylorus (Fig. 2). The pancreatojejunostomy and hepaticojejunostomy were identical to the reconstruction used for the Whipple resection (see Fig. 2). The intestinal food passage was created by an end-to-side duodenojejunostomy performed with two layers of running sutures (see Fig. 2). Both operations were carried out in combination with a standard lymph node dissection of the celiac trunk, the hepatoduodenal ligament, the superior mesenteric vein, the right side of the superior mesenteric artery, and the lymphatic tissue behind the head of the pancreas. Blood loss and operative time were assessed immediately after surgery by the surgeon and the anesthesiologist. All operative specimens were examined by intraoperative frozen section analysis and definitive histologic investigations after surgery.

Postoperative Management and Follow-Up. Tumor stage was determined according to the UICC classification and the TNM system. The secretion volume from the gastric tube and the volume of abdominal drainage were measured daily and the amy-

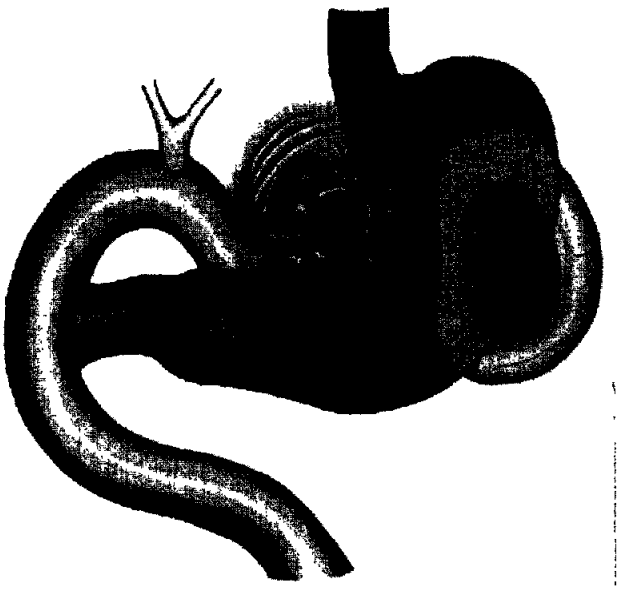

Fig. 2. Pylorus-preserving Whipple resection. This resection is basically identical to the classic Whipple procedure except that the stomach and proximal 3 to 4 cm of the duodenum are preserved. Therefore the gastroepiploic arteries along the greater curvature of the stomach as well as the vagal branches to the pylorus are preserved. In contrast, the right gastric artery is normally dissected to ensure oncologic radicality. The gastroduodenal artery is dissected near its origin followed by division of the duodenum approximately 3 to 4 cm distal to the pylorus. The reconstruction is identical to that used in the classic Whipple procedure. Intestinal continuity is achieved by means of an end-to-side duodenojejunostomy with a two-layer running suture.

lase concentration of the latter was determined every other day. All patients, regardless of the type of resection performed, underwent routine contrast imaging of the stomach 5 days after surgery prior to beginning of oral intake of food. Mortality and surgical and nonsurgical morbidity were recorded. Follow-up evaluations were conducted every 3 months following discharge and consisted of physical examination, routine laboratory tests including determination of CEA and CA19-9 values, estimation of tumor recurrence, and long-term survival.

The standard postoperative treatment followed generally accepted principles of surgical and supportive care including hemodynamic monitoring with a central venous catheter, urinary catheter, fluid balance, and adequate parenteral replacement of electrolytes. The nasogastric tube was removed when less than 300 ml fluid was secreted per day. Perioperative antibiotics and postoperative analgesics (intravenous and peridural) were administered according to the protocol or as needed, respectively. Patients were given nothing by mouth for the first 48 hours after surgery. Thereafter patients were allowed to consume a maximum of 300 ml of fluid per day until routine contrast x-ray examination of the stomach was performed. Either oral food intake or parenteral alimentation was then started where appropriate.

End Points of the Study

We analyzed multiple variables such as the complication rate, total morbidity, survival, and tumor recurrence. Therefore primary and secondary outcome variables have been determined according to the study protocol (Table I). Delayed gastric emptying was defined in our study as a persistent secretion over the gastric tube of more than 500 ml per day for more than 5 days after surgery or recurrent vomiting in combination with swelling of the gastrojejunostomy/duodenojejunostomy and dilatation of the stomach in contrast medium passage studies.[29] All patients with delayed gastric emptying received parenteral or enteral nutrition through a tube jejunostomy until they were able to tolerate solid food by mouth. A pancreatic fistula was defined as secretion of more than 30 ml of amylase-rich fluid (amylase concentration over 5000 IU) over the abdominal drainage tube for more than 10 days. Postoperative bleeding was defined as blood loss either through the

Table I. Primary and secondary outcome variables

Primary outcome variables
Total morbidity
Intraoperative complications
Postoperative complications
Intraoperative blood loss
Intraoperative blood substitution
Operating time
Secondary outcome variables
Tumor recurrence
Survival

Table II. Patient characteristics in intention-to-treat analysis (n = 114)

Demographics	Total (n = 114)	cWhipple (n = 58)	ppWhipple (n = 56)
Males	62 (54%)	30 (52%)	32 (57%)
Females	52 (46%)	28 (48%)	24 (43%)
Age (yr)	65 (range 26-86)	65 (range 33-86)	65 (range 26-83)
ASA class			
I-II	68%	66%	70%
III-IV	32%	34%	30%

abdominal drains or by hematemesis in combination with a decrease in the systemic hemoglobin concentration of more than 20 g/L to a value of less than 80 g/L within 24 hours, requiring at least two units of blood to prevent further blood loss. Although the incidence of postoperative ulcer disease after cWhipple and especially ppWhipple resection is an interesting and thus far not fully explored topic, patients included in the trial did not undergo routine endoscopy either postoperatively or during follow-up evaluation to objectively address this point. Based on our pretrial experience, which encompassed more than 200 pancreatic head resections at our institution, the occurrence of gastric ulcer disease was a rare event and routine endoscopy therefore did not seem justified. However, endoscopy was performed based on clinical suspicion of ulcer disease. All points of interest as well as all complications or adverse reactions were documented on the patient record forms. Data from all forms were then entered into a statistical database (SPSS, SPSS Chicago, Ill.).

Statistical Analysis

Based on our database where data from all patients undergoing a major pancreatic resection are recorded, the percentage of patients with one or more postoperative complications (applying the same definitions for delayed gastric emptying and pancreatic fistula as given earlier) following cWhipple resection was approximately 50% during the past 5 years. For the present study we hypothesized that the morbidity of the ppWhipple resection would be similar to that of the cWhipple resection. A power analysis indicated that 58 patients would have to be enrolled in each group to detect an alteration of 25% in morbidity at the two-sided 5% significance level with a power of 80%.

After enrolling more than 110 patients, we performed an interim analysis to compare early clinical (postoperative) results including operative time, blood loss, and blood transfusion. The results of long-term follow-up (tumor recurrence and survival) will be reported after the completion of the trial. For qualitative analyses, a chi-square test was applied and a Kruskal-Wallis H test or Mann-Whitney U test was used for quantitative variables. The analysis of survival was calculated according to the Kaplan-Meier method and the levels of significance were tested with a log-rank test.

RESULTS

Patient Characteristics

In total, 114 consecutive patients with suspected pancreatic or periampullary tumors were enrolled from June 1996 to April 1999. Primary and secondary outcome variables are detailed in Table I. Age, sex, and ASA classification of these 114 patients (intention-to-treat analysis) are presented in Table II. Because of the intraoperative findings (exclusion criteria), 37 patients (33%) had to be excluded. This was due to the presence of distant metastases in 19 patients and a locally unresectable tumor in six patients. In 10 patients a pylorus-preserving pancreatic head resection (ppWhipple procedure) was not possible because of tumor infiltration of the proximal duodenum (n = 1), the need for total pancreatectomy (n = 7), and resection of the pancreatic tail due to a tumor located in the body of the pancreas (n = 2). Therefore these patients were excluded from the trial. In two patients with suspected pancreatic cancer, frozen sections revealed chronic pancreatitis.

End Points of the Study

The remaining 77 patients (valid for efficacy) underwent pancreatic resection according to the randomization protocol. There were no significant differences between the two groups with regard to patient demographics in these 40 cWhipple and 37 ppWhipple operations (Table III). The histologic diagnosis of the patients is depicted in Table IV. Again there were no significant differences between the two groups. Operative time, blood loss, and the amount of

Table III. Patient characteristics in valid for efficacy analysis (n = 77)

Demographics	Total (n = 77)	cWhipple (n = 40)	ppWhipple (n = 37)
Males	36 (47%)	17 (43%)	19 (52%)
Females	41 (53%)	23 (57%)	18 (48%)
Age (yr)	66 (range 26-83)	67 (range 26-83)	67 (range 26-83)
ASA class			
I-II	65%	63%	68%
III-IV	35%	37%	32%

Table IV. Final histologic findings after duodenopancreatectomy (n = 77)

Characteristics	Total (n = 77)	cWhipple (n = 40)	ppWhipple (n = 37)	Significance
Histologic findings				
Pancreatic tumor	52 (67%)	30 (75%)	22 (59%)	NS
Ampullary tumor	9 (12%)	1 (2%)	8 (22%)	NS
Common bile duct tumor	14 (18%)	8 (20%)	6 (16%)	NS
Duodenal sarcoma	1 (1.3%)		1 (3%)	NS
Chronic pancreatitis	1 (1.3%)	1 (2%)		NS
Tumor stage (UICC)				
I	8 (10%)	4 (10%)	4 (11%)	NS
II	13 (17%)	5 (12%)	9 (24%)	NS
III	41 (53%)	25 (63%)	16 (43%)	NS
IV	1 (1.3%)	1 (3%)		NS
No cancer	13 (17%)	5 (12%)	8 (22%)	NS

NS = not significant.

Table V. Intraoperative findings and length of hospital stay (n = 77)

Parameters	cWhipple (n = 40)	ppWhipple (n = 37)	Significance
Operation time (median ± SE; min)	476 (range 240-780)	404 (range 240-645)	$P = 0.04$
Blood loss (median ± SE; ml)	2096 (range 800-6,000)	1453 (range 400-4,000)	$P = 0.03$
Blood transfusion (median ± SE; units)	3.6 (range 0-10)	2.1 (range 0-6)	$P = 0.05$
ICU stay (median ± SE; days)	2 (range 1-7)	2 (range 1-5)	NS
Hospital stay (median ± SE; days)	24 (range 8-67)	25 (range 12-61)	NS

blood transfused, however, were significantly reduced in the ppWhipple group ($P = 0.04$, $P = 0.03$, and $P = 0.05$, respectively). Length of stay in the intensive care unit and length of hospital stay were again unchanged in the two groups (Table V). In-hospital mortality was comparable in the two groups and was 5% after cWhipple and 2.7% after ppWhipple resections, respectively ($P = 0.61$). The deaths were caused by acute cardiac failure and endocarditis in the Whipple group. One patient died of Candida sepsis after a ppWhipple resection. Two patients (one in each group) required relaparotomy because of hemodynamically significant hemorrhage adding to a reoperation rate of 2% after cWhipple vs. 3% after ppWhipple ($P = 0.95$) resection. Postoperative cumulative morbidity (occurrence of delayed gastric emptying included) was significantly higher after cWhipple compared to ppWhipple resection (72.5% vs. 57%, respectively; $P = 0.05$), but individual surgical and medical postoperative complications were not significantly different between the two groups (Table VI). Especially the incidence of delayed gastric emptying, according to our

Table VI. Surgical and medical morbidity (n = 77)

Morbidity	Total (n = 77)	cWhipple (n = 40)	ppWhipple (n = 37)	Significance
Surgical				
Delayed gastric emptying*	30 (39%)	18 (45%)	12 (32%)	NS
Bleeding	5 (6%)	4 (10%)	1 (3%)	NS
Fistula†	2 (3%)	1 (2%)	1 (3%)	NS
Infection (wound/abscess)	6 (8%)	3 (7%)	3 (8%)	NS
Medical				
Pulmonary	14 (18%)	9 (22%)	5 (13%)	NS
Cardiocirculatory	6 (8%)	4 (10%)	2 (5%)	NS
Renal	6 (8%)	4 (10%)	2 (5%)	NS
Other	6 (8%)	4 (10%)	2 (5%)	NS
Relaparotomy	2.6%	2.5%	2.8%	NS
Cumulative morbidity	65%	72%	57%	$P = 0.05$
Mortality	3.9%	5%	2.7%	NS

*Delayed gastric emptying was defined as persistent secretion over the gastric tube of more than 500 ml/day for more than 5 days after surgery or recurrent vomiting in combination with swelling of the gastrojejunostomy/duodenojejunostomy and dilatation of the stomach in contrast medium passage studies. All patients with delayed gastric emptying were unable to tolerate solid foods by mouth for at least 10 days following surgery, and therefore received parenteral or enteral nutrition through a tube jejunostomy until solid foods were tolerated.
†One pancreatic fistula after ppWhipple and one biliary fistula after cWhipple resection.

definition as stated earlier, was not influenced by the operative procedure (45% after cWhipple vs. 32% after ppWhipple; $P = 0.17$). All patients suffering from delayed gastric emptying were unable to tolerate solid food for at least 10 days after surgery. Therefore all those patients diagnosed as having delayed gastric emptying according to our definition also fulfilled the more commonly used definition (inability to tolerate food during the first 10 days postoperatively) as used by many other surgeons. All these patients were successfully treated conservatively. There was one patient with a pancreatic fistula in the ppWhipple group and one with a biliary fistula after cWhipple resection. Both fistulas were treated nonoperatively.

Follow-Up Results. Based on the final histologic diagnosis, 13 patients with benign lesions were excluded from the follow-up study (5 after cWhipple and 8 after ppWhipple). Therefore 61 patients (33 after cWhipple and 28 after ppWhipple) were included in the final analyses of long-term survival and tumor recurrence. The histologic cancer classification of these 61 patients showed no difference between the two groups. There were 24 pancreatic and nine periampullary cancers in the Whipple group (73% and 27%, respectively). In the ppWhipple group, 18 patients had pancreatic cancer and 10 had periampullary cancer (64% and 36%, respectively). Median follow-up was 1.1 years with a range of 0.1 to 2.9 years. Seventeen patients developed tumor recurrence after cWhipple and 11 patients after ppWhipple resection (51% and 39%, respectively; $P = 0.3$). During follow-up, 11 patients in the cWhipple group died of tumor

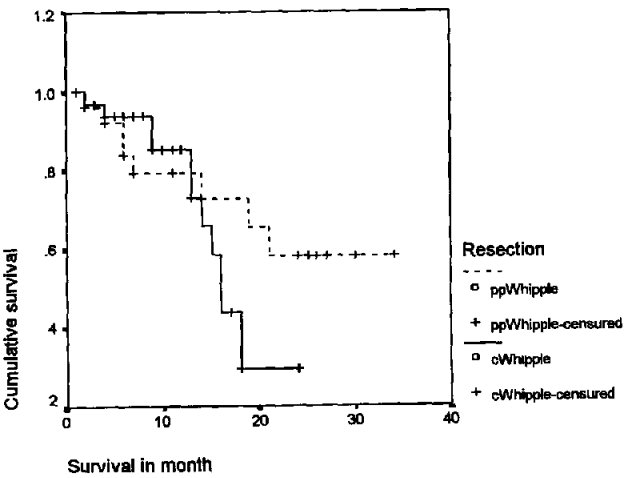

Fig. 3. Kaplan-Meier survival curves for all patients with histologically proved pancreatic or ampullary cancer undergoing either cWhipple procedure (n = 33) or ppWhipple resection (n = 28). The median survival following cWhipple and ppWhipple operations showed no significant difference and was 16 months and 24 months, respectively ($P = 0.29$). † = censored patients (patients still alive).

recurrence compared to eight patients after ppWhipple resection (33% and 29%, respectively; $P = 0.69$). No patient had to be reoperated. According to the Kaplan-Meier analysis, median survival time was 16 months after cWhipple and 24 months after ppWhipple resection (Fig. 3). There was no statistically significant difference in survival ($P = 0.29$; see Fig. 3).

DISCUSSION

During the past two decades, the mortality after pancreatoduodenectomy has markedly decreased, so that a mortality rate of less than 5% is currently considered standard.[2-4] With the introduction of octreotide in combination with refinements in surgical technique, a significant reduction in the high incidence of pancreatic fistulas with their well-known septic complications has been observed.[28,30] Despite these advances, there is still no generally accepted agreement as to which surgical procedure offers the best possible treatment for patients with cancer in the pancreatic head or the periampullary region. In Japan many surgeons favor an aggressive type of resection with radical lymph node dissection.[31-33] Most surgeons in the United States and Europe still favor the classic Whipple procedure as the standard type of resection.[5,34,35]

With the introduction of the pylorus-preserving Whipple technique by Watson,[10] and later by Traverso and Longmire,[11] an increasing number of surgeons now apply this procedure even for the treatment of pancreatic and periampullary cancers.[13-20,36] However, preservation of the pylorus in patients undergoing a duodenopancreatectomy for cancer remains a controversial issue. The arguments against the use of the pylorus-preserving Whipple procedure were the high incidence of anastomotic ulceration at the duodenojejunostomy, an increase in delayed gastric emptying because of disturbed perfusion, and neural innervation of the pylorus and very importantly a compromised radicality.[13,37] However, recent data could not substantiate any of these arguments.[13,14,16,20-22] In contrast to early reports, the occurrence of delayed gastric emptying after a ppWhipple resection is comparable to or lower than reported cases after a classic Whipple procedure in recent series.[19,20,23] The incidence of delayed gastric emptying strongly correlates with the rate of surgically related complications in one prospective but nonrandomized study.[23] Furthermore, in another prospective randomized trial comparing pylorus-preserving Whipple resection with the duodenum-preserving resection of the pancreatic head in patients suffering from chronic pancreatitis, none of the 20 patients in the Whipple group suffered from delayed gastric emptying.[38] Although there were no patients with pancreatic cancer in this trial, it seems that the extent of the surgical resection, such as extended lymph node dissection, in cancer patients and the occurrence of complications are far more important factors in determining the incidence of delayed gastric emptying than preservation of the pylorus alone.

In terms of long-term survival, only one small retrospective study reported a decrease in survival after preservation of the pylorus in patients with pancreatic and periampullary cancer,[13] whereas several other series found no difference in long-term outcome.[14-19] Furthermore, clinical observations and autopsy studies have shown that the pancreatic resection margin rather than the duodenum or pylorus or the adjacent lymph nodes is more likely to be infiltrated by the tumor.[39-42] There is increasing evidence that the long-term outcome after pylorus-preserving resection is comparable to that of the standard Whipple procedure.

Unfortunately it is still a matter of fact that despite "curative" intended resection (R0), most patients with pancreatic cancer will suffer from early local tumor recurrence or metastatic disease. Current long-term survival rates are averaging approximately 10% in nonselected large series.[5-7] Although it is intended to be "curative," for most of these patients pancreatic resection turns out to be a palliative treatment. Therefore quality of life for patients after pancreatic resection is becoming an important issue. The arguments favoring preservation of the pylorus in this context include improved postoperative weight gain and avoidance of postgastrectomy syndromes.* Furthermore, the ppWhipple technique seems to go along with a shortened operative time and reduced blood loss compared to the standard type of resection.[14,15,19] However, the results in terms of nutritional status and quality of life are also controversial. Whereas some authors observed improved weight gain and better quality of life after the ppWhipple procedure,[14,15] others were unable to show any marked superiority of the ppWhipple procedure.[45,46]

All of these divergent arguments, however, are based on retrospective or nonrandomized prospective studies. To achieve unbiased evidence, controlled and prospective randomized trials are urgently needed. Therefore we conducted this study, which is to our knowledge the first randomized prospective trial on this issue. We hereby report our initial clinical results with a mean follow-up of 1.1 years. The study remains ongoing and data concerning long-term follow-up, ability to work, and quality of life will be published in due time.

From the total of 114 patients enrolled in the preoperative randomization process, 77 patients qualified for the study (based on inclusion and exclusion criteria; see Tables I to IV). There were no differences in patient demographics or in the type of tumor or histologic classification between the two groups. One patient in whom pancreatic cancer was suspected on the basis of intraoperative frozen sections but who was later proved to have chronic pancreatitis on definitive histologic examination remained in the evaluation for

*References 13-15, 17, 19, 20, 36, 43, 44.

efficacy analysis. In agreement with previous reports from the literature,[14,15,19] we noted a significant decrease in operative time and intraoperative blood loss as well as less need for blood transfusions (see Table V). The mortality rate and the rate of reoperation were 3.9% and 2.6%, respectively, for the whole series and were unchanged in either of the two groups. These data compare well with recent data from the literature demonstrating that major pancreatic resections can be performed with acceptable risk.[3,19] Cumulative morbidity was significantly decreased after ppWhipple resection; however, specific surgical and medical complications did not reach a significant difference. In particular, the incidence of delayed gastric emptying was the same after both procedures. To achieve a definite distinction between the two procedures, we used a very sensitive definition for delayed gastric emptying, which may partly explain the considerably high incidence of this complication after both types of resections. Although other authors have used a less discriminating definition such as a minimum of 10 days of persistent gastric reflux after resection, for example,[23] our definition allows a more sensitive comparison between the two groups. There was only one patient out of 77 who had a pancreatic fistula, which was successfully treated by a conservative approach. This observation goes along with other reports showing that the perioperative administration of octreotide in combination with a standardized technique can significantly reduce the risk of postoperative pancreatic fistula.[28,30]

The majority of the nonsurgical complications were the cardiopulmonary disturbances commonly seen after major abdominal surgery. There were no differences in length of stay in the intensive care unit or length of hospital stay after cWhipple compared to ppWhipple resection. A total of 61 patients with pancreatic and periampullary cancer were analyzed for short-term survival (mean 1.1 years). There were no significant differences either in tumor recurrence (17 after cWhipple and 11 after ppWhipple, respectively) or in actuarial survival according to the Kaplan-Meier calculation with a median survival time of 16 months after cWhipple vs. 24 months after ppWhipple resection. Although the trial is ongoing, our initial results indicate that the "more conservative" ppWhipple resection does not seem to compromise long-term survival.

CONCLUSION

Based on our results, we have shown that the standard Whipple resection and the pylorus-preserving technique are both equally effective with comparable and acceptable perioperative risk. The pylorus-preserving type of resection offers the advantage of a shorter operative time, decreased blood loss, and fewer blood transfusions in combination with reduced perioperative morbidity. However, at present a definite answer addressing the superiority of either of these two procedures for the treatment of pancreatic and periampullary tumors cannot be given. It is hoped that the final analysis after completion of this study may provide a definitive conclusion.

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Small-Diameter Mesocaval Shunts: A 10-Year Evaluation

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The use of small-diameter portosystemic shunts for the treatment of bleeding esophageal varices caused by portal hypertension has emerged as an outgrowth of the development of polytetrafluoroethylene vascular grafts, which allow the use of a narrow lumen. We report our experience with this type of graft over a 10-year period. Thirty-three patients with good liver function (Child-Pugh class A) were electively operated. The average age of these patients was 45 years (range 17 to 71 years). Twenty-nine patients had liver cirrhosis, one had portal fibrosis, and three had idiopathic portal hypertension. Operative mortality was 3%, and the rebleeding rate was 15%. Postoperative encephalopathy was observed in 14 patients (11%), three of whom had grade III to IV encephalopathy. The remaining 11 patients, had mild encephalopathy that was easily controlled. Postoperative angiography showed shunt patency in 81% of the patients, reduction in portal vein diameter in 33% of the patients, and portal vein thrombosis in 6%. Good postoperative quality of life was observed in 63% of the patients. Survival according to the Kaplan-Meier actuarial method was 81% at 12 months, 56% at 60 months, and 36% at 10 years. These shunts are a good alternative for patients being considered for surgery in whom other portal blood flow preserving procedures (i.e., selective shunts, devascularization with esophageal transection) are not feasible. (J GASTROINTEST SURG 2000;4:453-457.)

KEY WORDS: Portal hypertension, surgery, small-diameter shunts

Surgery for bleeding esophageal varices due to portal hypertension has expanded widely over the past 50 years. A large number of operative procedures (i.e., shunts and devascularizations) have been developed and evaluated with varying results, depending on such factors as the length of the evaluation period, severity of the underlying liver disease, and the level of expertise of the surgical team. Several other forms of therapy have also been introduced including pharmacotherapy, sclerotherapy and band ligation, transjugular intrahepatic portosystemic shunts (TIPS), and liver transplantation,[1] and each modality has its own indications and selection criteria. It is currently accepted that surgical treatment has the lowest rebleeding rate and is indicated for patients with good liver function whose operations are elective.[2] Portal blood flow preserving procedures are also the operations of choice. Among these procedures, selective shunts are the most commonly used, leaving Sugiura-Futagawa extensive devascularization technique for those patients whose anatomy is unsuitable for a shunt.[3]

In the past two decades, small-diameter portacaval and mesocaval shunts have emerged as an alternative to selective shunts, showing distinct advantages over total shunts (i.e., shunts that divert all of the portal flow) with a low rate of rebleeding and a low incidence of encephalopathy.[4] We report herein our experience with small-diameter (10 mm) ring-reinforced polytetrafluoroethylene (PTFE) mesocaval shunts over a 10-year period.

MATERIAL AND METHODS

Patients with acute variceal bleeding are treated at our institution by means of pharmacotherapy, sclerotherapy, and/or banding. Once the acute episode is controlled, the patient's liver function is reevaluated along with other parameters as listed in Table I. If the

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Table I. Selection criteria for patients undergoing surgery for portal venous hypertension

History of variceal bleeding
Good cardiopulmonary function
Good renal function
Good liver function
Albumin >3.5 g/dl
Prothrombin time <2 seconds
Total bilirubin <2 mg/dl
No ascites
No encephalopathy
Good nutritional status

patient is found to be low risk, surgical treatment is considered. If liver function is poor, transendoscopic treatment and/or pharmacotherapy is recommended. Some of these patients are referred to the liver transplant program at our hospital.

Abdominal, arterial, and venous angiography is performed in all low-risk patients. The hepatic arteriogram is closely examined to rule out any malignancies related to liver disease. In the venous phase, the splenic, mesenteric, and portal veins are carefully evaluated, paying particular attention to diameter and position. The renal vein is also examined by means of venography, and its drainage and position are evaluated. The type of operation selected is based on the anatomic features of each patient. If the splenic vein is found to be of adequate diameter and position, and the renal vein also has good position and distance with adequate drainage, a distal splenorenal shunt is recommended. In patients who have no vessels considered suitable for a shunt, extensive two-stage esophagogastric devascularization with esophageal transection is recommended.[5] When neither a selective shunt nor devascularization (portal blood flow preserving operation) is feasible, and if an adequate mesenteric vein is found, a small-diameter mesocaval shunt is indicated.

Surgical Technique

After the abdomen is opened, a liver biopsy is done. The third portion of the duodenum is mobilized and both the superior mesenteric vein and infrarenal vena cava are dissected free. After placement of vascular clamps, venotomies are performed, and an end-to-side anastomosis of a ring-reinforced 10 mm PTFE graft is constructed. The edges are sutured in an everting fashion, with an oblique section of the graft on each side, which allows good positioning of the graft and a wide opening at the level of the anastomosis (between 15 and 20 mm).

Angiography is performed within the first postoperative month (between 1 and 2 weeks) and then yearly thereafter. We began using this operation at our hospital in 1989. To evaluate the long-term results of this operation, we reviewed the records of all patients operated on between 1989 and 1994, so that all patients had a follow-up period of at least 5 years.

The patients' clinical features were evaluated, as well as operative mortality, rebleeding rate, postoperative encephalopathy, angiographic assessment of the shunt, and portal blood flow. Kaplan-Meier survival curves were constructed.

Quality of Life

Quality of life was evaluated and rated good, fair, or poor according to the following preestablished criteria[6]:

Good = Patient is able to carry out routine daily activities. Patient is able to exercise at an age-appropriate level. Patient has no need for hospitalization. Patient visits the hospital only as required for follow-up studies.

Fair = Patient is able to perform normal daily activities but is unable to exercise. Patient requires occasional nonscheduled hospital visits and also requires long-term drug treatment (e.g., diuretics, cathartics).

Poor = Patient is unable to perform routine daily activities. Patient requires hospitalization for rebleeding and close monitoring by a physician during follow-up. Patient also requires drug treatment.

Evaluation was done by independent observers. Encephalopathy was evaluated by means of clinical parameters, motor function tests, and serum ammonium levels. Rebleeding was defined as the presence of hematemesis and/or melena with a decrease in the hemoglobin level requiring a blood transfusion.

RESULTS

Twenty-nine of the 33 patients included in the study had good liver function and were considered low risk. Hepatitis and alcoholic liver cirrhosis were the most common causes of variceal bleeding. The cause was unknown in three cases, and one case of portal fibrosis was found. Ten patients had prior sclerotherapy, and seven patients had a history of one prior attempt at surgical treatment. In all cases the superior mesenteric vein was found to be suitable for a shunt.

Operative mortality was 3%. One patient who had posthepatitis C cirrhosis died at the end of the fourth postoperative week. The cause of death was multiple organ failure secondary to pneumonia.

Table II. Angiographic findings after mesocaval shunt

	First month	First year	Fifth year
Total patients studied	31	29	14
Angiography	31	25*	14
Shunt patent	25 (81%)	23	14
Thrombosis	6 (19%)	2	0
Portal vein			
Patent	18	16	12
Thrombosed	2†	0	0
Patent with decrease in diameter	11	9	2
Portal flow			
Hepatopetal/encephalopathy	18/3	16/3	8/2
Hepatofugal/encephalopathy	11/16	9/6	4/2

*Patient with shunt thrombosis within the first month did not undergo subsequent angiography.
†Also had thrombosed shunts.

Rebleeding

Five patients (15%) had postoperative bleeding. One patient had bleeding on postoperative day 36, which was controlled with sclerotherapy. Another patient who had bleeding at 24 months' postoperatively was managed with transendoscopic therapy, and two patients with an obstructed shunt developed multiple organ failure and died 12 months and 36 months, respectively after the operation.

Encephalopathy

Encephalopathy was observed in 14 patients (42%). Ten of them were classified as grade I, and were easily controlled with dietary measures, neomycin, and lactulose. One patient had grade II encephalopathy and three patients had incapacitating encephalopathy (grade III in two cases and grade IV in one case). The patient with grade IV encephalopathy (reversible) was lost to follow-up and presumably died. Shunt closure had been planned for that patient.

Postoperative Angiography

Shunt patency was demonstrated in 81% of the patients, and in the remaining patients the shunts were found to be obstructed. Rebleeding occurred in two patients. Portal vein diameter was diminished in 11 patients (33%) and in two patients (6%) total portal vein thrombosis was demonstrated. Complete angiographic findings are presented in Table II.

Survival and Quality of Life

Quality of life was considered good in 21 patients (63%), fair in five patients (14%), and poor in three patients (9%). It was not possible to evaluate quality of life in the remaining four patients (1 operative death, 1 early death, and 2 patients lost for follow-up).

At the end of the study, 14 patients (42%) were alive and 14 (42%) had died. Mean length of follow-up was 72 months, and five patients were lost to follow-up. Survival was 81% at 1 year, 56% at 5 years, and 36% at 10 years.

Rebleeding, encephalopathy, and quality of life were calculated on the basis of 29 cases (until death or lost to follow-up). Four patients with very short follow-up were excluded (1 operative death, one early death [2 months postoperative], and two with short follow-up [lost to follow-up at 2 and 3 months postoperative]).

DISCUSSION

In the past five decades, surgery for portal hypertension has changed with regard to types of procedures and indications. Total shunts, which are effective to control rebleeding, have a negative effect on liver function because of portal blood flow deprivation. However, a well-known study from the Boston Inter-Hospital Liver Group showed no impact on survival.[7] Other types of therapy have also been developed and evaluated, such as transendoscopic sclerotherapy and band ligation,[8] pharmacotherapy,[9] liver transplantation,[10] and transjugular intrahepatic shunts.[11] Surgery for portal hypertension has a well-defined role and is almost universally accepted. Patients with good liver function who are electively operated are the ones who benefit most from this option. Some investigators have also shown that the most successful type of operation is one that preserves portal blood flow. Selective shunts have been shown, in most cases, to main-

tain portal blood flow, and these results have been reproduced in most centers.[12]

Small-diameter shunts have been used as an alternate treatment in selected patients. Sarfeh et al.[13] conducted hemodynamic studies with this type of shunt and summarized their long-term results. In various subsets of patients they have shown low rebleeding and encephalopathy rates, long-term patency, and preservation of liver function. The most widely studied type of operation is the 8 mm portacaval shunt. A prospective controlled randomized study has demonstrated its advantages over total shunt.[14]

Paquet et al.[15] reported good long-term results in their experience with small-diameter mesocaval shunts.[15] Scudamore et al.,[16] in their experience with 15 patients, reported no operative deaths or rebleeding, with a 13% rate of shunt dysfunction and a 20% incidence of postoperative encephalopathy.

Our results differ from those in other reported series studying small-diameter shunts and are most similar to those achieved with the transjugular intrahepatic portosystemic shunt (TIPS).[17] We found a low operative mortality rate (lower than the mortality reported for the TIPS), which is similar to that achieved with selective shunts and devascularization in low-risk patients. This is most likely because our patient population was a selected one.

The rebleeding rate after a 10 mm mesocaval shunt, in our experience, is 15%, which is higher when compared to the 8 mm portacaval shunt and lower when compared to the TIPS procedure, which approaches 25% to 30%. This can probably be explained by the technical differences between these shunts. The rebleeding rate is also lower when compared to that in most series studying the TIPS procedure but is higher than that of small-diameter portacaval shunts.

Most series studying small-diameter portacaval shunts show a lower rate of obstruction (<10%). We think that for mesocaval shunts, there is a greater likelihood of obstruction because the vein has a smaller diameter compared to the portal vein. Also, the mesenteric vein has thinner walls and the anastomosis is technically more demanding.

The rate of encephalopathy was high in our patient population, and this is most likely because of the hemodynamic characteristics of this type of shunt. Preservation of portal blood flow is dependent on two factors: flow and resistance, which correlate with each other. In the case of the TIPS procedure, the shunt is placed at the end of the portal vein, replacing the sinusoidal resistance. This is why most of the flow goes through the shunt, thus eliminating the sinusoidal phase. For the small-diameter portacaval shunt, sinusoidal resistance is the common denominator. If sinusoidal resistance is high, most of the flow will go through the shunt. Sinusoidal resistance is dependent on the subjacent liver disease as well as its degree of activity and is specific for each patient. In our series, we found it difficult to predict which patients would have preserved portal blood flow. Although most of the mesenteric veins evaluated had almost the same diameter, in some instances portal blood flow was preserved, in others it was partially preserved, and in still others it was totally lost. Also, the postoperative characteristics of the portal vein are very difficult to predict (Figs. 1 and 2).

Fig. 1. Selective splenic artery and venous angiography. Venous flow from the spleen is directed into the superior mesenteric vein (retrograde) and through the graft to the systemic circulation. Portal vein is not filled. Hemodynamics are similar to that of the total portacaval shunt.

It is likely that the increased frequency of encephalopathy observed with the mesocaval shunt is due to the quality of the blood shunted. Portacaval shunts (small diameter) derive mixed blood from the mesenteric and splenopancreatic area. Mesocaval shunts divert mainly mesenteric flow.

We have previously compared this type of shunt with the selective shunt, and demonstrated a significant difference favoring the distal splenorenal shunt in which a lower obstruction rate, a lower encephalopathy rate, and fewer postoperative portal vein alterations were found.[18]

We believe that patient selection is as critical as the type of operation selected. It is unwise to perform the same operation in all patients. The decision as to which procedure to choose should be based on the anatomic characteristics of the splanchnic vessels, as demonstrated by means of angiography (and in some cases magnetic resonance angiography). Selective shunts (distal splenorenal shunts) should not be used in cases where an inappropriate splenic vein is found as well as an inadequate renal vein, and a devascular-

Fig. 2. Selective mesenteric artery and venous angiography. The complete flow of the mesenteric vein is diverted through the graft to the systemic circulation. No flow is demonstrated through the portal vein.

ization is not advisable when the patient has a history of one or more previous abdominal operations that would make a devascularization procedure very difficult. For patients with thrombosis of the portal system, a devascularization type of procedure is preferred.

CONCLUSION

Small-diameter mesocaval shunts are a good alternate choice for patients in whom a selective shunt or a devascularization procedure is not feasible. Better results are achieved than those with the TIPS procedure, and these shunts can also be used as a bridge to liver transplantation, leaving the hepatic hilus intact.

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New Insights Into Impairment of Mucosal Defense in Portal Hypertensive Gastric Mucosa

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Portal hypertension (PHT) increases susceptibility of the gastric mucosa to injury. The aim of this study was to investigate whether PHT affects rat gastric mucosal defense mechanisms in vivo at the pre-epithelial, epithelial, and/or post-epithelial levels. PHT was produced in rats by staged portal vein ligation and sham-operated (SO) rats served as controls. The gastric mucosa was exposed, chambered, and continuously superfused with buffers under in vivo microscopy. We measured gastric mucosal gel layer thickness, surface epithelial cell intracellular pH (pH_i), mucosal blood flow, and mucosal/serosal oxygenation. In PHT rats, gastric mucosal gel layer thickness was significantly reduced (88 ± 16 μm in PHT rats vs. 135 ± 25 μm in SO rats; $P < 0.0001$), and the surface epithelial cell pH_i was significantly decreased (6.80 ± 0.11 in PHT rats vs. 7.09 ± 0.21 in SO rats; $P < 0.01$). Although total gastric mucosal blood flow was significantly increased in PHT rats by 72% ($P < 0.05$), the oxygenation of the gastric mucosal surface was decreased by 42% ($P < 0.05$) compared with SO rats. PHT impairs pre-epithelial (mucosal gel layer thickness), epithelial (pH_i), and post-epithelial (maldistribution of blood flow) components of the gastric mucosal barrier. These findings can explain the increased susceptibility of portal hypertensive gastric mucosa to injury. (J GASTROINTEST SURG 2000;4:458-463.)

KEY WORDS: Portal hypertension, gastric mucosa, mucosal defense, surface epithelial cell intracellular pH, mucosal oxygenation

Portal hypertensive (PHT) gastropathy is now recognized as a clinical entity that is frequently seen in patients with portal hypertension.[1-3] The PHT gastric mucosa has distinct morphologic and functional abnormalities and increased susceptibility to damage by noxious factors such as alcohol, aspirin, and ischemia/reperfusion.[4-6] The underlying mechanism of this increased susceptibility has not been fully elucidated.

The gastric mucosa possesses a number of defense mechanisms that can be classified into pre-epithelial, epithelial, and post-epithelial components. As a pre-epithelial component, an unstirred layer of mucus and bicarbonate maintains a "neutral" microclimate at the luminal surface of the surface epithelial cells.[7] The surface epithelial cells are able to secrete mucus, bicarbonate, prostaglandin E_2 (PGE_2), and prostacyclin.[8] Because of the presence of phospholipids on their surfaces, these cells are hydrophobic, "repelling" acid and water-soluble damaging agents. Mucosal microcirculation serves a post-epithelial component of the mucosal defense mechanism, supplying oxygen and nutrients to the entire mucosa and removing toxic substances.[9,10]

The aim of this study was to elucidate the underlying mechanisms of the increased susceptibility to injury of PHT gastric mucosa. We investigated whether portal hypertension affects rat gastric mucosal defense mechanisms in vivo. We used novel techniques to measure gastric mucosal gel layer thickness, surface

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epithelial cell intracellular pH (pH_i), and gastric mucosal blood flow and oxygenation corresponding, respectively, to pre-epithelial, epithelial, and post-epithelial components of the gastric mucosal defense mechanism.

MATERIAL AND METHODS

Animal Preparations

This study was approved by the Subcommittee for Animal Studies of the Long Beach Veterans Affairs Medical Center and the West Los Angeles Veterans Affairs Medical Center. Fifty Sprague-Dawley rats (8 weeks old; weighing 250 to 300 g) were used in the experiments. Rats were housed individually in wire-bottom cages with free access to standard rat chow (Rodent Diet No. 8504; Harlan Teklad, Madison Wis.) and water. The room where the animals were kept was maintained on a 12-hour light/dark cycle. Room temperature was kept at 18° to 22° C and humidity at 60% to 70%.

In rats anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally), portal hypertension was produced by staged portal vein occlusion and splenic vein ligation as previously described.[4] In brief, this technique involves initial constriction of the portal vein to approximately 50% of its diameter with a ligature at the first stage. At that time, a second ligature is applied to the portal vein, but it is not tied down to constrict the vein. The ends of the second ligature are exteriorized via the flanks and tied to the dorsum of the rat. The rats are then allowed to move freely in their cages with access to chow and water. After 3 days, the exteriorized ends of the second ligature are pulled firmly outward, which produces complete occlusion of the portal vein. Sham-operated (SO) rats underwent a similar operation but without occlusion of the portal and splenic veins.

We used 25 rats in the SO group and 25 rats in the PHT group. Four animals (1 in the SO group and 3 in the PHT group) were excluded from this study because they died after surgery or during the experiments. Three groups were identically prepared. In the first group of six SO and six PHT rats, the portal venous pressure was measured. In a second group of 10 SO and eight PHT rats, the surface epithelial cell intracellular pH (pH_i), gastric mucosal gel layer thickness, and mucosal blood flow were measured. In a third group of eight SO and eight PHT rats, the gastric mucosal/serosal oxygenation was measured.

Experiments were also performed 14 days after the operations. After a 24-hour fasting period with free access to water, rats were anesthetized with 1.25 g/kg urethane (Sigma Chemical Co., St. Louis, Mo.) intraperitoneally and then prepared for the experiments.

Portal Venous Pressure

Portal venous pressure was measured to assure the reproducibility of the method of PHT induction. After laparotomy, A polyethylene tube (PE-50 Intramedic, Baxter Diagnostics Inc., McGaw Park, Ill.) was introduced into a peripheral mesenteric vein tributary. With the vena cava as the reference point, portal venous pressure was measured from the height of the column of saline solution within the tube.

In Vivo Microscopic Preparations

An in vivo microfluorometric technique was used to measure intracellular pH_i.[11-13] The rat was placed supine on a plastic stage. Body temperature was maintained at 37° C by means of a heating pad and a heat lamp. The posterior wall of the corpus was then everted through the incision made at the forestomach. A concave brass disc (2.5 cm diameter, 5 mm depth) with a 5 mm center aperture was fixed watertight on the mucosal surface with a silicone plastic adherent (Silly Putty, Binney & Smith Co., Easton, Pa.). A thin coverslip was placed to make a chamber on the exposed mucosa. The chamber was continuously superfused with Krebs solution (pH 7.4) containing 100 μmol/L carboxyfluorescein diacetate (CF) fluorescence dye for 15 minutes to preload the surface epithelial cells with CF before the experiment was begun. The preloading time also allowed blood flow measurements to be stabilized. The experiments were performed subsequently for 30 minutes, under continuous perfusion with the same buffer. The CF-labeled surface cells in the chambered mucosa were viewed under a microscope and continuously monitored on a computer.

Measurements of Mucosal Gel Thickness

After CF loading, graphite particles were placed over the mucosa to delineate the top of the gel layer. The microscope was alternately focused from the fluorescent cell apical surface to the graphite layer. The vertical travel distance of the microscope objective was measured by the degrees of rotation of the fine focus knob, providing a value for the mucosal gel thickness.[13]

Measurements of Gastric Surface Epithelial Cell pH_i

After CF penetration into cells, the intensity of emitted fluorescence at 495 nm is pH dependent, whereas that at 450 nm is not.[11] Therefore the microscopic filters for the two fluorescence wavelengths were used alternatively to obtain pairs of images. The paired images of the identical areas were saved on the computer every 5 to 7 minutes during the experiment.

A small area of a gastric gland corresponding to three surface cells was selected for the measurements. Fluorescence intensity of the area was digitized and measured with an image analyzer (PDP-11 microcomputer [Digital Equipment Corp., Maynard, Mass.] with an imaging board [Imaging Technology Inc., Woburn, Mass]). Calibration and background compensation were performed as described previously.[11] The value of fluorescence intensity at 495 nm was divided by that at 450 nm, and the resulting ratio was converted to pH_i with our in vivo calibration curve.[11]

Gastric Mucosal Blood Flow Measurement

Mucosal blood flow was measured by laser Doppler flowmetry (Laserflo model BPM403A blood perfusion monitor; Vasomedics Inc., St. Paul, Minn.) with a probe (model H41-3667, Vasomedics Inc.) applied gently to the chambered mucosa. Data were recorded continuously by a chart recorder (model 3021, Yokogawa Electric Works, Tokyo, Japan) during the entire experiment. Gastric mucosal blood flow was expressed as perfusion units.[14]

Gastric Mucosal/Serosal Tissue Oxygenation

A specifically designed oxygen sensor (modified Clark electrode, Orange-1, Orange Medical, Costa Mesa, Calif.) adapted for application to small animal experimentation was used as described previously.[15] The sensor was placed on the anterior serosal surface of the distal stomach and P_{O_2} was measured. Through the incision in the forestomach, the sensor was placed on the luminal surface of the distal gastric mucosa. Special care was taken to place the sensor opposite the same area that was used to measure serosal P_{O_2}. A measurement was considered satisfactory when the following conditions were met: (1) it was stable for at least 10 seconds, (2) it was free of motion artifacts, and (3) the reading was reproducible.

Statistical Analysis

Values are expressed as mean ± standard deviation. Student's *t* test was used to analyze data in the SO and PHT rats. A *P* value of less than 0.05 was considered statistically significant. Pearson's correlation coefficient[16] was used to analyze differences in gastric mucosal gel layer thickness and surface mucous cell intracellular pH (pH_i).

RESULTS

All PHT rats showed evidence of splanchnic venous congestion with dilatation of mesenteric veins and thickening of the mesentery. Portal venous pressure in PHT rats was 26.1 ± 2.1 cm saline compared with 17.5 ± 1.0 cm in SO rats ($P < 0.00001$).

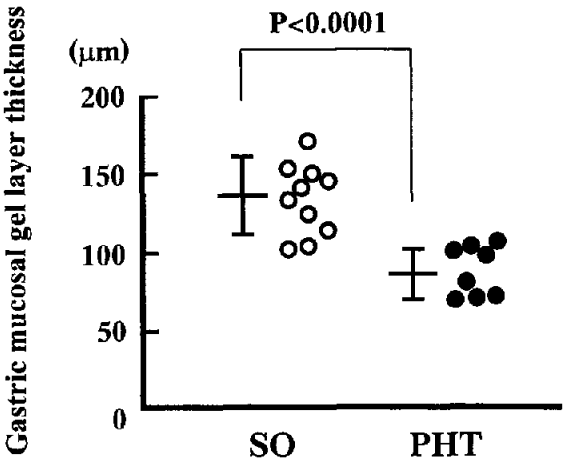

Fig. 1. Gastric mucosal gel layer thickness in SO (○) and PHT (●) rats. The technique is described in Material and Methods. Gastric mucosal gel layer thickness was significantly reduced in PHT rats compared with SO rats ($P < 0.0001$).

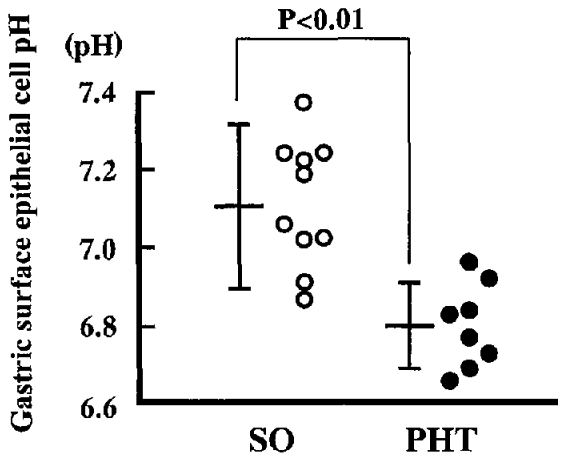

Fig. 2. Gastric surface epithelial cell pH_i in SO (○) and PHT (●) rats. The technique is described in Material and Methods. Gastric surface epithelial cell pH_i was significantly decreased in PHT rats compared with SO rats ($P < 0.01$).

Gastric mucosal gel layer thickness was significantly reduced in PHT rats (88 ± 16 μm in PHT vs. 135 ± 25 μm in SO; $P < 0.0001$) (Fig. 1). A gastric surface epithelial cell pH_i was significantly decreased in PHT rats (6.80 ± 0.11 in PHT vs. 7.09 ± 0.21 in SO; $P < 0.01$) (Fig. 2). Total gastric mucosal blood flow was significantly increased by 72% in PHT rats com-

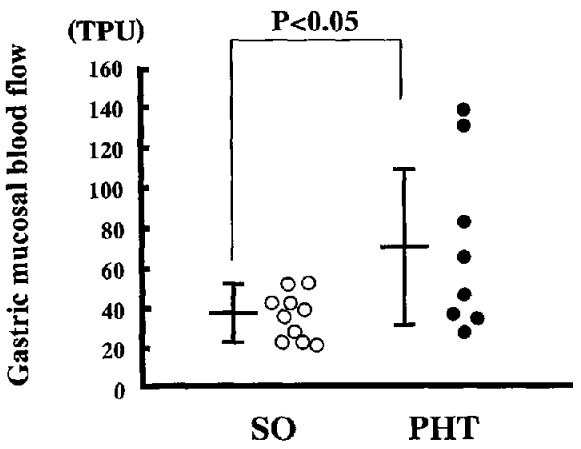

Fig. 3. Gastric mucosal blood flow in SO (○) and PHT (●) rats. The technique is described in Material and Methods. Gastric mucosal blood flow was significantly increased in PHT rats compared with SO rats ($P < 0.05$). TPU = tissue perfusion units.

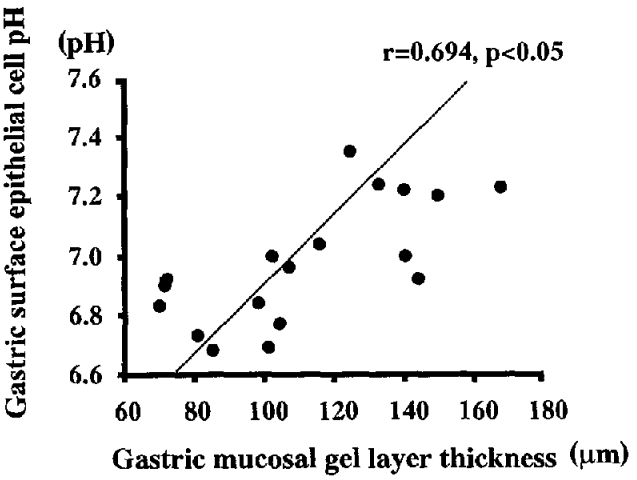

Fig. 4. Correlation between gastric mucosal gel layer and gastric surface epithelial cell pH. Pearson's correlation coefficient was calculated ($r = 0.694$, $P < 0.05$, n = 18).

Table I. Results of gastric tissue oxygenation measurements

	SO	PHT	*P* value
No. of animals	8	8	
Mucosal P_{O_2} (mm Hg)	45.3 ± 5.9	26.2 ± 7.1	<0.05
Serosal P_{O_2} (mm Hg)	54.7 ± 3.5	50.4 ± 4.8	NS

Values are mean ± standard deviation. NS = no statistically significant difference between the SO and PHT rats; SO = sham operated; PHT = portal hypertensive.

pared with SO rats ($P < 0.05$) (Fig. 3). There was significant correlation between gastric mucosal gel layer thickness and gastric surface epithelial cell intracellular pH ($r = 0.694$; $P < 0.05$) (Fig. 4).

Gastric mucosal and serosal oxygenation is summarized in Table I. Oxygenation of the gastric mucosal (but not serosal) surface was significantly decreased by 42% in PHT rats compared with SO rats ($P < 0.05$).

DISCUSSION

This study demonstrated that the gastric mucosal gel layer thickness in vivo is significantly reduced in the PHT gastric mucosa of rats with staged portal vein ligation compared with the normotensive gastric mucosa. Gastric mucus serves as a lubricant, retards diffusion of H^+ions and pepsin, inhibits pepsinogen activation, and exerts antibacterial and mucosal protective actions.[17,18] Therefore the results of this study revealed pre-epithelial mechanisms predisposing the PHT gastric mucosa to damage.

In cirrhotic rats with portal hypertension, the mucosal gel layer thickness is significantly reduced compared with normal rats.[19] However, the complications of cirrhosis may include not only portal hypertension but also a variety of hepatic functional disorders (e.g., reduced production of hepatocyte growth factor, increased ammonia), which might directly influence the gastric mucosal layer thickness. Since the animals in this study were not cirrhotic, then portal hypertension alone caused a reduction in the mucosal gel layer thickness. Prostaglandins, especially PGE_2, are known to stimulate mucus release[8] and PGE_2-like immunoactivity is significantly decreased in the gastric mucosa of patients with portal hypertension.[20] Our in vitro study demonstrated that the secretion of PGE_2 is decreased in epithelial cells isolated from PHT gastric mucosa of rats.[8] Therefore the reduced mucosal gel thickness in PHT gastric mucosa may be mediated, at least in part, by reduced generation of prostaglandins.

This study also identified, for the first time, in the portal vein ligation model that surface epithelial in-

tracellular pH is significantly reduced in the PHT gastric mucosa compared with the normotensive gastric mucosa. The gastric surface epithelial cells can maintain pH_i by slowing proton entry despite the enormous H^+ gradient, and enhanced pre-epithelial factors (mucosal gel thickness and bicarbonate secretion) may additionally assist the epithelial cells to maintain pH_i.[21] Our present study shows that there is a significant correlation between mucosal gel layer thickness and epithelial cell pH_i. Decreased gel layer thickness in PHT gastric mucosa might contribute to the reduced epithelial cell pH_i. Previously we demonstrated that in PHT rats there is a lower gastric transmural potential difference and greater permeability to acid back-diffusion.[4] Therefore the present study demonstrating decreased epithelial cell pH_i and reduced mucosal gel thickness in PHT gastric mucosa explains the increased permeability to acid back-diffusion found in the previous study. Impaired maintenance of epithelial cell pH_i might be the epithelial factor predisposing PHT gastric mucosa to increased susceptibility to injury.

Our present study confirmed previous reports,[22-25] indicating that total gastric mucosal blood flow is increased in PHT rats. However, we demonstrated in this study that oxygenation of the luminal mucosal surface is significantly reduced, which may reflect reduced blood flow in the upper mucosal layer. The oxygen sensor detects oxygenation of gastric mucosal surface within 100 μm, whereas laser Doppler flowmetry detects the blood flow in the full thickness of the gastric wall including mucosa and submucosa. Therefore we may assume that decreased superficial mucosal blood flow coexists with increased submucosal blood flow in PHT gastric mucosa. Furthermore, the severe edema of PHT gastric mucosa could explain this phenomenon. In our previous study we found that endothelial cells of gastric mucosal microvasculature in PHT rats have expanded cytoplasm, which reduces the capillary lumen by 3.5-fold. These changes reduce the flood flow and delivery of nutrients to the luminal regions of the mucosa.[26] The studies of Manabe et al.[27] and Hashizume et al.[28] have demonstrated that there is a significant increase in the number of submucosal arteriovenous communications in the PHT stomach. Mucosal microvasculopathy and the increased number of submucosal arteriovenous communications in PHT gastric mucosa might, therefore, be another explanation for the surface mucosal hypoxygenation.

Generation of nitric oxide by nitric oxide synthase (NOS) plays a major role in gastric mucosal defense by modulating the mucosal circulation.[29,30] However, excessive nitric oxide production has cytotoxic potential and increases gastric mucosal injury.[31,32] Several studies have shown that inhibition of NOS can correct the hyperdynamic circulation and nitric oxide might be a major contributor of the hyperdynamic circulation in portal hypertension.[33-35] Recently we demonstrated that endothelial NOS is overexpressed in PHT gastric mucosa of rats and that inhibition of NOS reverses the abnormal gastric microcirculation and the increased susceptibility to alcohol injury.[36] Moreover, we have demonstrated that tumor necrosis factor-alpha (TNF-α) might regulate endothelial NOS expression in the PHT stomach.[3] Therefore excessive nitric oxide production by overexpressed TNF-α and NOS might play an important role in the maldistribution of blood flow participating in postepithelial components of mucosal defense mechanism in PHT gastric mucosa. Furthermore, we have shown increased lipid peroxidation and overproduction of peroxynitrite by impaired oxygenation and excess of nitric oxide in PHT gastric mucosa.[37] Oxidative stress increases gastric mucosal injury by inhibition of mucus secretion in isolated gastric cells.[38] Thus increased oxidative stress in PHT gastric mucosa might also interfere with mucus secretion related to pre-epithelial components of the gastric mucosal barrier.

CONCLUSION

This study demonstrates that PHT reduces mucosal gel layer thickness and surface epithelial cell pH_i, reflecting the impairment of pre-epithelial and epithelial components of the gastric mucosal barrier, respectively. Although overall gastric blood flow is increased in PHT rats, mucosal surface oxygenation corresponding to the post-epithelial component is significantly impaired by maldistribution of blood flow. These data provide evidence of impairment of gastric mucosal defense in portal hypertension and can explain the increased susceptibility of PHT gastric mucosa to injury.

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Lipid Risk Profile and Weight Stability After Gastric Restrictive Operations for Morbid Obesity

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There are no longitudinal data that address weight loss stability and lipid levels in bariatric surgical patients. The goal of this study was to determine whether weight regain adversely affected reduction in lipid levels after gastric bariatric operations. Of 651 consecutive patients undergoing gastric restrictive surgery for morbid obesity, 227 (35%) had increased serum levels of total cholesterol (TC), triglycerides, or both preoperatively. High-density lipoprotein cholesterol (HDL-C) levels were subnormal (≤35 mg/dl) in 45 (20%) of the hyperlipidemic patients. Fasting lipid profiles were determined at 6-month intervals postoperatively. This series included the following three operations: gastroplasty (GP; N = 13), standard Roux-en-Y gastric bypass (RYGB; N = 205), and distal Roux-en-Y gastric bypass (DRY; N = 9). By 6 months postoperatively, patients had a ≥15% mean reduction in TC and a ≥50% mean reduction in triglycerides, both of which were significant in comparison with preoperative levels ($P \leq 0.05$). Mean HDL-C levels had increased significantly vs. preoperative levels by 12 months postoperatively ($P < 0.05$) and continued to increase through 5 years. By 18 months both HDL-C and TC were significantly lower after DRY than after GP or RYGB. In 91 patients who were followed for 2 years or longer (mean 48 ± 25 months), mean excess weight loss was 55% with mean body mass index reduced from 48 to 33 kg/m^2. This group was divided into patients whose weight remained stable (N = 54) and patients who regained ≥15% of their lost weight or lost less than 50% of excess weight (N = 37). Although mean excess weight loss and body mass index were significantly different between the two groups ($P < 0.0001$) at 2 years, there was no difference in the lipid profile (TC/HDL) between the two groups at any interval through 5 years. These results show that abnormal lipid profiles can be permanently improved after gastric bariatric surgery and are not adversely affected by mediocre weight loss or regaining ≥15% of lost weight. DRY appears to be a superior operation for TC reduction in comparison with GP and RYGB. (J GASTROINTEST SURG 2000;4:464-469.)

KEY WORDS: Obesity, gastrointestinal surgery, cholesterol, triglycerides, hyperlipidemia

Obesity and hypercholesterolemia have both been firmly established as independent risk factors for the development of coronary artery disease and premature cardiovascular death.[1-3] There is a considerable body of evidence which demonstrates that lowering total cholesterol (TC) and raising high-density lipoprotein cholesterol (HDL-C) levels decreases the risk of myocardial infarction.[2] Although increased mortality risk in patients with isolated hypertriglyceridemia is not clearly established, the 1984 National Institutes of Health consensus conference on hypertriglyceridemia concluded that serum triglycerides in the "borderline" 250 to 500 mg/dl range are associated with "an approximate twofold excess risk of cardiovascular disease" in patients with other risk factors such as hypertension, cigarette smoking, and obesity.[4] Because obesity is reported as "the most common secondary factor raising triglyceride levels" treatment is recommended in patients with "borderline" hypertriglyceridemia. Successful treatment of obesity generally results in satisfactory reduction of triglyceride levels.[4]

The present report is a prospective evaluation of 227 consecutively treated hyperlipidemic patients who had three types of gastric restrictive operations performed for treatment of morbid obesity over a 15-year

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period. The primary purpose of this study was to determine whether the magnitude of weight loss or later regaining of lost weight affected postoperative reductions in serum lipid levels. A second goal was to learn how the type of operation influenced changes in postoperative lipid profiles. The effect of diet composition on postoperative lipid levels was also evaluated in a subset of 78 patients.

CLINICAL MATERIAL AND METHODS

Between 1981 and 1996, a total of 651 patients had gastric restrictive operations for morbid obesity at Robert Wood Johnson University Hospital. The operations included 56 horizontal gastroplasties, 30 vertical banded gastroplasties, and 565 Roux-en-Y gastric bypasses. The technique common to all was a stapled partitioning incorporating a 20 ± 5 cc volume upper gastric pouch with an outlet stoma ranging from 9 to 11 mm in diameter. Roux-en-Y gastric bypass (RYGB) was performed using three lengths for the Roux limb including two proximal measurements in which the Roux limb length ranged from 50 to 150 cm. Twenty-three patients had a distal modification in which the defunctionalized afferent limb was anastomosed to the ileum 75 cm proximal to the ileocecal junction.

The preoperative lipid profiles were obtained on the day of surgery after an overnight fast. Two hundred twenty-seven patients (35%) had increased serum levels of TC, triglycerides, or both, including 13 who underwent gastroplasty and 214 who had a gastric bypass. Approximately half of the 424 patients with normal preoperative cholesterol and triglyceride levels had decreased levels of HDL-C prior to surgery. Serum lipid analysis was performed in the research laboratory of the Division of Cardiovascular Diseases using the Lipid Research Clinics methods.[5] The normal range for lipid values in this laboratory is 150 to 220 mg/dl for TC, 50 to 250 mg/dl for triglycerides, and 36 to 65 mg/dl for HDL-C.

The hyperlipidemic patients included 179 females and 48 males ranging in age from 20 to 62 years (mean 41 ± 8 years). Preoperative weight for these 227 patients ranged from 201 to 522 pounds (mean 297 ± 61 pounds). Mean body mass index was 48.4 ± 8.5 kg/m^2 and ranged from 35 to 79.4 kg/m^2. Fasting lipid profiles of the hyperlipidemic patients were followed at 6-month intervals postoperatively. One hundred seven (47%) of the 227 patients had isolated elevation of TC ranging from 225 mg/dl to 448 mg/dl, 18 (8%) of the 227 patients had isolated elevation of triglycerides ranging from 253 to 468 mg/dl, and 102 patients (45%) had increased serum levels of both cholesterol and triglycerides ranging from 225 to 386 mg/dl for cholesterol and 252 to 2260 mg/dl for triglycerides, respectively. HDL-C levels were low (≤35 mg/dl) in 45 patients (20%).

Follow-up ranged from 12 to 145 months (mean 40.8 ± 30.6 months). At each follow-up visit, weight and blood pressure were recorded and a detailed recall diet history was taken by the clinical nutritionist. Dietary fat intake was recorded using the one-day recall method and analyzed with the Nutritionist III computer program (N-Squared Computing, Silverton, Ore.). Total fat intake in grams was recorded and then broken down into saturated, monounsaturated, polyunsaturated, and cholesterol categories.

Ninety-one patients who were followed for 24 months or longer (mean 48 ± 25 months) were empirically placed into two weight loss outcome groups. Fifty-four lost ≥50% of their excess weight and maintained weight loss within 15% of the nadir. The remaining 37 patients either did not lose 50% of their excess weight or regained more than 15% of their lost weight after 24 months. Fifty percent excess weight loss has been used as a determinant of successful outcome in a number of previous reports of obesity operations.[6,7] Excess weight is the calculated difference between the preoperative weight and ideal weight for height.

Statistical comparisons between the two outcome groups were computed using unpaired Student's *t* test. Preoperative vs. postoperative data were compared using paired Student's *t* test and analysis of variance with the Student–Newman-Keuls test. Pearson correlation coefficients were computed between weight loss outcome as a percentage of excess weight lost vs. each of the serum lipids and postoperative calorie intake. Comparisons between the 227 patients with hyperlipidemia and the 424 patients with normal preoperative lipid levels were made using the chi-square test.

RESULTS

Weight loss results for the 91 patients followed for 24 months or longer are shown in Table I. Group 1 included 54 patients who lost at least 50% of their excess weight and maintained this loss throughout the study. Weight loss in these patients at 24 months postoperatively ranged from 51% to 88% of excess weight lost (mean 62.5%). Group 2 included the remaining 37 patients who either failed to lose 50% of their excess weight or regained ≥15% of their lost weight after stabilization. Mean excess weight loss in this group at 24 months was 38.7% and ranged from 27% to 49%. There were no differences in weight loss parameters between the two groups during the first 12 months postoperatively. However, by 18 months patients in group 2 started regaining weight. Weight and body mass index in group 2 continued to increase

Table I. Postoperative weight loss

Time	Group 1 N	Weight (pounds)	BMI (kg/m²)	% Excess lost	Group 2 N	Weight	BMI (kg/m²)	% Excess lost
Preoperatively	54	289 ± 43	47 ± 8	—	37	287 ± 60	47 ± 7	—
6 mo	34	214 ± 48	35 ± 7	48 ± 14	28	223 ± 41	35 ± 6	45 ± 15
12 mo	32	201 ± 56	33 ± 8	56 ± 18	29	199 ± 38	33 ± 6	56 ± 16
18 mo	19	188 ± 44	31 ± 7	64 ± 17*	23	205 ± 44	33 ± 6	52 ± 19*
24 mo	36	192 ± 51*	32 ± 8	62 ± 27*	25	217 ± 47*	35 ± 7	39 ± 19*
36 mo	19	205 ± 60	33 ± 7	58 ± 21*	20	222 ± 41	35 ± 6	39 ± 13*
48 mo	18	189 ± 46*	30 ± 6*	62 ± 20*	13	224 ± 40*	36 ± 6*	40 ± 22*
≥60 mo	19	191 ± 46	31 ± 6	63 ± 20*	20	174 ± 23	31 ± 4	45 ± 19*

N = number out of 91 study patients available for follow-up at each interval; BMI = body mass index.
*Indicates significant difference between the two groups ($P \leq 0.05$ by unpaired Student's t test).

Table II. Mean cholesterol, triglyceride, and HDL-C levels

Time	Group 1 N	TC	TG	HDL-C	Group 2 N	TC	TG	HDL-C
Preoperatively	54	248 ± 40	234 ± 118	48 ± 15	37	259 ± 40	291 ± 275	45 ± 13
6 mo	34	208 ± 33	140 ± 55	51 ± 15	28	209 ± 35	141 ± 55	47 ± 12
12 mo	32	203 ± 44	138 ± 73	49 ± 12	29	213 ± 30	131 ± 54	55 ± 13
18 mo	19	192 ± 48	111 ± 54	54 ± 19	23	213 ± 33	146 ± 93	54 ± 12
24 mo	36	206 ± 39	132 ± 69	57 ± 23	25	219 ± 97	137 ± 83	60 ± 17
36 mo	19	197 ± 39*	118 ± 59	58 ± 13	20	225 ± 33*	155 ± 122	56 ± 28
48 mo	18	204 ± 46	135 ± 84	56 ± 15	13	200 ± 27	133 ± 54	50 ± 10
≥60 mo	19	188 ± 23*	110 ± 47	62 ± 13	20	215 ± 26*	147 ± 64	60 ± 14

Lipid levels expressed in mg/dl.
N = number out of 91 study patients available for follow-up at each interval. Mean total cholesterol (TC) and triglyceride (TG) reductions at each interval were significant vs. preoperative levels ($P < 0.05$ by analysis of variance with Student–Newman-Keuls test). By 18 months, postoperative mean HDL-C levels were significantly increased vs. preoperative levels in both groups ($P < 0.05$ by analysis of variance with Student–Newman-Keuls test) and continued to increase through 24 months before stabilizing.
*Indicates significant difference in TC levels between groups 1 and 2 ($P < 0.05$ by unpaired Student's t test).

during the next 6 months before stabilizing after 24 months postoperatively. Weight parameters in both groups remained relatively stable after the second postoperative year. However, the difference in excess weight loss between the two groups was significant at each postoperative interval after 18 months ($P < 0.05$ by unpaired Student's t test).

Postoperative changes in serum lipid levels for the subgroup of 91 patients who were followed for 24 months or longer are shown in Table II. By 6 months postoperatively, there was a ≥15% mean reduction in TC and a ≥50% mean reduction in triglyceride levels, both of which were significant vs. preoperative levels. Differences in mean TC levels between the two weight loss outcome groups were significant at 2 and 4 years postoperatively. There was no significant difference in either triglyceride or HDL-C levels between the two groups at any postoperative interval.

Table II also shows changes in mean HDL-C levels over time. Unlike cholesterol and triglycerides, HDL-C levels did not change significantly during the period of most rapid weight loss in the first 6 months postoperatively. However, mean HDL-C levels were significantly increased vs. preoperative levels at 12 months postoperatively and continued to rise to more than 20% above mean preoperative levels after 18 months.

Fig. 1 shows calorie and fat intake over time in a subset of 78 patients in the two weight loss outcome groups. Mean calorie intake was lowest at 6 months postoperatively and then gradually increased by approximately 10% at each subsequent interval through 2 years postoperatively. There was no differences in either total calorie intake or dietary fat intake between the two outcome groups either before or after operation.

Lipid levels became normal in 67 (74%) of the 91 patients followed for 24 months or longer. Seventeen of the remaining 24 patients had "improved" lipid levels characterized by a mean 40% reduction in triglyc-

Variable	Weight Stable Group	Weight Gain >15% Group
	2558±333	2091±228
	788±80	895±136
	1111±145	1491±230
	38±2	39±2
	29±3	27±3
	33±2	37±3
	15±1	14±1
	10±1	10±1
	12±2	12±2

Fig. 1. Comparison of daily calorie and fat intake in the two outcome groups expressed as mean ± standard deviation. Difference between preoperative calorie intake and intake at 2 years postoperatively in the two groups was significant ($P \leq 0.03$ by unpaired Student's t test).

Table III. Comparison of lipid levels in restrictive vs. malabsorptive operations

Operation	N	TC	TG	HDL	TC/HDL
GP	11	212 ± 34*	143 ± 88	67 ± 16*	3.2
RYGB	34	200 ± 51*	123 ± 58	56 ± 13*	3.5
DRY	5	113 ± 42*	96 ± 37	38 ± 9*	2.8

Lipid levels expressed in mg/dl.
N = total number of patients available for follow-up at 18 months postoperatively; GP = gastroplasty; RYGB = conventional Roux-en-Y gastric bypass; DRY = distal Roux-en-Y gastric bypass; TC = total cholesterol; TG = triglycerides; HDL = high-density lipoproteins.
*Indicates significant difference between DRY vs. GP and RYGB ($P < 0.05$ by analysis of variance with Student–Newman-Keuls test).

erides and a mean 30% increase in HDL-C but with minimal changes in TC, which remained at a mean 231 mg/dl. Two of these 17 patients, who had lost ≥70% of their excess weight at 2 years postoperatively, had a greater than 25% reduction in TC, although mean levels remained elevated. Lipid levels were considered unimproved in seven patients (7.7%) because mean cholesterol and HDL-C levels remained virtually unchanged, although postoperative triglycerides had decreased by 25%. Mean weight and body mass index were significantly lower in the 67 patients with normal postoperative lipid levels vs. the 24 patients with elevated cholesterol levels at 2 years postoperatively (186 ± 36 pounds/30 ± 6 kg/m^2 vs. 215 ± 62 pounds/35 ± 9 kg/m^2; $P < 0.04$).

To determine whether a weight regain threshold exists for improvement of hyperlipidemia after bariatric operations, a post hoc analysis comparing patients who regained more than 30% (N = 13) or 50% (N = 7) of their lost weight vs. the remaining patients was performed. This analysis showed no significant difference in lipid levels between patients who regained either 30% or 50% of their lost weight vs. the remaining patients.

Our distal modification (DRY) of RYGB was designed to produce malabsorption in addition to restricted calorie intake. In DRY the afferent (bypassed) jejunum was the same length as in our conventional RYGB but was anastomosed to the ileum 75 cm proximal to the ileocecal junction. DRY generally produced loose, foul-smelling stools consistent with steatorrhea. Use of DRY was restricted to superobese patients ≥200 pounds overweight. Table III compares lipid levels at 18 months postoperatively among the three operations used in this series. TC levels after DRY were significantly lower in comparison with TC after conventional RYGB and gastroplasty. Conversely, HDL-C levels remained lower after DRY. Hence there was no difference in the postoperative lipid profile (TC/HDL) between restrictive and malabsorptive procedures.

DISCUSSION

Mechanisms of Postoperative Changes in Lipid Levels

The short-term effectiveness of surgically induced weight loss in lowering serum lipid levels has been documented by many investigators. Weight loss after jejunoileal bypass results almost entirely from dietary malabsorption. Conversely, weight loss after both gastroplasty and RYGB results predominantly from reduced calorie intake. A 1981 comparison of lipid levels after gastric and jejunoileal bypass reported substan-

tially greater decreases in cholesterol levels after jejunoileal bypass, although cholesterol after both operations was significantly decreased in comparison with preoperative levels.[5] Postoperative HDL-C levels have varied considerably after intestinal bypass, with Rucker et al.[8] reporting a mean decrease in HDL-C levels, Dobrea et al.[9] finding no change in postoperative HDL-C, and Goldberg et al.[10] showing a late increase in HDL-C levels at 2 years postoperatively.[8-10]

The precise mechanisms by which lipid profile changes occur after gastric restrictive operations is unclear. Many investigators believe post–gastric restriction lipid reduction is primarily due to factors associated with rapid weight loss.[11,12] Every report of lipoprotein changes after gastric restrictive operations has shown dramatic decreases in tryglycerides and relatively modest decreases in TC.[8,12-14] Since hyperinsulinemia stimulates hepatic triglyceride production, the marked decrease in postoperative triglyceride levels may be due to the decreased insulin resistance associated with weight loss.[4] Reduced low-density lipoprotein levels after gastric restrictive operations are probably due to decreased dietary cholesterol intake. The greatest postoperative reductions in cholesterol and triglycerides generally occur in the patients with the highest preoperative elevations. In the present series this observation was invariably accurate for triglycerides provided that satisfactory weight loss was maintained.

In the present study the malabsorptive DRY resulted in markedly reduced TC levels but little change in HDL-C postoperatively. Conversely, GP and RYGB produced relatively modest decreases in TC with a nearly 20% increase in HDL-C. Although lipid profiles (TC/HDL) in this study were considerably improved after both restrictive and malabsorptive procedures, the mechanisms responsible for these results are different. It is unclear whether malabsorption is in any way superior to restriction of calorie intake in sustaining long-term improvement in lipid profile.

In the subset of 78 patients who had detailed diet analysis, there was no correlation between unsatisfactory weight loss and increased postoperative fat intake. Moreover, neither total calorie nor fat intake correlated with serum lipid levels postoperatively. However, there was a significant correlation between the magnitude of reduction in calorie intake and weight loss outcome suggesting that reduction in total calories has a greater impact on weight loss than dietary composition.

Impact of Weight Loss on Lipid Levels

Our 1990 report showed a significant correlation between the magnitude of weight loss and lipid reductions after gastric restrictive operations.[15] In our earlier report we observed a gradual regression of improved lipid levels in patients who did not lose 50% of their excess weight. This finding led us to conclude that amelioration of obesity-related hyperlipidemia may be transient in patients with unsatisfactory weight loss. Conversely, in the present study neither loss of less than 50% of excess weight nor regaining $\geq$15% of lost weight adversely affected long-term improvements in the lipid profile. Moreover, the present study included 189 new patients with hyperlipidemia and all of the data from our previous study with longer postoperative follow-up. These results show that weight loss maintenance is not an essential component of improvement of lipid profiles after gastric restrictive operations. Closer scrutiny of these results suggests that the lack of difference in lipid profiles (TC/HDL) between the two outcome groups was primarily driven by the HDL-C levels, which were closely parallel throughout the study. Although the correlation between weight loss and TC reduction was not significant in the present study, both weight and body mass index were significantly lower in the 67 patients with normal TC and triglyceride levels vs. the remaining 24 patients with elevated TC levels at 2 years postoperatively. This finding suggests that greater weight loss is more likely to result in improvement of hypercholesterolemia after gastric bariatric operations.

Postoperative follow-up in most of the early reports of lipid profile changes after bariatric operations was relatively short with mean follow-up in the range of 12 months or less. Although Gleysteen et al.,[16] showed that salutary changes in lipid reductions are maintained at 5 years after RYGB, their study was predominantly comprised of patients who had normal preoperative lipid levels. There are virtually no data that address the relationship between weight stability and recidivism in hyperlipidemic patients following gastric restrictive operations. Weight loss after gastroplasty and RYGB usually stops between 12 and 18 months postoperatively. Because regaining of lost weight generally commences within 6 to 12 months of the nadir of weight loss, the present study focused on the stability of lipid levels at 2 years postoperatively and beyond. Our past hoc comparison of patients who regained 30% to 50% of lost weight vs. stable patients suggests that there is no weight loss threshold for improvement of lipid levels after bariatric operations.

The fact that lipid levels were available in only 91 of the 227 hyperlipidemic patients at $\geq$2 years postoperatively is due to several factors. Thirty-six patients had operations performed less than 2 years

from the study closure date. Many patients missed follow-up visits at specific times but were not lost to follow-up. Patients who had reoperations for staple line disruption (N = 4) or unsatisfactory weight loss (N = 3) were dropped from the study. Lipid levels were not measured in patients who did not fast prior to their visit as instructed. This occurred frequently. We lost contact with only 32 patients (14%) who participated in this study. Long-term follow-up is notoriously problematic in bariatric surgical patients. Difficulty with hands-on follow-up is the likely explanation for the paucity of longitudinal data on medical comorbidities after bariatric operations.

The salutary effects of surgically induced weight loss on a variety of obesity-related medical problems have been clearly demonstrated by many investigators. Several reports have shown that many patients experience dramatic improvement of medical problems with relatively modest weight loss.[17-19] Lack of correlation between quantity of weight loss and improvement of medical comorbidities embodies the dilemma of outcome assessment after bariatric operations. A patient who has lost only 35% of excess weight and experienced dramatic improvement of comorbidities cannot be considered a total failure. Although 37 of our patients (40%) did not achieve long-term "success" in terms of maintaining loss of 50% of excess weight, the overall long-term mean excess weight loss for the 91 study patients approximated 50%, which is consistent with other clinical reports with follow-up of 5 or more years.[20] Both the amount of weight loss and amelioration of medical problems should be included in outcome assessment of obesity operations.

Although there are no clinical studies which clearly show that substantial weight loss and improvement of hyperlipidemia in bariatric surgical patients improves longevity, there are data showing objective improvement in cardiac disease following partial ileal bypass.[21] Partial ileal bypass is a purely malabsorptive procedure, which failed to provide satisfactory long-term weight loss for patients with morbid obesity. Operations such as DRY, which combine malabsorption with restriction of intake, apparently provide both satisfactory weight loss and significant amelioration of hypercholesterolemia, which improves only modestly after restrictive procedures. This report shows a 92% incidence of improvement or resolution of hyperlipidemia in response to surgically induced weight loss at 2 years postoperatively. Improved lipid profiles were sustained for more than 5 years in most patients. These results suggest that abnormal lipid profiles in morbidly obese patients may be permanently improved after gastric bariatric operations.

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Can Perforated Appendicitis Be Diagnosed Preoperatively Based on Admission Factors?

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The optimal initial treatment for selected patients with perforated appendicitis may be nonoperative. For this reason it is important to be able to diagnose perforated appendicitis preoperatively. The purpose of this study was to determine the accuracy of diagnosing perforated appendicitis using only admission factors. The study population was comprised of 366 adult patients who underwent appendectomy for presumed appendicitis during 1997. Admission factors associated with perforated appendicitis were determined using univariate and multivariate analyses. These variables were then used to formulate a rule for the diagnosis of perforated appendicitis. Sensitivity and specificity were calculated for this rule. The admission factors analyzed were sex, race, age, days of pain, temperature, heart rate, symptoms, physical examination findings, and laboratory findings. Multivariate regression analysis revealed days of pain, temperature, and localized tenderness outside the right lower quadrant to be significant ($P < 0.05$). Using two or more days of pain, a temperature of ≥101° F (38.3° C), or localized tenderness outside the right lower quadrant as criteria to indicate perforation, we achieved a sensitivity of 86% and a specificity of 58% for distinguishing perforated from nonperforated appendicitis. We concluded that (1) perforated appendicitis cannot reliably be distinguished from nonperforated appendicitis based on admission factors, and (2) two or more days of pain, localized tenderness outside the right lower quadrant, and a temperature of ≥101° F (38.3° C) define a group of patients with appendicitis who have a high incidence of perforation. (J GASTROINTEST SURG 2000;4:470-474.)

KEY WORDS: Appendicitis, diagnosis, treatment, pathology

Traditionally appendicitis is treated by early operation, based on tenants established a century ago by Fitz[1] and McBurney.[2] Subsequent experience has shown that the complication rates incurred with surgery are dependent on whether or not the appendix is perforated.[3-7] Recent studies have shown the complication rate for perforated appendicitis to be 12% to 30%.[4-7] Contemporary efforts to lower the complication rates in patients with perforated appendicitis include radiologic-guided methods of abscess drainage and selective initial nonoperative management.

Initial nonoperative management for the small subgroup of patients with perforated appendicitis who present with a mass has been shown to be effective and safe with low complication rates in many studies.[8-14] Based on our early success with this technique, we have expanded its indications to include patients who have perforated appendicitis without masses, and who have likewise demonstrated low complication rates.[15,16] Nonoperative treatment of patients with appendicitis mandates accurate preoperative diagnosis of localized perforation. The purpose of this study was to determine the accuracy of diagnosing perforated appendicitis using only admission factors.

MATERIAL AND METHODS

During 1997, a total of 390 adult patients underwent appendectomy at our institution for presumed appendicitis. The study population consists of 366 patients. Twelve patients were excluded because of unavailable medical records and 12 patients were ex-

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Table I. Admission factors

	Nonperforated (n = 244)	Perforated (n = 76)	*P* value
Male:Female	70:30	70:30	NS
Age (yr)	28.5 (10.2)	32.4 (11.4)	<0.01
Days of pain	1.5 (0.9)	3.3 (3.6)	<0.01
Temperature (°C)	37.6° F (0.8)	38.2° F (0.9)	<0.01
Heart rate	89 (16)	103 (19)	<0.01
Nausea	71	74	NS
Vomiting	65	62	NS
Anorexia	58	59	NS
Diarrhea	9	20	0.01
Fever	45	54	NS
Localized tenderness (not right lower quadrant)	0.4	4	0.02
Rebound	56	55	NS
Guarding	45	51	NS
Mass	1	7	<0.01
White blood cells	14.8 (4.1)	14.4 (3.9)	NS
Neutrophil (%)	82 (9)	85 (7)	NS

Values are reported as means, with standard deviations in parentheses, or percentages; NS = not significant.

cluded because of diffuse peritonitis at the time of admission. Medical charts were reviewed retrospectively by a single person.

Data collected included age, sex, race, historical features (days of pain, nausea, vomiting, anorexia, diarrhea, fever), physical examination features (right lower quadrant tenderness, other quadrant localized tenderness, localized peritoneal signs, mass), temperature, pulse, white blood cell count, and percentage of neutrophils. The physical examination features and historical data were extracted from the initial surgical consultation notes. Abdominal tenderness was characterized as either localized right lower quadrant tenderness or localized tenderness most pronounced outside the right lower quadrant. The temperature and pulse recorded were the maximum values that occurred while the patients were in the emergency department. The recorded white blood cell count and percentage of neutrophils were the first values obtained from the emergency department.

The outcome variables used were perforated appendicitis and nonperforated appendicitis as determined pathologically. Acute appendicitis and gangrenous appendicitis were categorized as nonperforated appendicitis. Patients with normal appendices were not included in the univariate and multivariate analyses.

To compare the two groups, we used the Wilcoxon rank-sum test for quantitative data and chi-square analysis, or Fisher's exact test where appropriate, for qualitative data. A value of <0.05 was accepted as statistically significant. Those variables found to be significant using univariate analysis were used in multivariate logistic regression to identify the variables independently predictive of perforation. These variables were then used to formulate a rule to distinguish perforated from nonperforated appendicitis. We first defined cutoff values for these variables based on a qualitative inspection of our data. We then used these cutoff values to define a rule for diagnosing perforation. Sensitivity, specificity, and predictive values for diagnosing perforated appendicitis were then determined by applying this rule to our study population.

RESULTS

The pathologic diagnoses for the 366 patients in this study were normal appendix in 13%, acute appendicitis in 43%, gangrenous appendicitis in 24%, and perforated appendicitis in 21%. Our rate of negative appendectomy was 7% in males and 24% in females. Twenty-four percent of patients with appendicitis had perforation.

The group of patients with pathologically proved appendicitis (n = 320) had a mean age of 29 years (range 16 to 79 years). Only six patients were 60 years of age or older. Men comprised 70% of the group. The mean patient delay before seeking treatment (days of pain) was 1.9 days (range 5 hours to 28 days). A palpable mass was noted in 2% of patients at the time of presentation.

The differences between the groups with perforated appendicitis and nonperforated appendicitis are shown in Table I. Days of pain differed most between

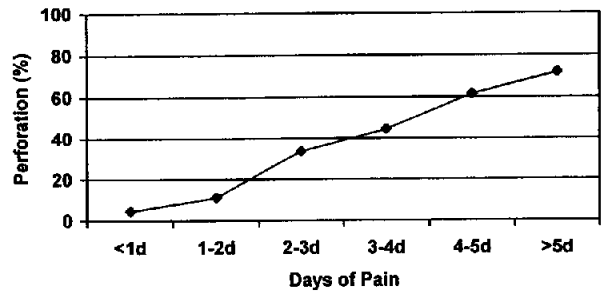

Fig. 1. Incidence of perforation relative to days of pain (n = 320).

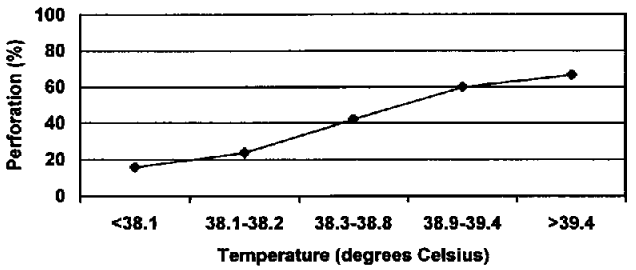

Fig. 2. Incidence of perforation relative to temperature (n = 320).

the groups (mean 3.3 days vs. 1.5 days). Age, temperature, and heart rate were greater in the perforated group, but the absolute differences were smaller. The finding of localized tenderness not in the right lower quadrant, a periappendiceal mass, and patient complaints of diarrhea were significantly associated with perforation. None of these findings were common, however.

Multivariate logistic regression was used to determine which of the variables were independently predictive of perforation. Days of pain, temperature, and abdominal tenderness localized outside the right lower quadrant were found to be significant ($P < 0.05$). Of note, the presence of a mass and patient age were not found to be independent predictors of perforation.

To formulate a rule for the diagnosis of perforated appendicitis, we incorporated the three variables found to be significant by multivariate analysis. We chose cutoff values for pain duration and temperature based on qualitative inspection of our data. The distribution of days of pain and temperature relative to incidence of perforation is shown in Figs. 1 and 2. Patients with 2 to 3 days of pain had a 33% incidence of perforation, as opposed to a 10% incidence for patients with less than 2 days of pain. Likewise, patients who presented with a temperature of 101 to 101.9° F (38.3 to 38.8° C) had a 42% incidence of perforation, as opposed to a 15% incidence for patients with a temperature of less than 101° F (38.3° C). Because pain duration of 2 days and temperature of 101° F (38.3° C) were associated with substantial increases in the incidence of perforation, these values were chosen as cutoff values to indicate perforation. The final variable found to be significant by multivariate analysis was localized tenderness outside the right lower quadrant. Three out of the four patients in our study population found to have localized tenderness outside of the right lower quadrant had perforated appendicitis.

We defined our rule for diagnosing perforation based on admission factors as follows: pain two or more days of pain **or** a temperature of ≥101° F (38.3° C) **or** localized tenderness not in the right lower quadrant. When we applied this rule to our patient population, we achieved a sensitivity of 86%, specificity of 58%, positive predictive value of 39%, and negative predictive value of 93%.

DISCUSSION

There have been many studies detailing the differences between patients with perforated and nonperforated appendicitis. The presence of a mass in the right lower quadrant and diffuse peritonitis are commonly accepted indicators of perforation. Number of days of pain and patient age have repeatedly been found to be associated with perforation.[3,4,6,7,17-23] Hale et al.[6] reviewed 4950 appendectomy patients and characterized the difference in admission factors between those patients with perforated and those with nonperforated appendicitis. Using multivariate regression they found temperature, age, white blood cell count, and sex to be significantly associated with perforation. Berry and Malt[21] likewise examined factors associated with perforation and found duration of symptoms, complaints of fever, and presence of a mass to be significantly associated with perforation. Although the differences between patients with perforated and nonperforated appendicitis have been well characterized, the diagnostic accuracy of admission factors for perforated appendicitis is unclear.

Our patient population is consistent with others reported recently in the literature. Our predominance of males, normal appendectomy rate, and perforation rate are consistent with what has been published in recent large studies of at least 800 patients.[6,22,23] Most recent studies report the mean days of pain for patients with nonperforated appendicitis to be less than 2 days and for patients with perforated appendicitis to be more than 2 days, similar to our findings.[3,7,19]

We found days of pain, temperature, and tenderness localized outside the right lower quadrant to be

independently associated with perforation. Of these variables, days of pain was the most useful for predicting perforation. Temperature was less useful because of the significant overlap of values in the perforated and nonperforated groups. Tenderness localized outside the right lower quadrant was less useful because of its low incidence. One might expect the presence of a palpable periappendiceal mass and age to be independent predictors of perforation. We found both to be significant with univariate analysis but not with multivariate analysis. Many previous studies have demonstrated a strong association between age and incidence of perforation.[3,4,6,7,17-20] Our inability to demonstrate this using multivariate regression analysis likely is because our patient population is largely comprised of middle-aged and young adults. Only six patients in our study population were 60 years of age or older, and no patients were younger than 16 years of age. The presence of a mass was not found to be an independent predictor of perforation likely because of its low incidence and the association of this variable with days of pain.

When we applied the rule of two or more days of pain **or** a temperature of ≥101° F (38.3° C) **or** localized tenderness not in the right lower quadrant to our study population, we achieved a sensitivity of 86% and a specificity of 58%. The positive predictive value for perforation was 39%, whereas the incidence of perforation in the entire appendicitis group was 24%. Because we used the same population of patients to develop the rule and test it, the results obtained are likely better than what would be obtained if the rule was tested on a different patient population. Even given this "best case" situation, it is apparent that perforated appendicitis cannot be reliably diagnosed preoperatively using only admission factors. Nonetheless, it seems clear that patients (middle-aged and young adults) who present with two or more days of pain or a temperature of ≥101° F (38.3° C) have a much higher incidence of perforation (39% vs. 24%) relative to the average population of patients with appendicitis.

We believe these data to be important for two reasons. First, these results support the belief that physicians cannot reliably diagnose perforation preoperatively based on patient history, physical examination, and laboratory data. Second, our results enable more rational patient selection for imaging studies and potential nonoperative management.

It is our belief that select patients with perforated appendicitis can be treated nonoperatively with less morbidity than with urgent operative treatment. This is relatively well accepted for patients with a palpable periappendiceal mass.[8-14] For patients without a palpable mass we must rely on CT scanning to document perforation if we are to embark on a nonoperative treatment strategy. We have previously shown that appendicitis patients with CT findings of periappendiceal phlegmon, abscess, or extraluminal gas can be treated nonoperatively with low morbidity.[15,16] In the subgroup of patients with perforated appendicitis who have a periappendiceal abscess, we have demonstrated initial nonoperative management to be associated with a significantly lower morbidity rate than with urgent operative management (unpublished).

Our current data suggest that appendicitis patients with duration of pain of two or more days or a temperature of ≥101° F (38.3° C) or localized tenderness not in the right lower quadrant constitute a group with a high incidence of perforation that may benefit from CT scanning and possible initial nonoperative management. Although some researchers have recently advocated routine CT scanning for patients suspected of having appendicitis,[24] we favor the selective use of CT scanning for patients with indeterminate findings for appendicitis[25,26] and patients being considered for initial nonoperative management.

CONCLUSION

Perforated appendicitis cannot reliably be distinguished from nonperforated appendicitis based on admission factors. Days of pain, maximum temperature in the emergency department, and localized abdominal tenderness outside the right lower quadrant are the most important admission variables for distinguishing perforated from nonperforated appendicitis in our group of adult patients with appendicitis. Patients with appendicitis who present with pain of two or more days' duration, a temperature of ≥101° F (38.3° C), or localized abdominal tenderness outside the right lower quadrant have a much higher incidence of perforation relative to the overall appendicitis population.

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Cholinergic Intrapancreatic Neurons Induce Ca^{2+} Signaling and Early-Response Gene Expression in Pancreatic Acinar Cells

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Pancreatic exocrine function has been demonstrated to be under neuronal regulation. The pathways responsible for this effect, and the long-term consequences of such interactions, are incompletely described. The effects of neuronal depolarization on pancreatic acinar cells were studied to determine whether calcium signaling and *c-fos* expression were activated. In pancreatic lobules, which contain both neurons and acinar cells, agonists that selectively stimulated neurons increased intracellular calcium in acinar cells. Depolarization also led to the expression of *c-fos* protein in 24% ± 4% of the acinar cells. In AR42J pancreatic acinar cells, cholinergic stimulation demonstrated an average increase of 398 ± 19 nmol/L in intracellular calcium levels, and induced *c-fos* expression that was time and dose dependent. The data indicate that intrapancreatic neurons induce Ca^{2+} signaling and early-response gene expression in pancreatic acinar cells. (J GASTROINTEST SURG 2000;4:475-480.)

KEY WORDS: AR42J cells, calcium, *c-fos*, pancreatic neurotransmission

The mechanisms underlying pancreatic secretion have been well studied, and cholecystokinin (CCK) has been recognized as a major stimulant of pancreatic exocrine secretion.[1] Although many early studies implied that the stimulatory effects of CCK occurred directly on the acinar cells, many recent studies have discovered that neuronal pathways may mediate the effects of CCK as well.[2,3]

Neuronal depolarization has been demonstrated to induce secretion from the pancreatic acinar cells.[4] The intracellular pathways that are involved in this interaction, as well as long-term consequences, are unknown. In several cell types, the actions of neurotransmitters and hormones involve mobilization of calcium as a second messenger and the expression of the immediate early-response genes, such as proto-oncogene *c-fos*.[5,6]

In the present study we report evidence that rat pancreatic lobules, containing both acinar cells and neurons, respond to neuronal depolarization with increased Ca^{2+} and *c-fos* expression. The administration of carbachol, an analog of acetylcholine, caused increased Ca^{2+} and *c-fos* expression in isolated acinar cells, and both of these effects were abolished by co-incubation with atropine.

MATERIAL AND METHODS

Sonicated salmon sperm DNA, trypsin-EGTA (4-[2-hydroxyethyl]-1-piperazine ethanesulfonic acid) (HEPES), Tween-20, penicillin/streptomycin solution, formaldehyde, carbachol, atropine, and veratridine were from Sigma Chemical (St. Louis, Mo.). *c-fos* cDNA, chicken gluteraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, and AR42J cells (passage 17) were purchased from the American Type Culture Collection (Rockville, Md.). Dulbecco's modified Eagle medium, fetal bovine serum, L-glutamine,

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phosphate-buffered saline, 10% normal goat serum, and Trizol total RNA isolation kit were purchased from Gibco Biological Research Laboratories (Grand Island, N.Y.).

Male rats weighing 200 to 250 grams were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.). Fura-2-actoxymethyl ester, fura-2 free acid, and pluronic were purchased from Molecular Probes (Eugene, Ore.). Maximum Nytran nylon membranes were obtained from Schleicher and Schuell (Keene, N.H.). Radiolabeled (α-^{32}P)dCTP (3000 Ci/mmol), Rediprime DNA labeling kit, horseradish peroxidase–conjugated donkey antirabbit immunoglobulin G, and enhanced chemiluminesence kit were purchased from Amersham (Arlington, Ill.). Quik-Hybe hybridization buffer was from Stratagene (La Jolla, Calif.). Midi select G-50 Sephadex spin columns from 5 Prime-3 Prime (Boulder, Colo.). Polyclonal *c-fos* antibody was from Calbiochem (La Jolla, Calif.).

All experiments were performed in standard solutions except where noted. Standard oxygenated physiologic buffer (pH 7.40) contained the following (in mmol/L): NaCl 103, HEPES 25, $NaHCO_3$ 25, glucose 11, KCl 4.7, $CaCl_2$ 2.6, L-glutamine 2, NaH_2PO_4 1.2, $MgCl_2$ 1.1, as well as 2% (volume/volume) essential amino acids, 1% (weight/volume) bovine serum albumin, 0.1% (weight/volume) soybean trypsin inhibitor, and aprotinin 680 KIU/ml. Standard control buffer (pH 7.40) contained the following (in mmol/L): NaCl 118, KCl 4.7, $NaHCO_3$ 15, glucose 11, HEPES 10, $CaCl_2$ 1.8, NaH_2PO_4 0.9, and $MgSO_4$ 0.8.

cDNA Probes

cDNA for chicken GAPDH[7] and for *c-fos* were purchased from American Type Culture Collection. Qiagen Maxi-Pre isolation kits were used to harvest the plasmids. A 920 base-pair Pst I fragment of *c-fos* and a 1000 base-pair Pst I fragment of GAPDH were isolated with restriction enzyme digestion and Qiaex II gel isolation kits on agarose gels.

Pancreatic Lobule Studies

Pancreatic lobules, containing both neurons and acini, were harvested from Sprague-Dawley rats after an overnight fast. After sacrifice, individual pancreatic lobules were removed with the aid of a dissecting microscope. The lobules were placed into oxygenated physiologic buffer at 37° C and allowed to equilibrate for 30 minutes. For the calcium imaging experiments, lobules were incubated in buffer supplemented with 2 to 3 μmol/L fura-2 at 37° C for 30 minutes and loaded into a lucite superfusion chamber for imaging as described below. For the immunostaining experiments, the lobules were exposed to veratridine (10^{-4} mol/L) or control solution for 120 minutes. Lobules were then fixed with 4% paraformaldehyde, rinsed, serially dehydrated in alcohol solutions, and sectioned at 5 μmol/L. Sections were subsequently deparaffinized and rehydrated. Ten percent normal goat serum was added to the solutions to block for nonspecific immunoreactivity. The preparations were then incubated overnight at 4° C with polyclonal *c-fos* antibody (1:200) prepared in rabbits against a synthetic peptide corresponding to residues 4 to 17 of human fos protein. Subsequently the sections were washed three times for 10 minutes each in 0.1 mol/L phosphate-buffered saline, then incubated 1 hour in 1:200 biotinylated donkey antirabbit immunoglobulin. The sections were then incubated with ABC reagent for 30 minutes, after which DAB solution was added, and then finally hematoxylin was added for counterstaining.

Tissue Culture

AR42J cells were maintained as subconfluent monolayers in Dulbecco's minimum essential medium supplemented with 10% fetal bovine serum, L-glutamine (2 mmol/L), and penicillin (100 IU/ml)/streptomycin (100 mg/ml) solution. Cells were grown at 37° C and 10% CO_2, and the culture medium was changed every other day. Cells were used between passages 20 through 30. For calcium imaging experiments, cells were trypsinized and plated on a 22 mm coverslip at a density of 2×10^6 cells in 1 ml. Cells were incubated at this concentration at 37° C for 2 hours to enable adherence, after which medium was added. These coverslips were incubated for 24 hours before calcium studies. For RNA experiments, cells were plated at 5×10^6 cells per 10 cm dish and grown to 80% confluence. Plates were serum deprived overnight and then treated with control conditions, or carbachol with or without atropine prior to RNA isolation.

Loading and Cell Preparation for Imaging

Cultured cells were incubated at 37° C in fresh warmed medium containing 2 to 3 μmol/L fura-2 actoxymethyl ester and 0.025% pluronic for 45 minutes. Loaded coverslips were washed and replaced in standard control buffer, then placed in a lucite superfusion chamber. The superfusion rate of the control buffer and experimental solutions was 1 ml/min at 37° C.

Calcium Measurements

A Zeiss Axiovert inverted microscope and Attofluor digital imaging system (Rockville, Md.) were used for single-cell $[Ca^{2+}]_i$ determinations. $[Ca^{2+}]_i$ was calcu-

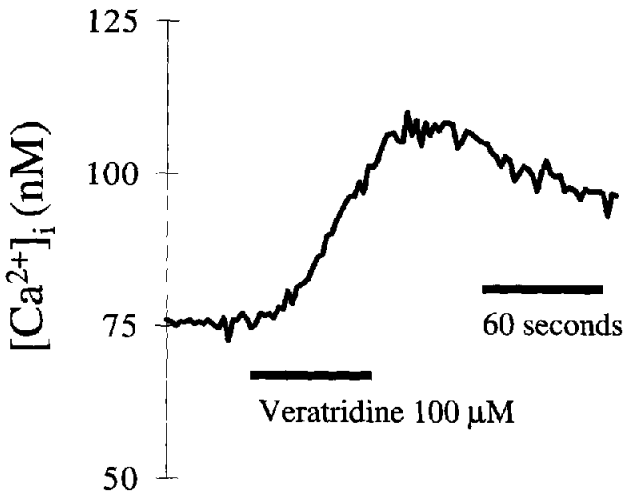

Fig. 1. Veratridine perfusion of pancreatic lobules increases $[Ca^{2+}]_i$. Tracing depicts the average of 48 acinar cells in the lobule preparation perfused with 100 μmol/L veratridine.

lated from the ratios of the fluorescence intensities of fura-2 at 334 and 380 nm wavelengths with an emission wavelength of 500 nm. Calibration of the system was performed with the following two-point standardization equation using fura-2 free acid: $[Ca^{2+}]_i = K_d[(R-R_{Lo})/(R_{Hi}-R)]\beta$, where K_d is the dissociation constant of the Ca^{2+}-fura-2 complex (225 nm), $R = F_{334}/F_{380}$, that is, the fluorescence at 334 nm divided by the fluorescence at 380 nm excitation, R_{Lo} = ratio at zero Ca^{2+} (1 mmol/L EGTA), R_{Hi} = ratio at high Ca^{2+} (1 mmol/L $CaCl_2$), and $\beta = F_{380}$ (zero Ca^{2+})/F_{380} (saturating Ca^{2+}). Frames were not averaged. A ratio pair was taken every second. The Attofluor digital imaging system calculated the changes in calcium concentration, and statistical analysis of the results was performed using Student's *t* test.

RNA Isolation and Northern Blot Analysis

Cells were harvested and total RNA was isolated using Trizol reagent. Equal amounts of RNA (20 μg/lane) were danatured and electrophoresed on 1.25% agarose-6% formaldehyde gels, transferred to nylon membranes, and immobilized by UV cross-linking (Stratalinker, Stratagene). cDNA probes were labeled with ^{32}P dCTP using a random hexanucleotide priming kit followed by purification on a G50 Sephadex spin column. Membranes were prehybridized for 30 minutes at 68° C with QuikHybe solution supplemented with 100 μg/ml salmon sperm DNA. Hybridization with the ^{32}P-labeled probes commenced for 60 minutes at 68° C. Membranes were washed in 2× sodium saline citrate (SSC)/0.1% sodium dodecyl sulfate (SDS) at room temperature, followed by 0.1 × SSC/0.1%SDS at 60° C, and then exposed to film (Kodak X-Omat AR, Rochester, N.Y.) with intensifying screens at −80° C. GAPDH cDNA was used subsequently under the same conditions to normalize the signals.

RESULTS

Effects of Veratridine on Ca^{2+} Release

Superfusion of pancreatic lobules with veratridine caused increases in $[Ca^{2+}]_i$ levels. Veratridine will open the fast sodium channels on the axons of neurons, inducing depolarization. Forty-eight pancreatic lobule cells exposed to 100 μmol/L veratridine demonstrated a rise in the $[Ca^{2+}]_i$ from an average basal level of 73 ± 2 nmol/L to a peak level of 112 ± 2 nmol/L after exposure (Fig. 1). In experiments using rat pancreatic AR42J cells, perfusion with 100 μmol/L veratridine had no effect on the $[Ca^{2+}]_i$.[8]

Effects of Veratridine on *c-fos* Expression

After exposure of pancreatic lobules to veratridine (100 μmol/L) for 120 minutes, 24% ± 4% of the acinar cells stained positively for *c-fos* (Fig. 2). Control lobules did not demonstrate any *c-fos* staining.

Effects of Carbachol on Ca^{2+} Release

Superfusion of fura-loaded pancreatic AR42J cells with carbachol increased $[Ca^{2+}]_i$. In 27 AR42J cells exposed to carbachol (10 μmol/L), the $[Ca^{2+}]_i$ rose from a basal value of 89 ± 5 nmol/L to a peak of 487 ± 19 nmol/L. The onset of this $[Ca^{2+}]_i$ peak was rapid, occurring within 5 seconds of perfusion by the agonist. The $[Ca^{2+}]_i$ then slowly returned to basal levels over the subsequent 400 seconds. These $[Ca^{2+}]_i$ responses to carbachol were dose dependent (Fig. 3), with maximal stimulation occurring at (1 μmol/L). The addition of atropine (10 μmol/L)

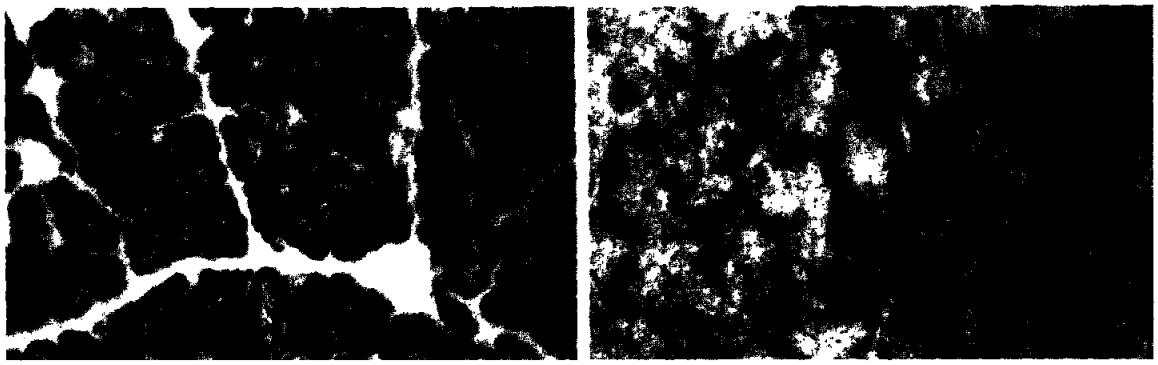

Fig. 2. Veratridine induces *c-fos* expression. Pancreatic lobules were harvested and exposed to control solution **(A)** or 100 μmol/L veratridine **(B)** for 120 minutes. Immunostaining with polyclonal *c-fos* antibody (1:200) resulted in 24% ± 4% of the veratridine-stimulated lobules demonstrating positive staining. Control solution had no effect.

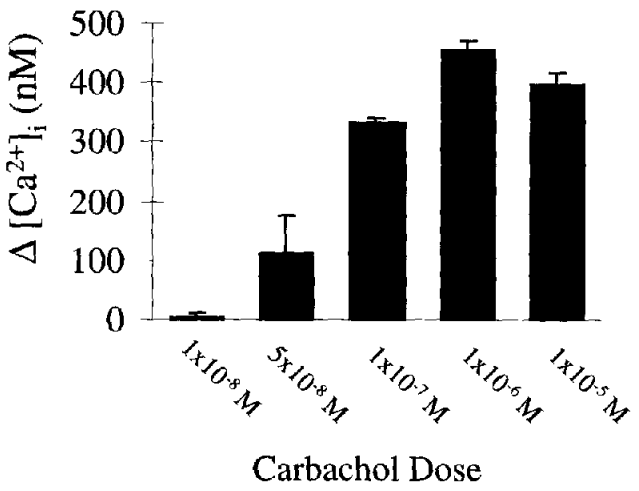

Fig. 3. Carbachol demonstrates dose-dependent increases in $[Ca^{2+}]_i$ in pancreatic AR42J cells. AR42J cells (n = 6 to 27) were perfused with increasing carbachol concentrations. Maximal change in $[Ca^{2+}]_i$ was seen at 1 μmol/L.

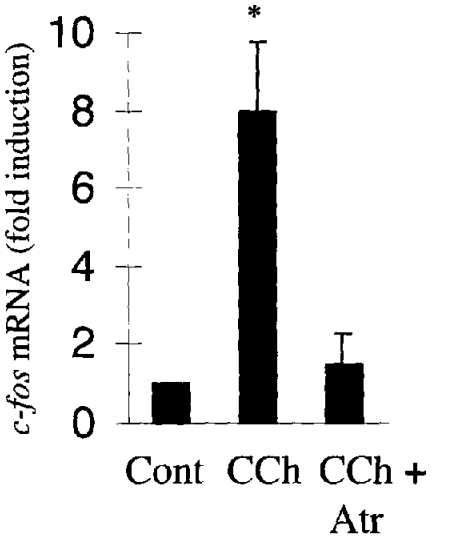

Fig. 4. Effect of atropine on carbachol (CCh)-induced *c-fos* expression. Ten μmol/L atropine completely abolished the observed *c-fos* induction from 10 μmol/L carbachol.

Table I. Time dependence of *c-fos* expression

CCh (10^{-5} mol/L) exposure	0 min	15 min	30 min	60 min	120 min
c-fos mRNA (fold induction)	1	10.1 ± 4.2*	18.0 ± 4.0*	2.1 ± 0.4	0.5 ± 0.2

Carbachol (CCh) induction of *c-fos* mRNA is time dependent. After treatment of AR42J cells, Northern blot analysis was performed, hybridizing for *c-fos* mRNA followed by cGAPDH to normalize for variations in technique. Results are expressed as fold induction of *c-fos* mRNA vs. control (no stimulus). Time-dependent increases were noted, which were significant at 15 minutes and maximal at 30 minutes.
*$P < 0.05$ by Student's *t* test.

Table II. Dose dependence of *c-fos* expression

CCh dose (μmol/L)	0	0.01	0.1	1	10	100
c-fos mRNA (fold induction)	1	1.6 ± 0.5	2.0 ± 0.5	3.9 ± 1.2	8.3 ± 1.2*	9.5 ± 3.0*

Dose-dependent increases in *c-fos* mRNA to carbachol (CCh), which were maximal at 10 μmol/L.
*$P < 0.05$ by Student's *t* test.

to the perfusion solution abolished the observed $[Ca^{2+}]_i$ increases.

Effects of Carbachol on *c-fos* Expression

Carbachol (10 μmol/L) stimulated the expression of *c-fos* mRNA in pancreatic AR42J cells that was time dependent (Table I). *c-fos* Expression was significant at 15 minutes, maximal at 30 minutes, and returned to baseline by 60 minutes. Pancreatic AR42J cells also expressed *c-fos* mRNA in a dose-dependent fashion to carbachol, which was maximal at 10 μmol/L (Table II). Atropine (10 μmol/L) completely abolished *c-fos* expression induced by 10 μmol/L carbachol (Fig. 4).

DISCUSSION

These studies demonstrate, in rat pancreatic lobules containing both acinar cells and neurons, that events leading to neuronal depolarization induce Ca^{2+} signaling and *c-fos* expression in acinar cells. In rat AR42J cells, the acetylcholine analog carbachol increases $[Ca^{2+}]_i$ and induces expression of *c-fos*. The effects of carbachol are abolished by coincubation with atropine.

Several recent studies support the role of neural regulation in pancreatic physiology. CCK has been considered one of the principal secretagogues for pancreatic acinar cells. Li and Owyang[3] have demonstrated that serum concentrations of CCK equivalent to those seen after a meal, when delivered directly, failed to elicit a response from purified acinar cells. This group also demonstrated that vagotomy will abolish CCK-induced pancreatic secretion and that the administration of atropine will abolish CCK responsiveness.[2] Although CCK receptors have been identified on acinar cell plasma membranes, they have also been localized on vagal afferents. This finding suggests that CCK may, at least partially, act in a reflex arc to stimulate acinar cell secretion through presynaptic actions.[9,10] Other studies have demonstrated the presence of neurons traveling between the duodenum and the pancreatic parenchyma.[11]

In the short term, changes in intracellular Ca^{2+} levels are associated with a variety of cellular responses in the gastrointestinal tract, such as motility and secretion.[12] Our data demonstrate that Ca^{2+} increases are present in the pancreatic acinar cells on neuronal stimulation. Veratridine, which opens fast sodium channels on neuronal axons and causes depolarization, was able to increase $[Ca^{2+}]_i$ in acinar lobule preparations. The Ca^{2+} response was due to the effects of neuronal depolarization, as veratridine could not elicit a Ca^{2+} response when applied directly to deinnervated pancreatic cells. In AR42J cells, which are representative of isolated acinar cells, carbachol was able to induce a dose-dependent calcium response that was blocked by coperfusion with atropine.

Cellular stimulation and altered cytosolic Ca^{2+} levels also lead to the initiation of gene programs, which can potentially affect long-term function. The protooncogenes *c-fos* and *c-jun*, which form a dimer that binds at the transcriptional regulatory element AP-1, have been shown to participate in cellular growth, differentiation, and development.[6,13] In the present study we demonstrated expression of *c-fos* mRNA and *c-fos* protein as indicators that neuronal depolarization could alter pancreatic physiology over a longer period of time. In AR42J cells, carbachol stimulation was able to induce *c-fos* mRNA production, which was initiated at 15 minutes, was maximal at 30 minutes, and returned to baseline by 60 minutes. This time course of carbachol stimulation is consistent with *c-fos* protooncogene expression, as reported by others.[6] That *c-fos* expression was dependent on a cholinergic pathway was demonstrated by dose dependence and by inhibition with atropine; this finding is consistent with those of other groups in earlier works.[14] The expression of *c-fos* mRNA was associated with production of the *c-fos* protein. This was demonstrated in the lobule preparations, as depolarization of the neurons with veratridine resulted in 24% positive staining by anti-*c-fos* antibody.

CONCLUSION

In rat pancreatic lobules, depolarization of intrapancreatic neurons causes increased Ca^{2+} and *c-fos* expression in the acinar cells. The administration of carbachol, an analog of acetylcholine, caused increased Ca^{2+} and *c-fos* expression in isolated acinar cells, and both of these effects were abolished by atropine coincubation. Intrapancreatic neurons affect pancreatic physiology in both the short term and long term.

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Number and Size of Stones in Patients With Asymptomatic and Symptomatic Gallstones and Gallbladder Carcinoma: A Prospective Study of 592 Cases

Attila Csendes, M.D., Marina Becerra, M.D., Jorge Rojas, M.D., Ernesto Medina, M.D.

The development of gallbladder carcinoma has been correlated with the presence of a single large gallstone in two retrospective studies. The objective of the present study was to determine the number and size of gallstones in patients with gallbladder carcinoma compared to asymptomatic and symptomatic female patients with gallstones. The following three groups of patients were included in this prospective trial: (A) 78 asymptomatic patients with gallstones; (B) 365 symptomatic patients with gallstones; and (C) 149 patients with gallbladder carcinoma. At the end of the operation, the resected gallbladder was opened and the number of stones counted. The maximum size of the stones was determined using calipers. Patients with gallbladder carcinoma were significantly older than patients in the other two groups ($P < 0.001$). In the group with asymptomatic gallstones, there were significantly more patients with one single stone, whereas in the group with gallbladder carcinoma there were significantly more patients with multiple stones (more than 11; $P < 0.01$). Patients with gallbladder carcinoma had significantly larger stones, regardless of the number of stones present ($P < 0.001$). We postulate that the increase in the number and size of the stones among patients with gallbladder carcinoma could simply be an effect of aging or it could be a reflection of the long-term presence of stones in the gallbladder rather than some particular chemical or physical influence. (J GASTROINTEST SURG 2000;4:481-485.)

KEY WORDS: Gallstones, gallbladder carcinoma, asymptomatic gallstones

Gallbladder carcinoma is highly prevalent in Chile.[1-4] It is the leading cause of death from malignant tumors among women, its incidence exceeds that of gastric, breast, and uterine carcinoma. The following three main factors[1,4,5] have been identified as being related to this type of carcinoma: (1) sex, with gallbladder carcinoma occurring with five to eight times greater frequency in women compared to men; (2) age, with a progressive increase in the prevalence of this carcinoma, particularly in those over 50 years of age; and (3) presence of gallstones, with this being related to almost 100% of our cases of gallbladder carcinoma. Several previous studies[6-8] have analyzed the relationship between the size of gallstones and the risk of gallbladder cancer, but all of these studies were retrospective and included few patients with conflicting results. The purpose of the present prospective study was to determine the number and size of the gallstones that were present in the following three groups of patients: those with asymptomatic gallstones, those with symptomatic gallstones, and those with gallbladder carcinoma.

PATIENTS AND METHODS

A total of 592 adult women were included in this prospective study, which lasted 5 years. Only women were included in our study because gallstones are three times more frequent in women compared to men and gallbladder carcinoma is six to eight times more frequent in women.[1,2] Patients were divided into three groups.

Asymptomatic Gallstones. Seventy-eight patients underwent ultrasonography, the results of which demonstrated one or more gallstones, with a normal extrahepatic common bile duct. In Chile the policy of operating on patients with asymptomatic gallstones is based on results of two prospective studies which have

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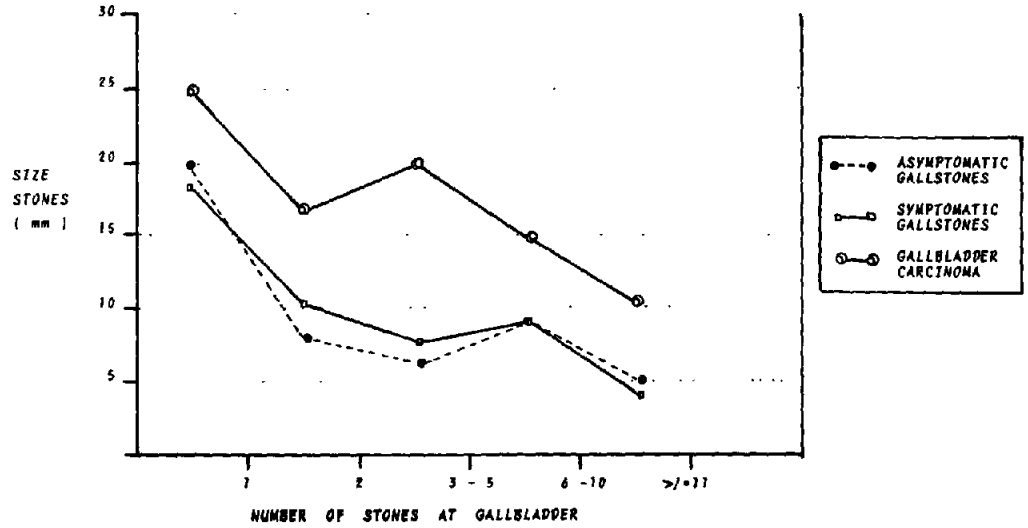

Fig. 1. Number of stones in the gallbladder among patients with asymptomatic gallstones, symptomatic gallstones, and gallbladder carcinoma.

Table I. Age distribution

Group	No.	≤60 yr	>61 yr
A. Asymptomatic gallstones	78	53 (68%)	25 (32%)
B. Symptomatic gallstones	365	291 (79.7%)	74 (20.3%)
C. Gallbladder carcinoma	149	56 (37.6%)	93 (62.4%)

Age ≤60 years: A vs. B, $P < 0.02$; A vs. C, $P < 0.000$; B vs. C, $P < 0.000$.
Age >61 years: A vs. B, $P < 0.02$; B vs. C, $P < 0.000$; A vs. C, $P < 0.000$.

Table II. Number of stones in the gallbladder

No. of stones	Group A: Asymptomatic gallstones (n = 78)	Group B: Symptomatic gallstones (n = 365)	Group C: Gallbladder carcinoma (n = 149)	*P* value
1	43 (55.1%)	129 (35.3%)	46 (30.8%)	A vs. B and C <0.001 B vs. C <0.3
2	6 (7.7%)	42 (11.5%)	12 (8%)	NS
3-5	3 (3.8%)	41 (11.2%)	7 (4.7%)	B vs. A and C <0.015 A vs. C <0.8
6-10	2 (2.6%)	49 (13.4%)	19 (12.7%)	A vs. B and C <0.02 B vs. C <0.8
≥11	24 (30.8%)	104 (28.5%)	65 (43.6%)	A and B vs. C <0.001 A vs. B <0.2

NS = not significant.

Table III. Number and size of stones in the gallbladder

	Mean size of gallstones (mm)			
No. of stones	Group A: Asymptomatic gallstones (n = 78)	Group B: Symptomatic gallstones (n = 365)	Group C: Gallbladder carcinoma (n = 149)	*P* value
1	19.7 ± 9.4	18.4 ± 10.1	25.4 ± 11.8	A and B vs. C <0.006
2	7.5 ± 4.5	10.3 ± 8.1	15.7 ± 13.0	A and B vs. C <0.002
3-5	6.6 ± 2.5	8.8 ± 5.3	19.5 ± 13.8	A and B vs. C <0.001
6-10	8 ± 1.4	8.5 ± 5.8	14.8 ± 11.1	A and B vs. C <0.01
>11	7.8 ± 6.0	7.4 ± 6.0	10.7 ± 8.1	A and B vs. C <0.008

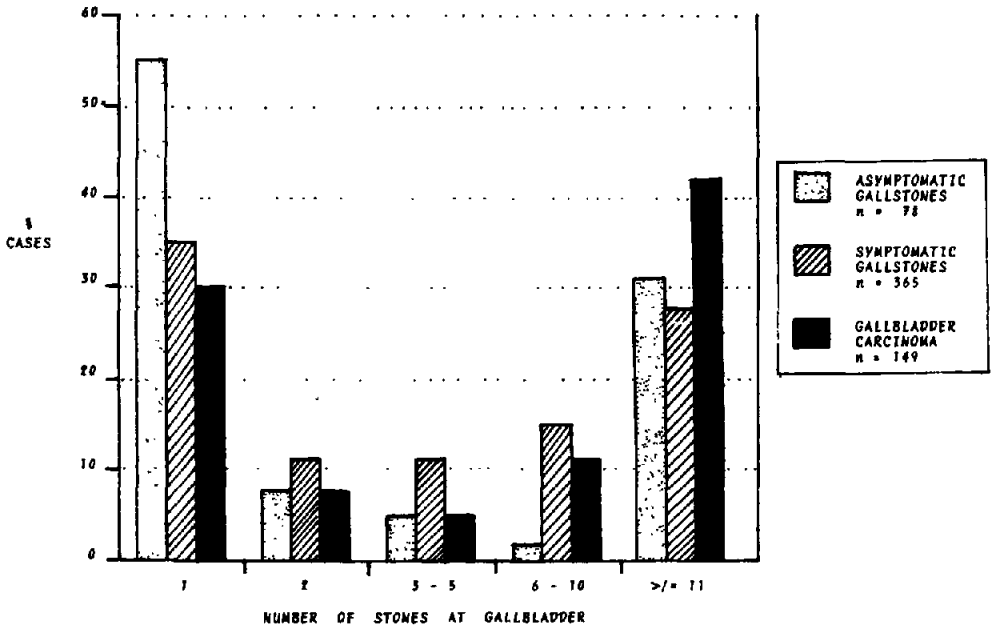

Fig. 2. Relationship between size of stones and number of stones in the gallbladder among patients with asymptomatic gallstones, symptomatic gallstones, and gallbladder carcinoma.

shown that in 50% of patients with asymptomatic gallstones complications develop within 5 to 8 years after diagnosis.[9,10] In addition, the prevalence of occult undiagnosed gallbladder carcinoma among patients referred for cholecystectomy is approximately 3%, which is very high for this disease.[2,3,5]

Symptomatic Gallstones. A total of 365 patients had a history of biliary cholic pain with or without jaundice. Ultrasonography in all of them demonstrated one or more stones in the gallbladder with a normal extrahepatic common bile duct.

Gallbladder Carcinoma. In 149 patients, carcinoma of the gallbladder was demonstrated by histologic examination after cholecystectomy.

Patients with acute pancreatitis, common bile duct stones, acute cholangitis, and stricture of the common bile duct were excluded, as were patients under 18 and over 80 years of age.

In all patients, open or laparoscopic cholecystectomy was performed as an elective procedure. Patients with gallbladder carcinoma all underwent a classic or extended cholecystectomy. A complete histopathologic examination was also performed in all patients to confirm or rule out presence of adenocarcinoma of the gallbladder. At the end of the operation, the gallbladder was opened in the operating room, and the number of stones that were present was carefully recorded. If more than one stone was found, the smallest and largest stones were measured twice by means of calipers rather than a visual approximation. For the purpose of the present investigation, the largest measurement was used for statistical analysis.

Statistical Analysis

Chi-square and Fisher's exact tests were used for statistical evaluation. A value of $P < 0.05$ was considered significant.

RESULTS

The age distribution of the three groups is shown in Table I. Mean age for asymptomatic gallstones was 46.8 years (range 19 to 72 years), for symptomatic gallstones 42.6 years (range 70 to 80 years), and for gallbladder carcinoma 62.3 years (range 34 to 80 years). In the group with symptomatic gallstones, there were significantly more patients under 60 years of age compared to the asymptomatic group. Patients with gallbladder carcinoma were significantly older than those in the other two groups, and the proportion of patients over 61 years of age was also greater ($P < 0.000$).

The number of stones present in the gallbladder is shown in Table II and Fig. 1. In the group with asymptomatic gallstones, there were significantly more patients with one stone compared to the other two groups. In the group with gallbladder carcinoma, there were significantly more patients with multiple stones (more than 11) compared to the groups with asymptomatic and symptomatic gallstones.

The number of stones correlated to the size of the stones is shown in Table III and Fig. 2. Patients with gallbladder carcinoma had significantly larger stones in the gallbladder compared to the other two groups, regardless of the number of stones present.

DISCUSSION

Gallbladder carcinoma in women has increased progressively in Chile over the past 20 years, and it is now the leading cause of death from malignant tumors.[1-3] One of the most important factors related to the development of this type of cancer is the presence of gallstones, inasmuch as it is extremely rare to find a patient with gallbladder carcinoma who does not have gallstones.[4,5] The results of the present prospective study suggest that among patients with gallbladder carcinoma more of them have multiple stones compared to patients with asymptomatic and symptomatic gallstones. In addition, they have significantly larger stones, regardless of the number of stones present, compared to patients without carcinoma.

Three previous studies have evaluated the relationship between the size of stones and the presence of gallbladder carcinoma. The first study was carried out by Diehl,[6] who reviewed the results from 10 hospitals over a 5-year period. He retrospectively reviewed the data from 81 patients with gallbladder carcinoma, 80 patients with gallstones, and 66 control subjects. However, he found useful data in only 45 patients with carcinoma and 66 patients with gallstones. He concluded that patients with gallbladder carcinoma are more likely to have one large stone. Lowerfels et al.[7] conducted a retrospective evaluation comparing 1894 patients with gallstones to 25 patients with gallbladder carcinoma. These investigators concluded that patients with carcinoma who were over 50 years of age had more single stones with a stone size 10 mm greater than stones in patients without carcinoma. They also noted that 12% of patients with gallstones who were over the age of 50 years of age had stones larger than 3 cm, whereas 40% of patients with gallbladder carcinoma had stones of a similar size. Moumand et al.[8] reviewed the results from 18 hospitals during a 7-year period. They collected data from only 43 patients with gallbladder carcinoma and 98 patients with gallstones, which was insufficient to draw definite conclusions. They did not find any significant differences between the two groups with respect to size. As can be seen, all three of these studies were retrospective, they contained very few cases of gallbladder carcinoma (Lowerfels compared 25 patients with carcinoma to 1844 with gallstones), and the data collected were incomplete in many instances.

Thus we established a prospective protocol because we had a high incidence of gallbladder carcinoma, including the results in patients with asymptomatic gallstones, who have not been mentioned previously. We also determined the size of the stones with the use of calipers at the end of the operation, in an attempt to be as precise as possible. Our results clearly demonstrate that with regard to the number of stones, patients with gallbladder carcinoma had significantly more stones in the gallbladder compared to patients with benign disease, whereas in the asymptomatic group there were more patients with a single stone. With respect to the size of stones, patients with gallbladder carcinoma had stones that were larger, regardless of the number of stones present, whereas patients with benign disease had a very similar size pattern.

The exact role of gallstones in the development of gallbladder carcinoma is difficult to determine. The chemical composition of the gallstones in our study was very similar to what is found in other Western countries. In a prospective study of 107 patients who underwent cholecystectomy, 90% of the stones had more than 75% of their weight attributed to the presence of cholesterol.[11] We have not studied the chemical composition of gallstones among patients with gallbladder carcinoma, but it would probably be similar. We know that control subjects have no bacteria in their gallbladder bile.[12,13] We have also recently demonstrated for the first time that in nearly one third of control subjects, gallbladder mucosa shows abnormal histologic findings, mainly chronic cholecystitis, in the absence of stones.[14] These histologic changes occurred more frequently in women. In contrast, among patients with asymptomatic gallstones, all of them had gallbladder mucosa showing histologic abnormalities. Therefore it seems that chronic inflammatory changes are present before stones appear, being that the bile is sterile. We have also analyzed the chemical composition of gallbladder bile in control subjects and in patients with gallstones.[15] Among control subjects, 27% had crystals of cholesterol in their gallbladder bile, indicating a supersaturated bile in these cases. It is possible that these microcrystals could produce some inflammatory changes in the gallbladder mucosa prior to the appearance of stones. The theory that an infected gallbladder can produce stones seems to be incorrect given that histologic changes in the gallbladder mucosa can occur in a sterile environment before the appearance of stones; on the other hand, in the presence of symptomatic gallstones only 22% of them show aerobic bacteria in the gallbladder bile.[12] We have also evaluated the bacteriology of gallbladder bile in patients with gallbladder carcinoma compared to gallstones.[16] Patients with carcinoma had significantly more organisms in their bile compared to those with symptomatic gallstones, regardless of age. However, *Salmonella typhi* was present in only 4% of cases, both in benign and malignant disease. As a consequence, we do not believe that this bacteria has any great significance in the development of gallbladder carcinoma.

Patients with gallbladder carcinoma were 20 years older than those with symptomatic gallstones.[5] We

postulate that it is possible that the increased size of the stones could simply be a reflection of the long-term presence of stones in the gallbladder. We have demonstrated a progressive increase in histologic changes in the gallbladder mucosa according to age and the presence of stones.[5] Therefore it is possible that chronic physical trauma or irritation of the gallbladder mucosa over a period of years, combined with an increase in the presence of enteric bacteria in older patients, could promote epithelial dysplasia and ultimately progression to carcinoma. Based on these facts, it is possible that the increase in the number and size of stones among patients with gallbladder carcinoma could simply be due to the effects of aging rather than to some particular chemical or physical influence.

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Outcome of Laparoscopic Anterior 180-Degree Partial Fundoplication for Gastroesophageal Reflux Disease

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Although Nissen fundoplication controls gastroesophageal reflux disease effectively, it is associated with an incidence of side effects. For this reason we have investigated the use of a laparoscopic 180-degree anterior fundoplication as a technique that has the potential to control reflux, but with less associated postoperative dysphagia and fewer gas-related side effects. Good short-term (6-month) outcomes have been previously reported within the context of a randomized trial. This report details the technique we used and describes the outcome of this procedure with longer follow-up in a much larger group of patients. The outcome for patients with gastroesophageal reflux disease who underwent a laparoscopic anterior 180-hemifundoplication was determined. Clinical follow-up was carried out prospectively by an independent scientist who applied a standardized questionnaire yearly following surgery. This questionnaire evaluated symptoms of reflux, postoperative problems including dysphagia, gas bloat, ability to belch, and overall satisfaction with clinical outcome. From July 1995 to May 1999, a total of 107 patients underwent a laparoscopic anterior hemifundoplication. Four patients underwent further surgery for recurrent heartburn, and persistent troublesome dysphagia occurred in one. At 1 year 89% of patients remained free of reflux symptoms, and at 3 years 84% remained symptom free. Of those with symptoms of reflux, approximately half of them had only mild symptoms. The overall incidence and severity of dysphagia for liquids and solids was not altered by partial fundoplication. Epigastric bloating that could not be relieved by belching was uncommon, and only 11% of the patients at 1 year and 10% at 3 years following surgery were unable to belch normally. Overall satisfaction with the outcome of surgery remained high at 3 years' follow-up. Laparoscopic anterior partial fundoplication is an effective operation for gastroesophageal reflux, with a low incidence of side effects and a good overall outcome. (J GASTROINTEST SURG 2000;4:486-492.)

KEY WORDS: Laparoscopy, surgery, anterior partial fundoplication, esophagitis

Gastroesophageal reflux disease is a commonly encountered disorder in Western countries, with symptoms occurring in up to 40% of the adult population.[1,2] As the majority of patients treated nonoperatively will relapse following cessation of medication,[3,4] fundoplication has an important place in the treatment armamentarium. Since the Nissen fundoplication was originally described in 1956,[5] this procedure has been shown to offer a good long-term outcome for patients with reflux disease.[6,7] However, it can be followed by postoperative problems such as dysphagia, gas bloat, and inability to belch.

Laparoscopic Nissen fundoplication was first reported by Dallemagne et al.[8] in 1991, and since then extensive experience with laparoscopic antireflux surgery has been reported from many centers.[9-11] The results of many early reports have been promising. However, the laparoscopic Nissen procedure is still associated with the problems of dysphagia, gas bloat, and inability to belch.[7,12] Recent studies evaluating both open and laparoscopic surgery suggest that an anterior partial fundoplication is associated with less postoperative dysphagia, improved ability to belch, and reduced risk of gas bloat syndrome.[13,14] This has

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been verified, at least in the short term, by early outcomes in a randomized trial reported from our institution of anterior partial vs. Nissen fundoplication.[15] However, there have been no reports of the outcome in a larger group of patients undergoing laparoscopic anterior partial fundoplication with longer term follow-up. The aim of this study, therefore, was to evaluate clinical outcome in patients undergoing anterior fundoplication, and the efficacy of this technique, with follow-up of up to 3 years.

MATERIAL AND METHODS

The outcome was determined for all patients who underwent a laparoscopic anterior partial fundoplication for gastroesophageal reflux disease in the Department of Surgery of Royal Adelaide Hospital between July 1995 and May 1999. Preoperative, operative, and postoperative data were collected prospectively, with all pre- and postoperative clinical data collected by an independent nonmedical scientist who applied a standardized clinical questionnaire.

Operative Technique

Laparoscopic 180-degree anterior partial fundoplication was performed using the technique described below. If the procedure could not be completed using a laparoscopic approach, then an identical procedure was performed through an upper midline incision.

The anterior fundoplication procedure begins in a similar manner to the laparoscopic Nissen procedure, which has been described in detail elsewhere.[16] The patient is placed in the lithotomy position with the table tilted head up, and the surgeon operates from between the patient's legs. Four laparoscopy ports are placed: an 11 mm port in the midline above the umbilicus for the laparoscope, an 11 mm port in the left midclavicular line just below the costal margin for the surgeon's dissecting and suturing instruments, and two 5 mm ports in the right midclavicular line just below the costal margin for the insertion of a grasping forceps that is to be held in the surgeon's left hand and insertion of another forceps in the left flank for gastric retraction by the assistant. In addition, a Nathanson liver retractor (Cook Medical Technology, Queensland, Australia) is passed through a 5 mm stab wound in the epigastrium just below the xiphisternum.

Dissection of the hiatus is usually achieved by a blunt dissection technique, with diathermy or scissors dissection applied when needed. The transparent windows in the peritoneum of the lesser omentum above and below the hepatic branch of the anterior vagus nerve are initially opened, while preserving the nerve, so that the right pillar of the diaphragmatic hiatus is reliably identified. The esophageal hiatus is then opened between the edge of the right pillar and the esophagus using a blunt dissection technique. This plane is bloodless if correctly identified.

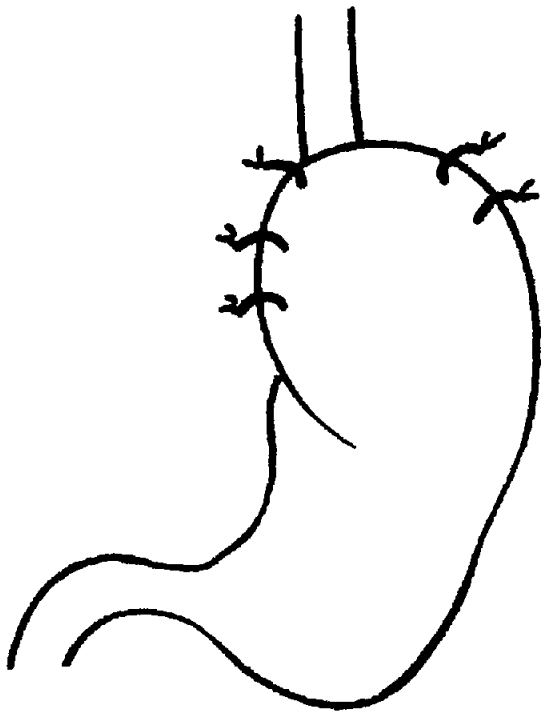

Fig. 1. Diagram of completed anterior fundoplication. In addition to posterior hiatal repair, the anterior esophagus is completely covered by the anterior fundus. The three sutures on the right side also incorporate the right hiatal pillar, as well as the esophageal wall superiorly and the gastroesophageal junction inferiorly. The two apical sutures anchor the fundoplication to the diaphragm anteriorly.

The phrenoesophageal ligament is then opened across the front of the hiatus, and dissection is continued along the anterior edge of the left pillar as posterior as possible. Dissection of the esophagus is then undertaken, so that it is fully mobilized distally, and a straight grasping instrument is passed from right to left behind the esophagus, and in front of the left pillar. A nylon tape is passed around the esophagus to lift it forward, while posterior esophageal dissection is completed, to adequately expose both hiatal pillars. The hiatus is routinely repaired posteriorly using interrupted 2-0 monofilament nonabsorbable sutures.

A 180-degree anterior partial fundoplication is then fashioned by fixing the anterior wall of the gastric fundus to the front of the esophagus and the diaphragmatic hiatus (Fig. 1). The anterior fundus is manipulated so that a piece is selected that sits loosely across the front of the esophagus, without tension. This has always been possible without dividing the

short gastric vessels. The right side of the fundus is sutured to the adjacent left side of the esophagus with two or three interrupted sutures to accentuate the angle of His. A top suture is placed through the top of the fundus, the esophagus immediatley below the hiatus, and the apex of the hiatus, and is then tied. Next the fundus is sutured to the right lateral wall of the abdominal esophagus and to the right hiatal pillar to complete the hemifundoplication. This is usually performed using two interrupted sutures that each incorporate the fundus, the muscle of the right lateral esophageal wall, and the right hiatal pillar. Some surgeons begin with the most posteroinferior suture, which usually incorporates the right (and sometimes also the left) hiatal pillar at the level of, or immediately below, the uppermost suture used for the posterior hiatal repair. Others prefer to begin proximally on the right. Other sutures are sometimes placed between the upper edge of the fundoplication and the apex of the hiatal rim, one on the left side and one on the right (crown sutures). These sutures do not incorporate the esophageal wall. This procedure accentuates the angle of His, repairs the hiatus, anchors a 3 to 5 cm length of esophagus below the diaphragm, and fashions a tension-free partial fundoplication, which is sutured to both the esophagus and the hiatal ring.

Patients usually begin taking fluids by mouth on the evening following surgery, and a soft diet is begun on either the first or second postoperative day. Most patients are discharged from the hospital on the second or third day following surgery.

Patient Assessment

Clinical follow-up data were collected prospectively. This was achieved preoperatively 3 months following surgery and yearly thereafter by applying a standardized questionnaire. The symptoms of heartburn, dysphagia for solids, and dysphagia for liquids were graded using individual visual analogue scales (0 = no symptoms; 10 = severe symptoms). Overall satisfaction with the postsurgical outcome was also assessed by means of another visual analogue scale (0 = totally dissatisfied; 10 = totally satisfied). In addition, any other postoperative problems including epigastric bloating, inability to belch, and inability to relieve bloating were determined.

RESULTS

Patients

A total of 107 patients with reflux esophagitis underwent a laparoscopic anterior partial fundoplication in the Department of Surgery of Royal Adelaide Hospital. Sixty-three patients were men and 44 were women, with a mean age of 48 years (range 18 to 85 years). Median follow-up was 2 years (maximum 3 years). One hundred one patients were available for follow-up at 3 months, 84 at 1 year, 61 at 2 years, and 32 at 3 years following surgery.

Fifty-four patients were enrolled in a prospective randomized trial and therefore were not selected for the partial fundoplication procedure because of abnormal preoperative motility studies. These patients comprised the "middle" half of our experience with this technique. For the other half of the series, there was a tendency to use the partial fundoplication procedure for patients with abnormal preoperative peristalsis at manometric assessment.

One hundred one patients (94%) had surgery to correct reflux symptoms that were not adequately controlled by medical therapy, and six patients who had good preoperative symptom control requested surgery so that they could stop taking medication. Preoperatively 65% of patients undergoing partial fundoplication experienced dysphagia (of varying severity) when swallowing solid foods and 32% reported dysphagia for liquids.

Preoperative Investigations

Esophageal manometry was performed in 91 patients (85%), with the remaining 16 patients selected for the partial fundoplication procedure because they were unable to tolerate manometry and esophageal peristalsis was uncertain at the time of surgery. In 47 patients (52%) peristalsis was normal and in 44 it was deficient (30 had less than 80% propagation of peristaltic waves, seven had distal peristaltic amplitude less than 25 mm Hg, and seven had deficient propagation and strength of peristaltic waves). Nine of these patients (10%) had an adynamic esophagus (total absence of primary peristalsis in the body of the esophagus). The lower esophageal sphincter resting pressure was less than 10 mm Hg in 63 patients (71%) and greater than 10 mm Hg in 26 patients; in two patients the sphincter could not be measured because of difficulties with placement of the manometry catheter due to a very large hiatal hernia.

Fifty-one patients underwent 24-hour ambulatory pH monitoring before surgery, and abnormal acid exposure in the distal esophagus was demonstrated in 46 (90%). Of the remaining five patients, three had endoscopic evidence of ulcerative esophagitis and two had no esophagitis at endoscopy. The latter two had typical reflux symptoms preoperatively, including regurgitation of acid, and had excellent relief of symptoms following fundoplication.

Endoscopy was carried out preoperatively in all patients, with 92 (85%) demonstrating esophagitis of

Table I. Pre- and postoperative heartburn and dysphagia symptom scoring

	Heartburn	Dysphagia for liquids	Dysphagia for solids
Preoperative	6.3 (5.6, 7.1)	1.5 (0.7, 2.1)	2.6 (1.8, 3.3)
3-5 mo	0.5 (0.2, 0.9)*	0.6 (0.3, 1.0)	1.5 (1.1, 1.9)
1 yr	1.1 (0.6, 1.6)*	1.1 (0.5, 1.6)	1.7 (1.1, 2.3)
2 yr	1.5 (0.9, 2.2)*	0.4 (0.1, 0.7)	1.4 (0.8, 1.9)
3 yr	1.6 (0.6, 2.6)*	1.1 (−0.03, 2.2)	1.2 (0.3, 2.1)

All data are expressed as mean (95% confidence intervals). Student's *t* test was used to compare preoperative and postoperative scores.
*$P < 0.0001$; for all other comparisons $P > 0.05$.

varying severity. A barium meal examination was performed in 54 patients (50%) preoperatively, and a hiatal hernia was demonstrated in 35 (65%).

Intraoperative Outcomes

Operative times ranged from 35 to 150 minutes (median 60 minutes). Three laparoscopic procedures (3%) were converted to open procedures because of the following difficulties: esophageal perforation in two patients due to dissection difficulties in those with dense periesophagitis, and difficulty defining the hiatal anatomy because of a very large hiatal hernia in the third patient.

Early Postoperative Outcomes, Complications, and Reoperation

Postoperative hospital stay ranged from 1 to 10 days (median 3 days). Nine patients (8%) had postoperative complications. Five were relatively minor and required minimal intervention including two chest infections, one wound infection, one deep vein thrombosis, and one case of urinary retention. The other four patients required early reoperation within the first 4 days of their original procedure. Two of these procedures were for repair of an acute paraesophageal hiatal hernia and the other two were for severe dysphagia due to a tight esophageal hiatus. In both of the procedures for dysphagia, a single hiatal repair suture was removed, which corrected the problem. There were no postoperative deaths in this series.

Late reoperation was required in another five patients between 8 months and 3½ years after the first procedure. This was completed laparoscopically in two patients who had recurrent reflux; one fundoplication was converted to a Nissen fundoplication and one anterior fundoplication was refashioned. In two additional patients, laparoscopic revision was attempted, but the procedure was converted to an open Nissen fundoplication in both because of recurrent reflux. One of these patients later required an open Ivor Lewis esophagectomy following the development of early esophageal cancer in preexisting Barrett's esophagus. The fifth patient underwent an open distal esophageal mucosectomy procedure following the development of severe dysplasia in Barrett's esophagus.

Long-Term Postoperative Clinical Outcome

Complete relief of reflux symptoms was initially achieved in all patients. However, recurrent reflux symptoms developed in nine patients (8%) overall. Four of them eventually underwent reoperation as described earlier, and the other five have some reflux symptoms (predominantly heartburn). Two of these five are taking no medications, two take occasional antacid tablets, and one patient is on a proton pump inhibitor. In none of the patients did symptoms of reflux become worse following partial fundoplication. The patients who underwent additional surgery had recurrent reflux demonstrated by either endoscopic evidence of ulcerative esophagitis, or an abnormal 24-hour pH study. Management of the other patients was based on clinical symptoms alone. Hence anterior fundoplication completely relieved all symptoms of reflux in 89% of patients followed for 12 months or longer. Furthermore, only five (6%) required reinstitution of acid suppression or reoperation for this problem. Five (16%) of 32 patients followed for 3 years or more developed recurrent reflux symptoms of any degree, with only three of them (9%) requiring acid suppression or reoperation. The mean heartburn score was significantly lower than the preoperative score at all follow-up intervals after surgery (Table I).

Postoperative dysphagia scores for both liquids and solids were slightly less than the preoperative scores at all follow-up intervals (see Table I), suggesting that anterior fundoplication was not associated with any long-term increase in the symptom of dysphagia. Twenty-five percent of patients at 3 months' follow-up, 8% at 12 months' follow-up, and 7% at 2 years' follow-up had a greater degree of dysphagia (as measured by the dysphagia score) compared to their preoperative status. On the other hand, 32% at 3 months, 31% at 12 months, and 31% at 2 years had dysphagia scores

that were better than their preoperative scores, suggesting that anterior fundoplication was associated with a net overall reduction in the incidence and severity of dysphagia. Only one patient required dietary modifications because of long-term troublesome dysphagia. Overall 81% of patients at 1 year, 90% at 2 years, and 85% at 3 years claimed no dietary restrictions.

Occasional epigastric bloating was common at all follow-up intervals (62% at 1 year, 64% at 2 years, and 75% at 3 years). This was comparable to an incidence of 52% for this symptom preoperatively. Most patients were able to relieve this symptom by belching. At 1 year 89% of patients reported a normal ability to belch; this figure was 88% at 2 years and 90% at 3 years. All patients claimed to be able to belch normally before surgery. An overall good or excellent outcome was achieved in 89% at 1 year, 89% at 2 years, and 91% at 3 years.

DISCUSSION

With the recent increase in interest in antireflux surgery, which has followed the development of laparoscopic techniques for the correction of gastroesophageal reflux disease, the medical community appears to have become more aware of the potential for troublesome side effects following the Nissen fundoplication procedure. This has led to the investigation of a range of modifications to Nissen's original procedure, which seeks to improve outcome in patients following antireflux surgery.[12] This is of importance because the perception that laparoscopic antireflux surgery is a good alternative to long-term acid suppression for reflux is ultimately dependent on the surgical community being able to achieve consistently excellent results following fundoplication, with a low incidence of side effects. Whereas in the past there has been a tendency for surgeons to concentrate their efforts on long-term efficacy and the risk of recurrent reflux following fundoplication, patients and referring physicians may also consider that the risk of adverse outcomes such as persistent dysphagia and gas-related symptoms might be even more important when deciding whether surgery is an appropriate treatment option, particularly in the era of effective medical treatment.

Such modifications to the Nissen fundoplication as division of the short gastric vessels,[17,18] as well as the routine use of posterior partial fundoplication, have failed to improve the overall outcome for patients undergoing surgery within previously published prospective randomized controlled trials,[19-21] and in particular there is no evidence from these trials that dysphagia is less likely to occur following a posterior partial fundoplication procedure. More recently, however, we reported the early outcome of a trial of anterior partial fundoplication vs. Nissen fundoplication, which demonstrated a reduced incidence of dysphagia and gas-related problems, yet equivalent control of reflux, at 6 months' follow-up.[15] Findings in this trial supported the conclusions drawn from previous short-term follow-up data from other small uncontrolled experiences with laparoscopic anterior partial fundoplication,[14,22] as well as longer term outcomes reported from a larger group of patients who underwent an open approach to anterior 120-degree fundoplication.[13] The operative technique for this variant of fundoplication differed from our approach in that a strip of esophageal wall on the right anterolateral aspect was not covered by the fundoplication in the procedures described by both Watson et al.[13] and Snow et al.[22] Nevertheless, there are probably enough similarities between our approach and that described by Watson et al.[13] to allow some comparisons, although we believe that our technique is simpler to perform.

Arguing against the routine use of anterior fundoplication is the possibility that an unacceptably high rate of recurrent reflux will ensue. Supporting this is the argument that the cardiomyotomy and Dor patch technique applied to the treatment of achalasia can be associated with a high rate of subsequent reflux.[23] However, postoperative reflux rates of approximately 6% to 11% have also been reported for this combination.[24,25] In addition, most surgeons who apply a Dor patch for treatment of achalasia do not combine it with hiatal repair and do not anchor the fundoplication to the hiatal rim on the right side. By anchoring the fundoplication to the hiatal rim on the right side and at the apex of the hiatus, and adding a posterior gastropexy and hiatal repair, the anterior fundoplication technique described in our report may achieve a more effective antireflux barrier. An average 3.5 years of follow-up data for the procedure previously described by Watson et al.[13] has also confirmed that this approach can achieve an acceptable long-term outcome.

The present series adds to the limited data reported for anterior fundoplication procedures. It reports a large series of prospectively followed patients who underwent a laparoscopic anterior fundoplication, with follow-up data now extending up to 3 years. This experience provides information about the longer term outcome following the laparoscopic approach. The recurrent reflux rate of approximately 10% may be considered high, but it compares favorably with experiences reported with the Nissen procedure. Hinder et al[26] reported a recurrent reflux rate of 8% at early clinical follow-up following a laparo-

scopic Nissen fundoplication procedure with division of the short gastric vessels, and Hunter et al.[27] reported a clinical recurrence rate of 7% at 12 months' follow-up after the Nissen fundoplication. It is possible in both our study and others reporting outcomes following the Nissen procedure that some asymptomatic patients may have an abnormal 24-hour pH profile postoperatively. However, we like others find it difficult to persuade asymptomatic patients to undergo postoperative 24-hour pH testing. The clinical rate of recurrent reflux reported in these series is higher than we would like, but the advantage of anterior fundoplication is that it achieved a high rate of patient satisfaction, which was aided by the low incidence of long-term side effects.

The anterior fundoplication technique achieves a reasonably durable outcome, at least in the midterm, although four of our patients underwent revision surgery for recurrent reflux symptoms. This incidence of reoperation for reflux was offset by a reduction in the incidence of late reoperation for persistent dysphagia, a problem that has been reported in a number of studies of the Nissen fundoplication.[28,29] Interestingly, however, two patients underwent laparoscopic revision within a few days of their original procedure for severe dysphagia because of overtightening of the hiatus, highlighting the fact that not all dysphagia following a fundoplication is caused by a tight wrap. Although the results in our series suggest a high overall reoperation rate of 8%, only the four procedures (3.7%) for recurrent reflux can be attributed to late failure of the original fundoplication. Four of the other five procedures were performed within a few days of the original procedure for repair of a paraesophageal hernia, or to widen the hiatus. We have previously advocated a policy of routine radiologic contrast studies within a few days of surgery, followed by early laparoscopic reexploration if problems are suspected,[9,30] and these early procedures were all in this category. All four patients were discharged within 5 days of the original procedure, and an excellent long-term outcome was obtained in all instances. The remaining "reoperation" followed the development of carcinoma in a patient with Barrett's esophagus.

We have reported manometric and/or pH monitoring outcomes in a smaller group of patients who entered our previously reported randomized trial.[15,31] Apart from anterior fundoplication producing a more "physiologic" lower esophageal sphincter, the outcomes were similar to those of patients undergoing a Nissen fundoplication. Furthermore, there was no disagreement between the objective and clinical outcomes, and for this reason we think that the clinical data in the current report accurately reflect the efficacy of the anterior fundoplication procedure. Ultimately, at least from the individual patient's perspective, it is the clinical outcome that matters, not the data derived from postoperative manometry or pH monitoring.

The overall reduction in dysphagia compared to the preoperative status was particularly encouraging, as a relatively high proportion of patients undergoing anterior fundoplication had poor esophageal peristalsis at preoperative manometric assessment. Dysphagia is often under-recognized as a preoperative problem, and in our experience it is present, at least to some extent, in approximately 30% to 40% of patients before they undergo surgery for reflux.[15,17] Although our data suggest a high rate of gas bloat symptoms following surgery, this symptom has also been common before surgery in our patients, with the preoperative incidence being approximately 50% in previous reports.[15,17] Furthermore, this symptom was not troublesome as the majority of patients were able to relieve it by belching normally.

We believe that the procedure of anterior fundoplication has a number of advantages over more commonly performed antireflux procedures. The procedure is a little easier to perform than the Nissen procedure, and it obviates the need for surgeons to agree about whether or not the short gastric vessels should be divided. The procedure is relatively quick to perform, and furthermore no esophageal bougie is required, reducing the potential for esophageal perforation during the laparoscopic antireflux procedure. The anterior fundoplication procedure has also been easier to revise at laparoscopic reoperation than the Nissen procedure,[29] should this be required later on. This is because no stomach lies behind the distal esophagus, simplifying the dissection required at reoperation.

Before anterior fundoplication is routinely applied, however, it is important to conclusively demonstrate that it is superior to the Nissen procedure. The early data from our randomized trial certainly supports its efficacy.[15] However, we would like to see others undertake trials of this procedure, and the long-term outcome (beyond 5 years) is obviously important. Despite this, the results of the present study support a wider application and investigation of this technique of laparoscopic anterior fundoplication.

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Impact of Complete Gastric Fundus Mobilization on Outcome After Laparoscopic Total Fundoplication

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With the objective of further optimizing the outcome of antireflux surgery, we have studied the importance of dividing the short gastric vessels when performing a laparoscopic total fundoplication. Ninety-nine consecutive patients with chronic gastroesophageal reflux disease (GERD) were enrolled in the trial. Forty-seven patients (25 men, age 52 ± 1.6 years [mean ± standard error]) were randomized to undergo a laparoscopic Nissen-Rossetti total fundic wrap with intact short gastric vessels, whereas 52 patients (29 men, 48 ± 1.4 years) had complete division of these vessels. Quality of life was assessed by means of the psychological general well-being and gastrointestinal symptom rating scale indices. The 6- and 12-month follow-up data are reported. Two patients were converted to open surgery. Mobilization of the fundus significantly prolonged the operative time (120 vs. 104 minutes, $P = 0.05$); otherwise the complication rates were similar in the two groups. Both procedures were equally effective in controlling gastroesophageal reflux at 6 and 12 months' postoperatively. Division of the short gastric vessels had no significant impact on the point prevalence of postfundoplication complaints at the given follow-up time points. Quality of life was significantly improved by both operative procedures and remained "normal" throughout the follow-up period. Dividing all short gastric vessels had no impact on the functional outcome during the first year of recovery after a total laparoscopic fundoplication. (J GASTROINTEST SURG 2000;4:493-500.)

KEY WORDS: Fundoplication, laparoscopy, Nissen-Rossetti fundoplication, gas bloat, dysphagia, gastroesophageal reflux disease, 24-hour pH monitoring, quality of life, endoscopy

Fundoplication procedures have repeatedly[1,2] been shown to effectively and durably control gastroesophageal reflux disease (GERD). The efficacy of the operation has been documented by symptom assessments, endoscopy, and 24-hour pH monitoring. Although a number of randomized clinical trials have not been able to demonstrate obvious clinical advantages in favor of a particular antireflux operation, a recently published series suggested somewhat better functional results after a posterior partial fundoplication as compared with a total fundic wrap.[3] The 360-degree fundoplication, as originally described by Nissen,[4] seems, however, to be the most frequently performed antireflux procedure on a global basis. This procedure has gained even greater popularity with the introduction of the laparoscopic technique.[5] However, some adverse consequences of total fundoplication seem to be unavoidable. Dysphagia and gas bloat–like complaints such as inability to belch, postprandial distention and pressure, and rectal flatus have been mentioned quite frequently and are considered by some to significantly hamper the postoperative outcome.[6-9] A number of different modifications of the original fundoplication procedure have been described not only to simplify the operative technique but also to improve the functional results. One technical issue that has been debated over the years is whether all short gastric vessels should be divided to ensure the construction of a fundic wrap floppy enough to avoid gas bloat and obstructive complaints.[10-13] We have conducted a randomized clinical trial to assess the clinical importance of dividing all short gastric vessels when performing a laparoscopic, total fundoplication. To further substantiate and expand the significance of the observations, we also assessed the quality of life by use of well-validated instruments. An interim analysis

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Table I. Clinical characteristics of patients enrolled in the trial

	Short gastric vessels intact (n = 47)	Divided short gastric vessels (n = 52)
Males/females	25/22	29/23
Age (yr)	52 ± 1.6 (range 26-73)	48 ± 1.4 (range 31-68)
Body mass index (kg/m^2)	27 ± 0.5	27 ± 0.5
GERD		
<1 yr	0	0
1-4 yr	13	15
≥5 yr	33	35
Missing data	1	2
Endoscopic grade[15]		
0	16	16
1	8	11
2	16	15
3	4	7
4	1	2
Missing data	2	1
24-hour monitoring (% time pH <4)		
Preoperative value	11.4 ±1.5	10.3 ± 1.2

Values are mean ± standard error.

of the short-term outcome of the trial has recently been reported.[14]

PATIENTS AND METHODS

Consecutive patients with chronic GERD, who were referred to our institution for antireflux surgery, were enrolled in the trial. The study protocol was approved by the local ethics committee, and informed consent was obtained from all subjects before they entered the study. These patients had not previously had any major upper abdominal open surgical procedures. All patients underwent a complete preoperative evaluation including esophageal manometry, 24-hour pH monitoring, endoscopy, and symptom assessment. At the time of endoscopy, some patients were being treated with antisecretory drugs, which jeopardized a valid assessment of the original severity of the esophagitis.[15] Demographic data and GERD history in the two study groups are presented in Table I. At the time of manometry we observed no differences in esophageal motor characteristics between the two study groups. Patients were selected for a total wrap on the basis of the motor function of the body of the esophagus.[16]

Ambulatory 24-hour pH monitoring was carried out using an antimony pH electrode positioned 5 cm proximal to the lower esophageal sphincter, the level of which was determined by means of station pull-through manometry.

At the time of surgery, patients were randomized to undergo fundoplication either with or without division of the short gastric vessels before any steps were taken to assess the feasibility of one procedure over the other. Randomization was carried out by means of a computerized program taking into consideration the severity and duration of the disease and the sex, age, and body mass index of each patient.

Pre- and postoperative symptoms were assessed according to a predefined protocol, where symptoms were assigned a score ranging from 0 to 3 (0 = no symptoms; 3 = severe incapacitating symptoms). Dysphagia was scored using a four-point grading scale with the following characteristics: grade 0 = no dysphagia; grade 1 = some obstructive complaints, but none that restricted the patient's normal daily activities or required special attention; grade 2 = mild-to-moderate dysphagia requiring some modification of diet in the form of a partial adjustment to semisolid foods; grade 3 = mandatory semisolid diet but allowing unrestricted fluid intake; and grade 4 = complete dysphagia.

The gas bloat score was used as a composite assessment of postfundoplication complaints, taking into account the patient's overall record relating to the ability to vent air from the stomach, the sensation of postprandial distention, and complaints of rectal flatulence. Complaints of gas bloat were assigned scores ranging from 0 (no symptoms) to 3 (severe and incapacitating).

Surgical Technique

Laparoscopic total fundoplication was performed using a standard operative technique. All operations were performed by two experienced surgeons (J.D. and H.L.). For patients undergoing dissection, the procedure began with dissection of the hiatal pillars

followed by full esophageal mobilization and a posterior crural repair with the use of nonabsorbable sutures. In patients randomized to undergo division of the short gastric vessels, this was the next step in the procedure. The vessels were dissected and in half of the patients these vessels were secured by metal clips and in the remaining patients the dissection was carried out using an ultrasound scalpel (Ultrascission, Ethicon Endo-Surgery, Cincinnati, Ohio). The division always began at the level of the inferior pole of the spleen and progressed in an oral direction along the greater curvature of the stomach until complete mobilization of the fundus had been achieved including division of all short gastric vessels and all tissues between the posterior portion of the stomach and the left crus. This procedure was completed in all patients irrespective of whether or not the surgeon considered it possible to encircle the esophagus with the loose fundic portion of the stomach at any particular step of the dissection.

In those patients who did not undergo division of the short gastric vessels, the anterior wall of the gastric fundus was pulled behind the esophagus for construction of the fundoplication. Occasionally, when the short gastric vessels had not been divided, the originally selected portion of the fundus used to encircle the esophagus was subsequently found to create too much tension on the wrap. By repositioning the instruments and then grasping an adjacent portion of the fundus, a looser wrap could then be constructed. Care was taken to ensure that the completed fundoplication was free of tension and to further guard against this, a 52 F bougie was inserted into the esophagus during construction of the wrap. Two to three polyester (TiCron, Davis & Geck, New York, N.Y.) interrupted sutures were used to secure the wrap, which made it approximately 1.5 cm long.

In the event that a laparoscopic procedure had to be converted to an open operation, because of intraoperative complications, the original randomization was followed.

Postoperative Care

Nasogastric tubes were not routinely used. Patients were allowed liquids by mouth on the first postoperative morning and solid food the following day. Discharge from the hospital was encouraged after the second postoperative day and in the later part of the study even on the first postoperative day.

Quality-of-Life Assessment

Quality of life was assessed preoperatively and at 1, 6, and 12 months postoperatively by means of several instruments. One generic instrument, the psychological general well-being (PGWB) index, includes 22 items divided into six dimensions—anxiety, depressed mood, positive well-being, self-control, general health, and vitality.[17] The dimensions contain three to five items each of which is graded using a six-point Lickert scale.[18] Higher scores indicate greater well-being. A gastrointestinal sy.mptom rating scale (GSRS), which is a disease-specific, well-recognized instrument, was also applied; it includes 15 items divided into five dimensions—diarrhea syndrome, indigestive syndrome, intestinal obstruction, abdominal pain syndrome, and reflux syndrome. The GSRS uses a seven-point Lickert scale, and higher scores indicated more pronounced symptoms.[19]

We began enrolling patients in the trial before the technical, logistic, and other practical issues relating to the acquisition of data on quality of life had been fully resolved. Therefore only the last 71 patients had a complete set of data from their preoperative to their postoperative condition. Thirty-seven patients (20 males and 17 females) in the mobilized group and 34 (17 males and 17 females) in the nonmobilized group, completed the quality-of-life assessments, and these groups were also comparable with regard to all other relevant preoperative characteristics.

Follow-Up

Follow-up of each patient was carried out by an independent evaluator (A.B.) at 3, 6, and 12 months after the operation, and the evaluator was unaware of the patients' group association. Postoperative outcome during the first year was assessed by means of symptom scoring and 24-hour pH monitoring; the latter was done after 6 months.

A particular approach was termed a clinical failure if the original procedure had to be abandoned during the index operation for any of the following reasons: if technical difficulties were experienced during the operation, if reoperation was required during the follow-up period, or if unacceptable and disabling functional postoperative symptoms were documented that required special attention or treatment.

Statistical Analysis

All data are given as mean ± standard error of the mean (SEM). Differences between study groups were assessed by means of Student's t test and analysis of variance (ANOVA).

RESULTS

Forty-seven patients (25 males, mean age 52 ± 1.6 years) were randomized to the group that had a total fundic wrap with intact short gastric vessels and 52

Table II. Perioperative and postoperative course

	Short gastric vessels intact (n = 47)	Divided short gastric vessels (n = 52)
Operative time (min)	104 ± 4.7	120 ± 4.9
Postoperative stay (days)	1.8 ± 0.2	2.8 ± 0.5
Early postoperative complications		
Surgical reintervention (bleeding, leakage, dysphagia)	0	3
Pneumothorax	0	1
Hemothorax	1	0
Postoperative transfusion	0	2
Postoperative pneumonia	0	4
Severe dysphagia	1	2
Esophageal food impaction	1	0
Late postoperative complications		
Reflux relapse	1	1
Obstructive ileus	0	2
Study failure		
Original procedure abandoned	1	0
Conversion to open procedure	0	2

Values are mean ± standard error.

Table III. Control of GERD assessed at 6 and 12 months postoperatively

	Short gastric vessels intact		Divided short gastric vessels	
	6 mo	12 mo	6 mo	12 mo
No. of patients investigated	44	42	48	42
Heartburn	1	2	0	2
Regurgitation	1	2	0	0
Dysphagia				
None	30	26	38	28
Grade 1	10	13	9	7
Grade 2	3	2	1	3
Grade 3	1	0	0	0
Missing data	3	0	4	1
Gas bloat				
Grade 0	12	15	22	17
Grade 1	19	13	13	18
Grade 2	8	13	9	6
Grade 3	1	0	2	1
% Time pH <4 (mean ± SE)	1.2 ± 0.3		1.9 ± 0.7	

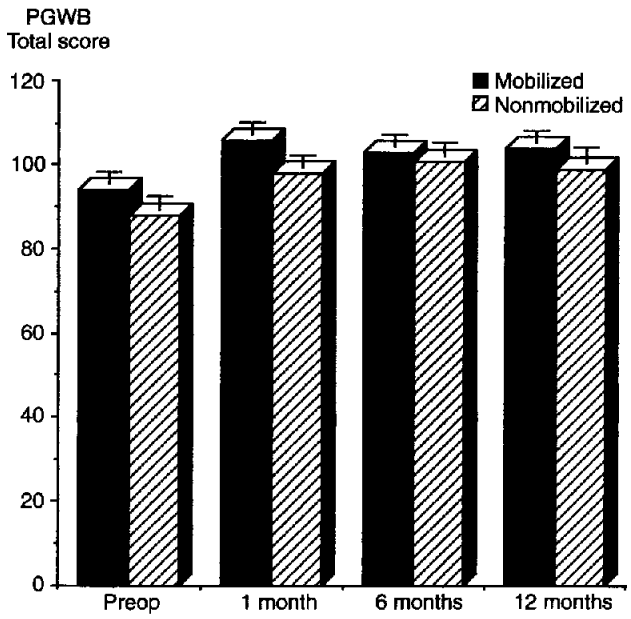

Fig. 1. Quality of life as assessed by the generic psychological general well-being (PGWB) index. Mean and standard error are given. Data are reported preoperatively and at 1, 6, and 12 months after antireflux surgery.

patients (29 males, mean age 48 ± 1.51 years) were randomized to wrap with complete division of all short gastric vessels. Ninety-seven of the operations were exclusively performed laparoscopically. Two patients had to be converted to laparotomy—one because of massive adhesions after a previous cholecystectomy and one because of subcutaneous emphysema. In both cases, the allocated procedure was completed and the study protocol was subsequently followed.

The operating time was 120 ± 5 minutes in the group undergoing mobilization compared to 104 ± 5 minutes for patients whose short gastric vessels were left intact ($P = 0.05$). One patient who, at the time of laparoscopy, had been randomized to the intact short gastric vessel group, had to undergo a full mobilization of the fundus in order to achieve a tension-free wrap. This patient was therefore, according to the protocol, classified as a failure for that particular operative approach. One patient in the group undergoing mobilization had severe postoperative bleeding and another patient had leakage from the mobilized fundus; both of them required surgical reintervention. Otherwise the postoperative recoveries proceeded uneventfully. In total, the number of perioperative and postoperative complications did not significantly differ between the two groups (Table II).

During the first postoperative year, the level of GERD control did not differ between the two study groups when they were assessed by means of 24-hour pH monitoring and symptom scoring (Table III). Functional outcomes for the two study groups are presented in detail in Table III and showed that almost 10% of the patients in each group had slight dysphagia, which persisted for the entire 12 months of the study. None of the patients had severe dysphagia at the 12-month follow-up. Approximately 70% of the patients had some difficulty venting air from the stomach, whereas one patient complained of incapacitating gas bloat at 6 months and one patient in the mobilized group remained disabled after another 6 months of follow-up.

Quality of life, as assessed by the generic PGWB, showed a significant improvement after the operation ($P < 0.0001$), an effect that was maintained throughout the entire study period (Fig. 1). In this context it is worth mentioning that quality of life was assessed in the preoperative setting when patients were not receiving any medical treatment. We were unable to demonstrate any significant difference between the two operative procedures.

The disease-specific GSRS scores were also significantly improved following the antireflux operation ($P = 0.005$). This was evident both when the total score and the reflux dimension were recorded (Fig. 2, *A* and *B*). "Normalization" of the GSRS scores was observed as early as 1 month after the actual laparoscopic operation. When the indigestion dimension was specifically analyzed within the GSRS instrument, a somewhat different picture emerged with scores remaining unchanged in patients with intact short gastric vessels, whereas a steady improvement was observed in those whose short vessels were divided (Fig. 2, *C*). This difference, however, never reached statistical significance.

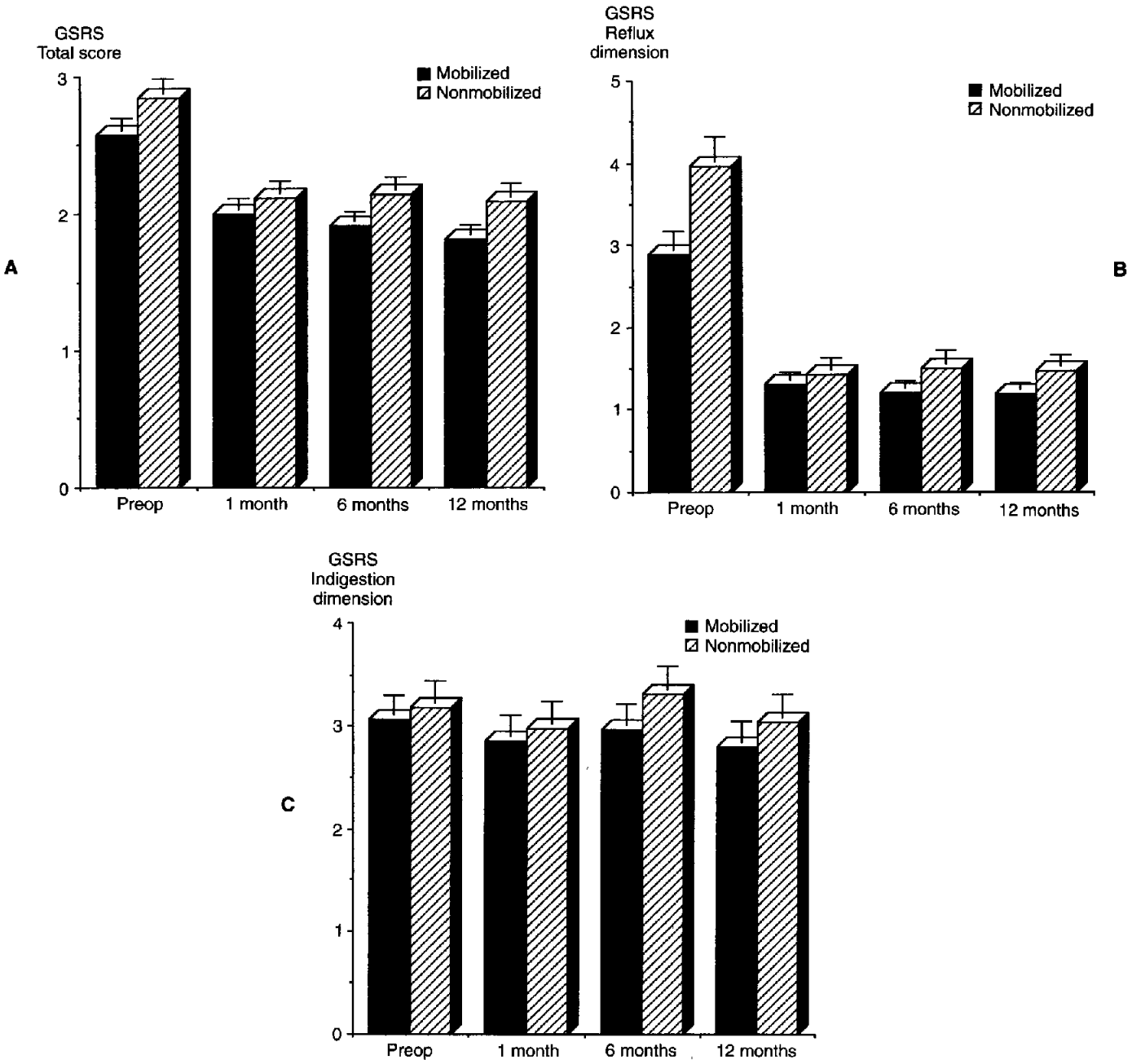

Fig. 2. Quality of life as assessed by the disease-specific gastrointestinal symptom rating scale (GSRS). **A,** Data on the total score; **B,** Reflux dimension; **C,** Indigestion dimension. Mean ± standard error are given before surgery and at 1, 6, and 12 months after antireflux surgery.

DISCUSSION

The importance of dividing all short gastric vessels when constructing a laparoscopic total fundoplication was addressed by the present randomized clinical study. Our experience supports that of other investigators who mostly found that it is technically feasible to encircle the esophagus with the wrap just by using only the anterior portion of the fundus without dividing any short gastric vessels.[20,21] Although it has frequently been recommended, no controlled data have established the importance of having a large bougie inserted into the esophagus to ensure the construction of a loose fundic wrap. We used a similar device in the present study to standardize the operative technique as much as possible and also to counteract potential criticism in the event of an unfavorable out-

come in the group with intact short gastric vessels. In fact, recent clinical observations have indicated an obvious risk of esophageal perforation with the use of similar bougies.[22]

In accordance with reports from other institutions, our results showed no difference in the efficacy with which these two operative approaches control reflux. Previous information from the literature would indicate that the length of the wrap as well as the floppiness of the fundoplication are important with regard to functional outcome.[6,10,11] In fact, Hunter et al.[23] reported an astonishingly high rate of dysphagia in patients who have a laparoscopic total wrap without division of the short gastric vessels compared to patients who have either a posterior partial fundoplication or a total fundoplication with sectioning of all short gastric vessels. On the other hand, during the era of open fundoplication, other investigators have demonstrated a high level of reflux control in patients undergoing a Nissen-Rossetti fundoplication without a disturbingly high frequency of complaints of obstruction or gas bloat.[20,21] It could, of course, be inferred that the lack of tactile feedback during the laparoscopic operation would introduce a limitation as to the ability of the operating surgeon to assess the floppiness of the fundoplication when a Nissen-Rossetti total fundic wrap is constructed.[24]

Not unexpectedly, we recorded a longer operative time for patients whose short gastric vessels were all divided. The dissection time could, of course, be shortened by using modern techniques such as the ultrasound scalpel or scissors. Two of our patients who were allocated to undergo complete mobilization of the fundus experienced significant complications (bleeding, hemorrhage, and leakage), which probably could have been prevented by using a similar ultrasound scalpel. Of greater clinical importance, however, was the fact that there was no difference in postoperative functional outcome between the two study groups. Some obstructive complaints were recorded in the early postoperative period, which subsided with the passage of time. In only one patient was it not possible to carry out the originally allocated procedure without compromising the need to always construct a tension-free wrap.

Recently two randomized clinical studies were published, one of which used the laparoscopic technique, that demonstrated essentially similar results to those in the present report.[25,26] There were some differences in the design and execution of the trials. First, only two surgeons were performing the actual procedures compared to seven in the Australian study.[26] Second, the mobilization of the fundus in our patients was probably more complete, and also included the posterior portion of the stomach. This is illustrated by the fact that considerably more than three gastric vessels were, in our experience, always divided in those patients. Accordingly we never performed a complete mobilization of the fundus by dividing less than five or six short gastric vessels. Despite these minor differences in the design and execution of our study, the outcomes were almost identical. Although the level of reflux control was very high in this study and others in which similar laparoscopic surgical procedures were used, our results and those of others[27] show that postfundoplication complaints in the form of obstructive symptoms, inability to belch, and complaints of gas bloat remain a significant problem. Technical aspects regarding the design of the wrap might be of significance to further optimize the surgical outcome.[28]

Despite these postfundoplication complaints, our patients reported a significant improvement in their quality of life as early as 1 month after the laparoscopic operation, again with no difference between the operative approaches. This "normal" quality of life was maintained throughout the study period. Although it can be argued that it is important to add quality-of-life assessments to the conventional efficacy variables in GERD, based on the patient's own assessment of the situation, we can conclude that there seems to be an obvious parallel between the outcome of conventional efficacy variables and quality-of-life assessments during the first year after laparoscopic total fundoplication.

We may therefore conclude that collective data from randomized controlled clinical trials suggest that mobilization of the gastric fundus is not followed by a better functional outcome after a total fundoplication.

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Identification and Comparative Analysis of Human Colonocyte Short-Chain Fatty Acid Response Genes

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Short-chain fatty acids (SCFAs) butyrate, propionate, and acetate produced during fiber fermentation promote colonic differentiation and can reverse or suppress neoplastic progression. We sought to identify candidate genes responsible for SCFA activity on colonocytes and to compare the relative activities of independent SCFAs. cDNA was generated from polyA$^+$ mRNA isolated from control Caco-2 cells and cells treated with equimolar butyrate, propionate, and acetate. GeneCalling, a restriction-based differential mRNA expression platform linked to a DNA sequence database lookup, was applied. A total of 30,000 individual genetic sequences were analzyed for differential expression among the three SCFAs. Differentially expressed peaks corresponding to cancer-related genes were isolated, sequenced, and cross-referenced to the GenBank human database. Gene identities were independently confirmed by oligonucleotide poisoning. More than 1000 gene fragments were identified as being substantially modulated in expression by butyrate. Butyrate tended to have the most pronounced effects and acetate the least. Five fragments selected for further study were fully sequenced and proved 100% homologous with human sequences for clusterin, amyloid precursor–like protein 2, and caudal homeobox 2 protein, not previously known to be modulated by SCFAs. In each case, a similar order of potency for the three SCFAs studied was observed. The common SCFAs appear to exert different effects. This study suggests the diversity of the SCFA response at the molecular level and facilitates identifying genes important in the biologic activity of dietary fiber. (J GASTROINTEST SURG 2000;4:501-512.)

KEY WORDS: Acetate, butyrate, Caco-2, colon cancer, gene expression, propionate, short-chain fatty acids

Epidemiologic and experimental data have suggested that dietary fiber may inhibit colorectal carcinogenesis.[1] Although a recent report suggests that dietary fiber may not protect against colon cancer in women, this study did not distinguish among different types of fiber.[2] Others have previously reported that fiber does protect against colon cancer in women.[3] Such discrepant results may reflect differences among fibers, since fiber effects vary with the nature of the fiber studied.[4] Competing (or complementary) explanations for this effect include direct antineoplastic activity by fiber components, dilutional reduction of luminal carcinogen concentrations, fiber-associated alterations in colonic transit that decrease colonic enterocyte exposure to luminal carcinogens, and production of short-chain fatty acids (SCFAs) such as butyrate by bacterial fermentation of dietary fiber.[5] SCFAs act directly on colonocytes[6] and inhibit microbial enzymes, which produce carcinogenic metabolites by decreasing luminal pH.[7]

SCFAs such as butyrate are the most prevalent anions in the colonic lumen.[8] Butyrate is the dominant energy source for colonocytes, accounting for 70% of their oxygen consumption.[9] We[10,11] and others[12-15] have observed that butyrate and other SCFAs slow proliferation, inhibit motility, and promote differentiation in colonocyte cell lines. These SCFA effects may contribute to the apparent protective activity of

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fiber against colon cancer. Indeed, butyric acid derivatives might be potentially useful in the treatment of solid tumors such as colon cancers.[16]

SCFAs such as butyrate modulate gene expression by altering histone acetylation[16] and DNA methylation.[17] Butyrate has been reported to be more potent than other common SCFAs such as acetate and propionate in its effects on histone acetylation,[18] suggesting a specificity of effect. Our previous studies in the well-differentiated human Caco-2 cell line have also suggested differential potency of common SCFAs. Butyrate and propionate appeared generally more potent than the more abundant acetate in modulating the Caco-2 phenotype.[10,11] We have observed similar effects in human mucosa.[19] Such differences in SCFA potency may also have clinical significance, since different fibers are fermented to yield different proportions of these SCFAs.[5]

This study is a preliminary attempt to further explore differences in the effects of common SCFAs at the genomic level while simultaneously identifying the Caco-2 genes that are most substantially altered by SCFAs. Most reports of SCFA-modulated genes have targeted specific genes related to cellular processes previously associated with neoplasia or other biologic phenomena. Instead, we used high-throughput differential gene expression (DGE) to sample all facets of SCFA-induced colonocyte response without bias. DGE includes all methods that simultaneously compare the relative abundance of multiple mRNA transcripts in two experimental samples. DGE technologies can be divided into two fundamental classes: closed architecture and open architecture. DNA chip microarrays[20,21] typify closed-architecture systems. They survey transcriptomes by measuring hybridization to a prefabricated microarray. Because gene chips only sample those genes for which sequence information is already known, novel genes are not identified. Also, because hybridization to a chip probe can occur at less than 100% sequence identity, gene chips may not distinguish among highly homologous members of a gene family. Open-architecture DGE, including differential display,[22] serial analysis of gene expression (SAGE),[23] and GeneCalling, the method described here,[24] do not require prior construction of a species-specific sequence database. This allows a more comprehensive survey of the transcriptome that includes characterization of novel gene sequences. GeneCalling is a high-throughput transcript-profiling technique that provides comprehensive sampling (both known and novel sequences) of cDNA populations with the sensitivity to detect 1.5-fold differences in mRNA abundance between two sample groups. An oligonucleotide-poisoning polymerase chain reaction (PCR) confirmation step eliminates the high false positive gene identification rate and the requirement for extensive candidate gene sequencing inherent in other open-architecture platforms.

RNA was isolated from unsupplemented Caco-2 cells or cells treated with 10 mmol/L butyrate, propionate, or acetate for 24 hours, converted to cDNA, and subjected to GeneCalling. Each treatment course was performed in triplicate, generating three independent cDNA samples per regimen. GeneCalling involves restriction digestion of each cDNA by selected pairs of restriction endonucleases (REs), ligation of fluorescent-tagged linker adapters, PCR amplification, high-resolution gel electrophoresis, and detection of each restriction fragment. Ninety-six independent pairs of REs were used and 30,000 individual gene fragments were assayed. Each differentially expressed peak was compared to a virtual restriction digest of GenBank human database (www.ncbi.nlm.nih.gov). Gene identities for each fragment included all human genes predicting a DNA sequence fragment whose length and terminal nucleotide sequences matched the REs used and the detected length of the differentially expressed peaks. The specific gene associated with a peak was confirmed by oligonucleotide poisoning, a gene-specific competitive PCR reaction.

Expression of more than 1000 genetic sequences was altered by butyrate at a differential expression amplitude of ± 1.5-fold or greater. We present here the comparative behavior following propionate and acetate treatment for 20 fragments most significantly altered by butyrate treatment. We also present the subsequent sequence identification fragments and independent confirmation by oligonucleotide poisoning of five gene fragments that were particularly intriguing based on the magnitude and directionality of their SCFA effects. For one gene, clusterin, we also performed confirmatory studies using reverse transcription–polymerase chain reaction (RT-PCR) with primers directed at clusterin to amplify only that gene and thus further illustrate the different effects of butyrate and propionate on gene expression. Northern blot analyses on clusterin and amyloid-like precursor protein 2 were also performed.

METHODS

Cell Culture

Caco-2 cells were maintained at 37° C in 5% carbon dioxide in Dulbecco's minimal essential medium supplemented with 10% fetal calf serum, 10 μg/ml transferrin (Boehringer Mannheim, Indianapolis, Ind.), 2 mmol/L glutamine, 1 mmol/L pyruvate, 10 mmol/L HEPES, 100 units/ml penicillin G, and 0.1 mg/ml streptomycin. Cells (1×10^8) were treated with 10

mmol/L sodium butyrate, 10 mmol/L sodium propionate, 10 mmol/L sodium acetate (Sigma, St. Louis, Mo.), or no additive for 24 hours at 37° C. Independent triplicate flasks were cultured and harvested for each treatment group.

GeneCalling Chemistry

Total cellular RNA was isolated with Trizol (GiBCO-Biological Research Laboratories, Baltimore, Md.), using one-tenth volume of bromochloropropane (Molecular Research Corp., Cincinnati, Ohio). polyA$^+$ RNA was prepared from 100 μg total RNA using oligo(dT) paramagnetic beads (PerSeptive Biosynthesis, Boston, Mass.) and then converted to cDNA using standard protocols. For each cDNA sample, triplicate GeneCalling chemistry reactions were executed in parallel for each of 96 six-cutter/four base-pair overhang restriction enzyme pairs as described.[24] The reactions were maintained at 16° C for ligation of PCR primers. Each unique four base-pair overhang requires a specific linker sequence in tandem with a common PCR amplification sequence. Twenty cycles of amplification (30 seconds at 96° C, 1 second at 57° C, 2 minutes at 72° C) were followed with 10 minutes at 72° C. PCR product purification was performed using MPG streptavidin beads (CPG, Lincoln Park, N.J.), separated with a magnet, dried, and resuspended in 3 μl of 80% formamide, 4 mmol/L EDTA, and 5% ROX-tagged molecular size standard (ABI, San Francisco, Calif.). In addition, every other lane received 5% TAMRA (ABI) as interlane bleed control. Following denaturation (96° C for 3 minutes), samples were loaded onto 5% polyacrylamide, 6 mmol/L urea, 1 × TBE ultrathin gels (Long-Ranger, FMC, Philadelphia, Pa.), and electrophoresed for 60 minutes at 3500 V on a Niagara instrument (CuraGen, New Haven, Conn.).

GeneCalling Data Analysis

Chromatograms were processed using the internet-based Open Genome Initiative (OGI) software. Gel images were visually inspected for quality, and each lane was tracked to delineate the path of best fit. Each lane contains a GeneCalling sample plus two sizing DNA ladders (labeled with ROX and TAMRA fluorochrome) spanning the range from 50 to 500 base pairs. Linear interpolation between the ladder peaks resolved the GeneCalling sample trace peaks into base pairs. Traces were submitted as point-by-point length against amplitude addresses to the GeneScape Oracle 8 database and organized by treatment group and restriction endonuclease pair. The nine traces corresponding to each treatment group/restriction endonuclease pair were superimposed and visually evaluated for alignment fidelity. Misaligned traces were excluded from differential gene expression analysis. Binary comparisons between traces from each SCFA-treated sample set and the untreated Caco-2 control were performed. To identify differentially expressed peaks, corresponding traces from each sample set were compared on a point-by-point basis to define areas of amplitude difference. Once a region of difference was identified, the local maximum for the corresponding traces of each set was identified. The variance of the difference was determined by the following:

$$\sigma^2_{\Delta}(j) = \lambda_1(j)^2\sigma^2_{Total}(j{:}S_1) + \lambda_2(j)^2\sigma^2_{Total}(j{:}S_2)$$

where $\lambda_1(j)$ and $\lambda_2(j)$ represent scaling factors and (j:S) represents the trace composite values over multiple samples. The probability that the difference is statistically significant was calculated by the following:

$$P(j) = 1 - \int_{-\Delta}^{\Delta} dy \frac{1}{\sqrt{2\pi\sigma^2_{\Delta}}} \exp\left(\frac{-y^2}{2\sigma^2_{\Delta}}\right)$$

where y is the relative intensity. Results are presented here as mean ± standard error.

GeneCalling Gene Confirmation

Oligonucleotide Poisoning. Restriction fragments that map in end sequence and length to known human genes and human expressed sequence tag (EST) assemblies were used as templates for the design of unlabeled oligonucleotide primers. An unlabeled oligonucleotide designed against one end of the restriction fragment was added in excess to the original reaction, and reamplified for an additional 15 cycles. This reaction was then electrophoresed and compared to a control reaction reamplified without the unlabeled oligonucleotide to evaluate the selective diminution of the peak of interest.

Gene Isolation. One microliter of the GeneCalling chemistry reaction containing the peak of interest was electrophoresed on an Elchrom Mini Gel (Elchrom Scientific, Lake Park, Fla.) at 55° C, 120 V to resolve the base pair corresponding to the peak of interest. The desired band length was excised from the gel lane, eluted into 10 mmol/L $MgCl_2$, and PCR amplified for 25 cycles of 30 seconds at 96° C, 60 seconds at 57° C, and 2 minutes at 72° C. The product was ligated to pCR2.1 cloning vector (Invitrogen, Carlsbad, Calif.) and electroplated into DH10B *E. coli*. Inserts were then PCR amplified and sequenced using standard protocols for fluorescent dye-terminator sequencing on ABI377 sequencing units. Outputted sequences were queued for oligonucleotide poisoning, and only those sequences that successfully

Table I. Effects of short chain fatty acid treatment on band intensity for 20 gene fragments

Band ID	Control	Acetate	Propionate	Butyrate
d0v0_324.6	184.7 ± 25.3	157.2 ± 11.4	178.5 ± 35.9	379.0 ± 26.3*
i0u0_152.2	101.8 ± 7.7	173.1 ± 11.0*	327.0 ± 13.0*	294.8 ± 10.7*
d0p0_93.2	43.3 ± 3.78	73.1 ± 4.3*	86.7 ± 3.5*	92.78 ± 3.2*
i0m0_183.5	102.6 ± 5.12	146.6 ± 5.2*	225.2 ± 8.4*	280.5 ± 14.8*
d0p0_124.2	227.7 ± 13.7	296.6 ± 15.7*	440.2 ± 11.8	423.1 ± 13.9*
i0u0_156	295.7 ± 45.4	315.6 ± 6.9	433.3 ± 9.0*	355.5 ± 10.8
i0r0_109.3	75.4 ± 9.9	79.0 ± 4.0	209.1 ± 6.4*	309.3 ± 9.1*
l0n0_67.8	133.4 ± 3	116.4 ± 5.9	109.5 ± 6.9	41.5 ± 1.5*
f0n0_323.5	118.5 ± 17.6	6.7 ± 2.3*	1.6 ± 0.2*	3.2 ± 0.7*
i0q0_165	10.4 ± 0.81	5.8 ± 0.5*	290.2 ± 63.3*	360.6 ± 33.8*
f0k0_433.1	107.8 ± 17.2	413.3 ± 43.7*	9.7 ± 3.2*	5.9 ± 0.9*
r0h1_57.2	144.0 ± 19	4.8 ± 1.1*	5.7 ± 1.2*	6.6 ± 1.7*
w0i0_328.0	23.5 ± 1.71	33.5 ± 0.3	212.0 ± 9.2*	368.8 ± 80.2*
g0s0_388.9	9.6 ± 3.59	27.8 ± 0.6*	36.3 ± 7.5*	147.3 ± 13.6*
h0r0_275.0	20.2 ± 2.34	11.0 ± 3.4*	1.9 ± 0.1*	1.3 ± 0.3*
s0v0_412.3	62 ± 4.2	75.0 ± 3.13	4.2 ± 0.4*	3.6 ± 0.4*
m1l0_341.4	107.3 ± 4.62	124.5 ± 19.4	9.7 ± 1.2*	6.2 ± 0.8*
p0t0_333.5	6.7 ± 0.99	10.5 ± 1.34	41.2 ± 6.3*	104.8 ± 4.7*
i0q0_246.3	30.6 ± 1.1	20.6 ± 0.9*	6.4 ± 1.2*	4.9 ± 0.7*
y0i0_392.1	6.5 ± 0.8	9.8 ± 0.9	32.5 ± 3.6*	101.6 ± 4.5*

*$P = 0.005$; n = 3.

poisoned the peak of interest were assigned to the corresponding expression difference.

Confirmatory PCR for Clusterin

In an additional series of experiments, new Caco-2 cells were treated as above with either control culture medium or medium containing 10 mmol/L acetate, propionate, or butyrate. Total RNA was isolated from 5×10^7 cells using Trizol (Gibco) as described above. cDNA was synthesized by RT-PCR from 5 μg of total Caco-2 cell RNA (260/280 >1.8) in a 50 μl reaction over 27 cycles. For each template, 2 units of Elongase enzyme mix were used for a 50 μl PCR. High-stringency 30mer primers for clusterin were designed using Oligo 4.0 software and used to PCR the clusterin gene sequence from this cDNA.

Northern Blot Analysis

One microgram of polyA$^+$ RNA prepared from each of Caco-2 cells treated with 10 mmol/L butyrate, 10 mmol/L propionate, 10 mmol/L acetate, and untreated Caco-2 as described earlier was transferred to Hybond plus membranes (Amersham, Piscataway, N.J.) and hybridized using standard techniques. Probes for cloned fragments were reamplified from isolated *E. coli* colonies containing the appropriate insert as described above and subcloned into the pCR2.1 vector (Invitrogen). Probes for GeneCalled fragments were obtained following PCR amplification of the fragment from sample cDNA prepared as described earlier using primers designed from the predicted database sequence designed to overlap the restriction enzyme sites of the gene fragment confirmed by oligonucleotide poisoning and to allow subcloning into the pCR2.1 vector. Then 10^6 dpm/ml of RNA probe in 1 ml of Zip-Hyb was added to the Northern blot for a 2-hour incubation at 65° C. Following hybridization, the buffer was removed and the blots were washed and exposed to phosphor screens (Molecular Dynamics, Sunnyvale, Calif.) overnight. The screens were scanned on a Storm 840 (Molecular Dynamics) at 50 μm resolution.

RESULTS

Initial comparisons suggested that 1425 of the 30,000 gene sequences analyzed were altered by at least one SCFA. Of these 1425 gene sequences, SCFA treatment promoted expression of 900 and decreased expression of 515. The rest showed mixed patterns of induction or suppression varying with the SCFA. Twenty of the most substantially and statistically significantly affected fragments were selected for comparative SCFA analysis (Table I). Expression of

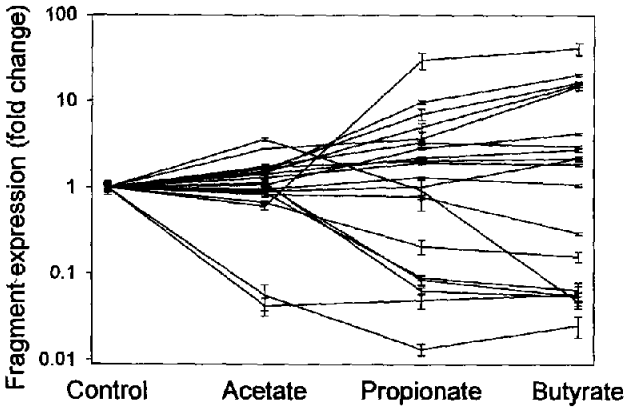


Fig. 1. Effect of SCFA treatment on gene fragment expression levels. mRNA isolated from Caco-2 cells treated with 10 mmol/L butyrate, propionate, or acetate was compared with cells maintained simultaneously in culture under control conditions. Triplicate data from each of three separate experiments were normalized to control values for expression of each gene fragment for each experiment so that gene fragment expression could be represented as a "fold change" over the control value. Each line represents data from a different gene fragment. Although SCFAs tended to stimulate expression of some gene fragments and inhibit expression of others, butyrate and propionate tended to exert more substantial effects than acetate for each of these gene fragments.

g0s0_388.9 and w0i0_328.0 was stimulated 14.8 ± 1.4 and 20.3 ± 0.6-fold, respectively (P <0.0001) by butyrate and to a lesser but still significant extent by propionate and acetate. Expression of the f0n0_323.5 and r0h1_57.2 fragments was essentially ablated by each of the SCFAs (P <0.0001). Substantial differences were observed for several genes among the effects equimolar concentrations of each SCFA. For instance, w0i0_328.0 induction was 1.5 ± 0.01-fold by acetate (P <0.01), 9.7 ± 0.4-fold by propionate (P <0.0001), and 20.3 ± 0.6-fold by butyrate (P <0.0001). When these data were expressed as the ratio of expression of each fragment after SCFA treatment to expression in untreated control cells, a more global pattern became apparent (Fig. 1). In general, butyrate appeared more potent than propionate, which appeared more potent than acetate. Although the magnitude of the effects differed substantially among the SCFAs and among the various gene fragments, the direction of the effects was generally similar. For 16 of the gene fragments selected for study, all three SCFAs either stimulated or inhibited expression of a given fragment. In contrast, SCFA effects on two fragments, i0q0_165 and f0k0_433.1, seemed inconsistent with this trend. Butyrate induced i0q0_165 expression 41.5 ± 6.6-fold and propionate 30.2 ± 6.6-fold (P <0.0001 for each), but acetate inhibited expression by 39.3% ± 5.0% (P <0.0001). Since i0q0_165 expression was very low in control and acetate-treated cells, this statistically significant inhibition might lack biological significance. However, f0k0_433.1 expression was ablated by butyrate (95.4% ± 0.7% inhibition, P <0.0001) but promoted by acetate (3.6 ± 0.2-fold, P <0.0001).

We selected five gene fragments, i0r0_109.3, i0u0-152.2, d0p0_124.2, i0m0_183.5, and l0n0_67.8, for further study on the basis of the magnitude and reproducibility of the SCFA effects.

Fig. 2 depicts the GeneCalling traces for fragment i0r0_109.3. Although both butyrate and propionate appeared to promote i0r0_109.3 expression, butyrate stimulation (4.2 ± 0.1-fold) appeared more substantial than propionate stimulation (2.8 ± 0.1-fold). This difference was statistically significant (P <0.0001). This fragment was identified as apolipoprotein J (clusterin) by oligonucleotide poisoning. This identification was confirmed by PCR for apolipoprotein J using separately targeted primers in a separate series of three experiments in which Caco-2 cells were treated with 10 mmol/L acetate, propionate, and butyrate prior to RNA isolation and RT-PCR using primers designed specifically for apolipoprotein J (clusterin) and a housekeeping gene (actin). Although bands of approximately equal intensity were observed for the housekeeping gene despite SCFA treatment (see Fig. 5, *A*, upper band labeled *control*), substantial differences were observed in the intensity of the apolipoprotein J band (see Fig. 5, *A*, lower band labeled *Apolipoprotein J*).

Expression of gene fragments i0u0_152.2, d0p0_124.2, and i0m0_183.5 was each also more substantially promoted by butyrate and propionate than acetate (see Table I). Oligonucleotide poisoning showed these fragments to be homologous with overlapping regions of the gene for human amyloid precursor–like protein 2 (Fig. 3). Northern blots confirmed that butyrate increased expression of apolipoprotein J and amyloid precursor–like protein 2 (see Fig. 5, *B*).

Finally, l0n0_67.8 expression was substantially inhibited by butyrate (P <0.0001), but only slightly inhibited by propionate and acetate (Table I, P <0.01 for acetate and P <0.005 for propionate). Oligonucleotide poisoning showed this fragment to be 100% homologous with a sequence in the human caudal homeobox protein 2 gene (Fig. 4).

Text continued on p. 510.

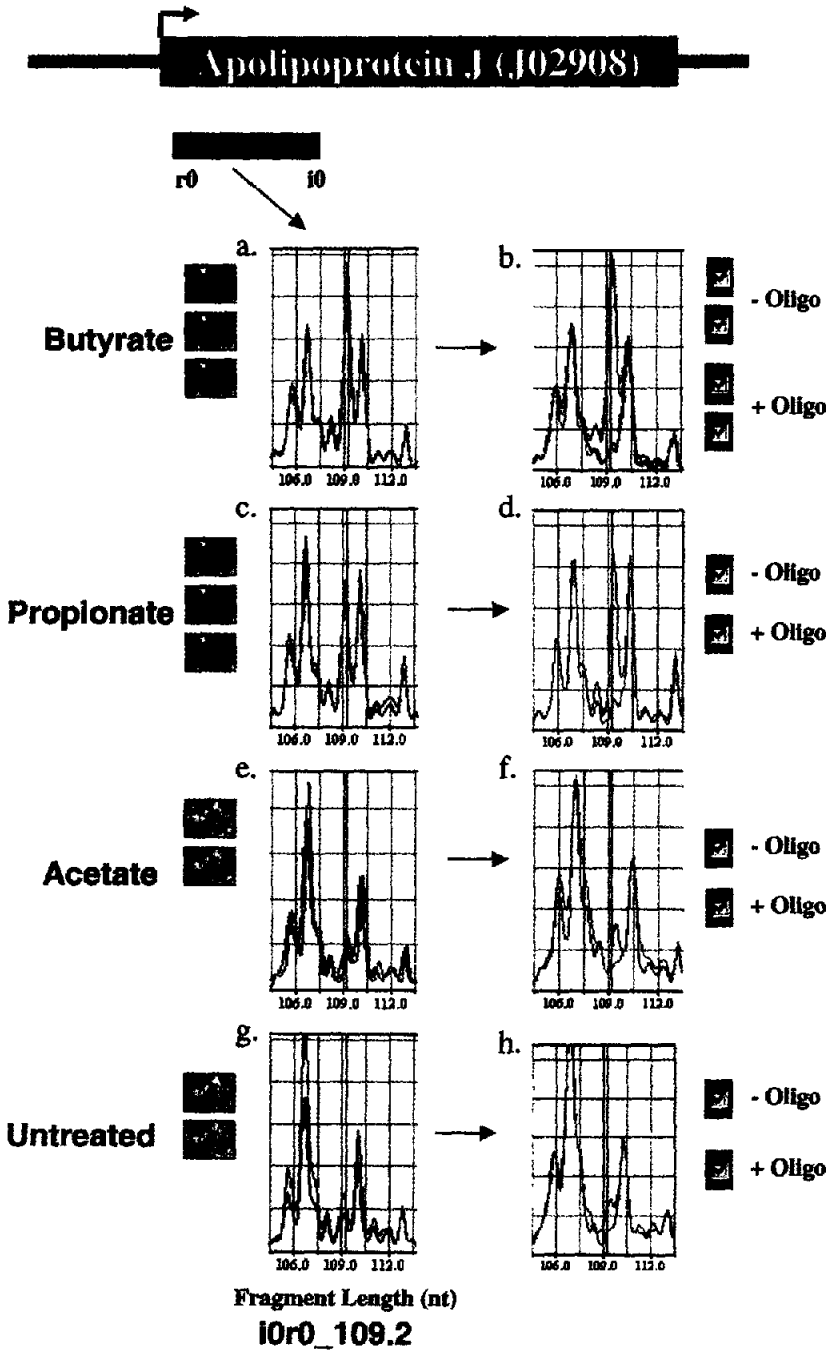


Fig. 2. GeneCalling profiles (a, c, e, g) and corresponding oligonucleotide poisoning chromatograms (b, d, f, h) for the i0r0_109.2 fragment corresponding to apolipoprotein J (ApoJ; clusterin, GenBank accession No. J02908) across the three SCFA treatment groups and the untreated control group. Each chromatogram represents the composite of the individual GeneCalling traces generated for that iteration of Caco-2 SCFA treatment. The total number of chromatograms for each peak reflects the number of SCFA-treated samples (up to three) where more than two traces passed quality control tests. The level of ApoJ induction increases with increasing SCFA chain length. The relative location of the i0r0_109.2 fragment within the ApoJ mRNA sequence is represented. The labels i0(Bg1II) and r0 (EcoRI) are arbitrary symbols for the restriction endonucleases used.

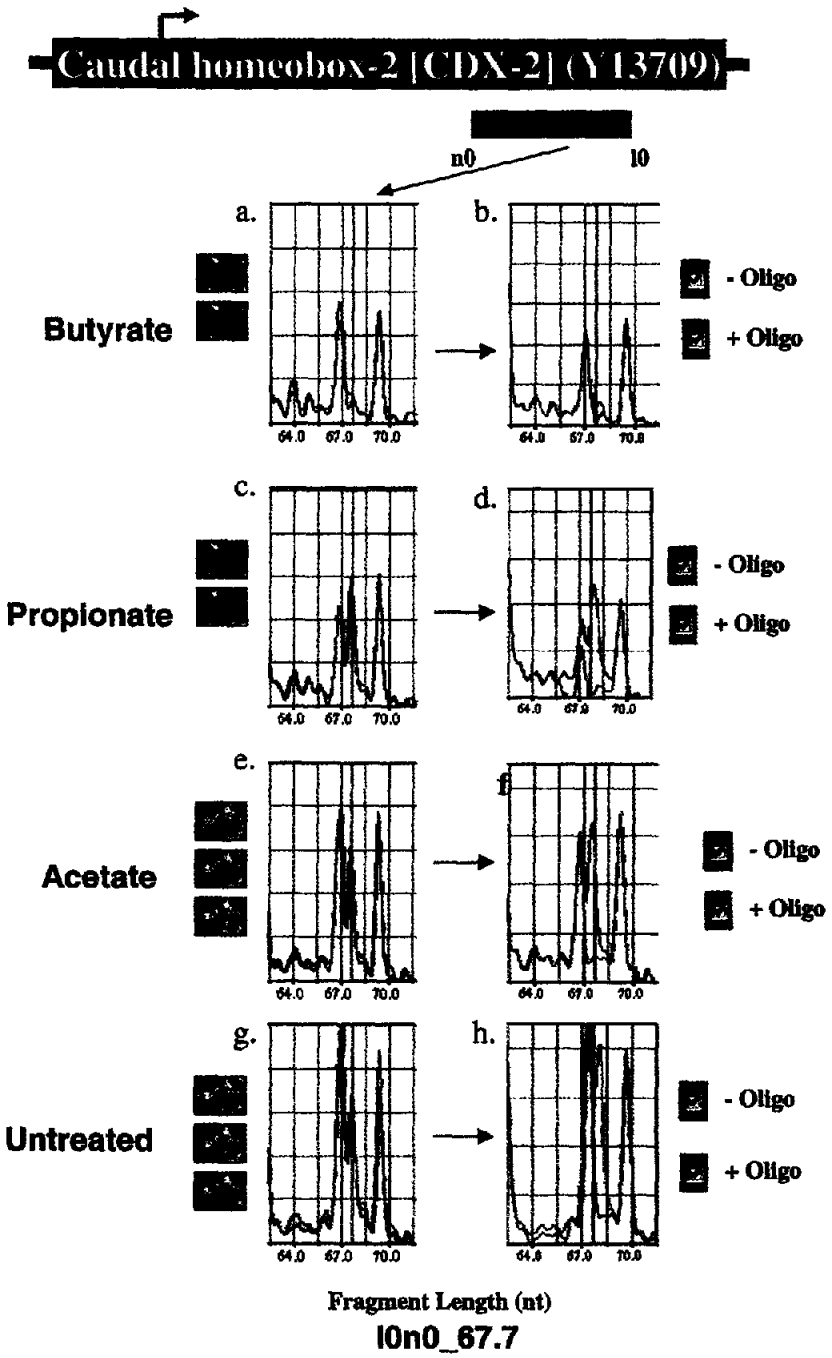


Fig. 3. GeneCalling profiles (a, c, e, g) and corresponding oligonucleotide poisoning chromatograms (b, d, f, h) for the l0n0_67.7 fragment corresponding to caudal-type homeobox 2 (Cdx-2, GenBank accession No. y13709) across the three SCFA treatment groups and the untreated control group. Cdx-2 is increasingly suppressed with increasing SCFA chain length. The relative location of the l0n0_67.7 fragment within the Cdx-2 mRNA sequence is represented. The labels l0 (BspE1) and n0 (Bsp14071) are arbitrary symbols for the restriction endonucleases used.

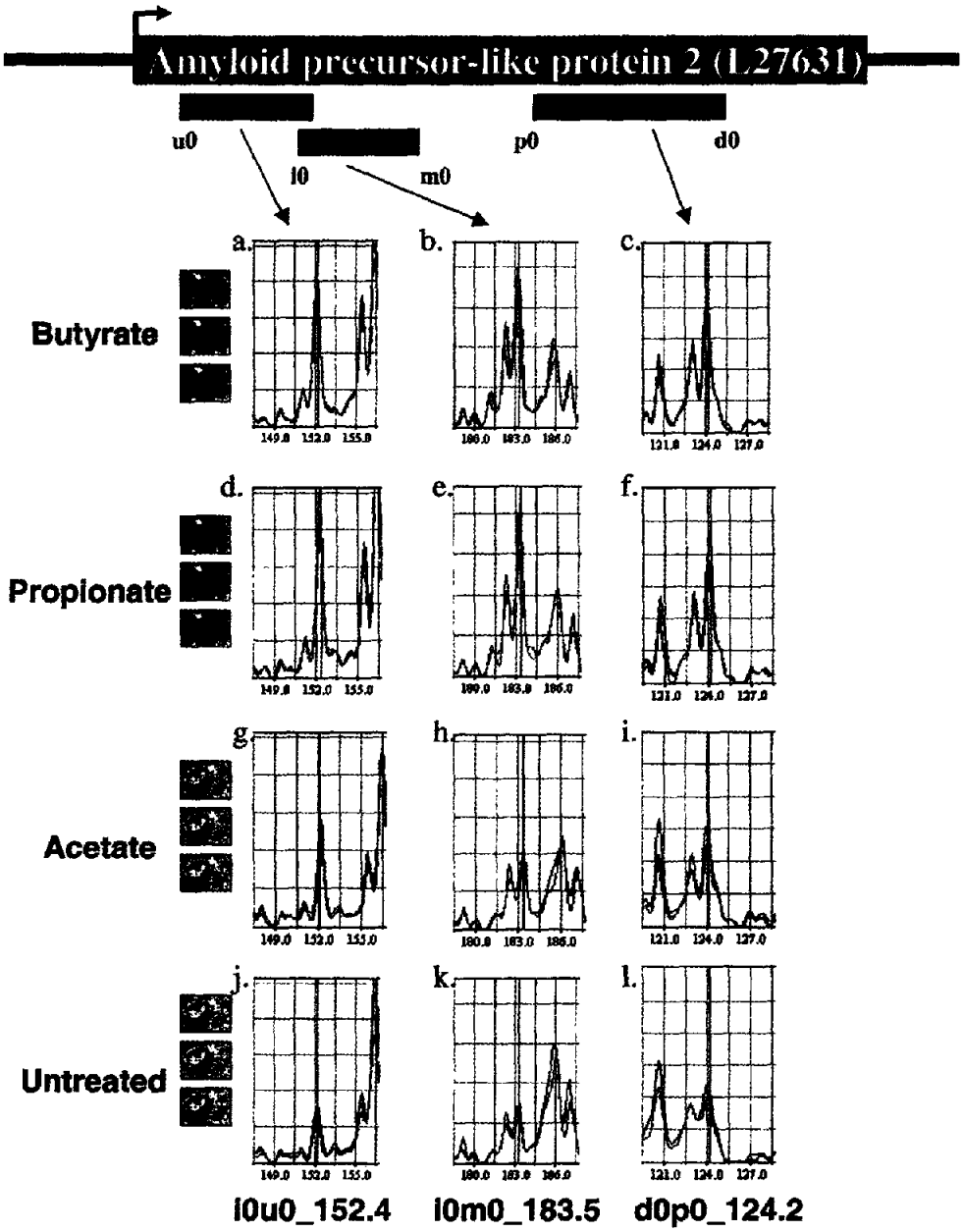
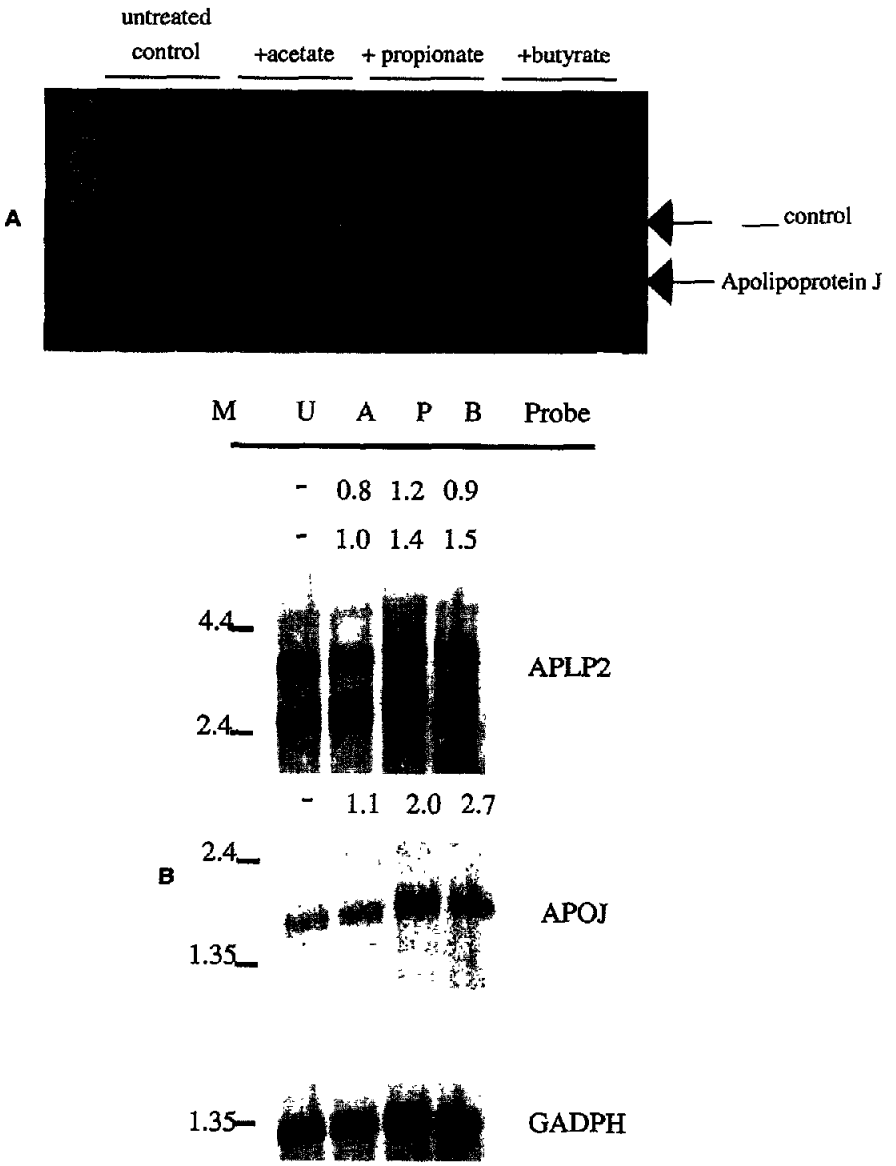


Fig. 4. Example of multiple band differences detected by GeneCalling corresponding to the same gene. Three of the differences caused by induction of amyloid precursor–like protein 2 (APLP-2) are depicted for butyrate-treated (a, b, c), propionate-treated (d, e, f), acetate-treated (g, h, i), and untreated Caco-2 control groups (j, k, l). The labels m0 (BspHI), i0 (BglII), u0 (NcoI), d0 (AcsI), and p0 (XhoII) are arbitrary symbols for the restriction enzymes used.



Samples	Probes
U - untreated	APLP2 - Amyloid precursor-like protein 2
A - acetate treated	APOJ - Apolipoprotein J
P - propionate treated	GADPH - glyceraldehyde-3-phosphate dehydrogenase
B - butyrate treated	- control

Fig. 5. A, RT-PCR was used to verify that apolipoprotein J is expressed in Caco-2 cells maintained under control conditions or with supplementation of the culture medium with 10 mmol/L acetate, 10 mmol/L propionate, or 10 mmol/L butyrate. Three separate dishes were used for each condition. These experiments were performed with different cells and at a different time than those represented in Figs. 1 to 4. mRNA was isolated from each dish and cDNA synthesized by RT-PCR. PCR for apolipoprotein J was then performed using high-stringency 30-mer primers combined with primers for GAPDH as a control, and the resulting PCR products were resolved by agarose gel electrophoresis. **B,** Northern blots show the effect of SCFA on expression of amyloid precursor–like protein 2 *(APLP2)* and apolipoprotein J *(APOJ)* as compared with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) control. Untreated *(U)* Caco-2 cells were compared with cells treated with 10 mmol/L acetate *(A)*, propionate *(P)*, and butyrate *(B)* for 24 hours prior to lysis and mRNA extraction in experiments performed separately from the treatments used for Figs. 1 to 4. APLP2 appears as a doublet. The numbers above each blot represent arbitrary densitometric units normalized to control (U) band intensity values.

DISCUSSION

We have previously extensively characterized regulation of intestinal epithelial differentiation in Caco-2 cells. Although derived from a human colon cancer, Caco-2 cells differentiate spontaneously in culture[25] and are closely regulated by matrix proteins, soluble bioactive agents, and intracellular signals.[26-30] These cells are thus an excellent model to study SCFA effects on human colonic mucosal cells.[10,11] This study extends previous reports of butyrate effects on colonocyte oncogenes to previously unstudied genes and demonstrates substantial differences among common SCFAs.

The 10 mmol/L concentration for each SCFA produces maximal or near-maximal effects on Caco-2 proliferation[11] and is consistent with changes in colonic luminal SCFAs that might be induced by diet changes.[5] Although differences in SCFA potency could be concentration dependent, this is not true for Caco-2 proliferation,[11] and higher butyrate concentrations are difficult to study in culture since downregulation of integrins interferes with Caco-2 adhesion.[10]

Butyrate modulates gene expression both directly and indirectly. At the gene-specific level, butyrate modulates oncogene expression in colonic cell lines by various mechanisms at the transcriptional level including shifts in promoter usage and blockage of transcriptional elongation as well as at the post-transcriptional level.[12-14] In addition to direct effects on individual genes, butyrate induces global changes in chromatin structure by blocking histone deacetylase. This increases acetylation of certain fractions of the H3 and H4 histone classes, which in turn alter the topology of the chromatin to which they are bound and thus modulate the activity of promoter sites such as TATA boxes upstream from many different genes.[31] Altering dietary fiber intake in rats alters colonic mucosal histone acetylation parallel to luminal butyrate levels.[32] The modulation of the acetylation of N terminal lysines of nucleosomal core histones not only modulates the chromatin structure and thus gene transcription, but also appears to provide for communication between the chromatin and cellular signal transduction pathways, thus providing a mechanism for heritable epigenetic information.[33] Butyrate also induces global decreases in DNA methylation and selective hypermethylation, which may also alter gene expression.[34]

This study explores the effects of three common SCFAs, butyrate, propionate, and acetate, at biologically relevant concentrations[5] on expression of a diverse set of 30,000 random Caco-2 gene fragments; 95% of the Caco-2 expressed transcriptome.[24] This preliminary report represents the most substantial positive results and does not address genes that might have been modulated less substantially or statistically significantly. Thus this report is not inconsistent with reports of SCFA modulation of other genes or proteins in Caco-2 cells or other colonocytes.

This study also focuses only on expression at the mRNA level by beginning with polyA$^+$ mRNA as the starting point. Differences in post-transcriptional processing also regulate protein levels. Nevertheless, this study suggests that the effects of SCFAs on Caco-2 gene expression are likely to be far more diverse than the few oncogenes currently known to be SCFA responsive. Indeed, we have to this point identified five gene fragments with 100% sequence identity over at least 40 distinct nucleotides to Caco-2 genes not previously known to be SCFA responsive. These are apoliprotein J, APLP2, and human caudal homeobox protein 2.

The apolipoprotein J gene product is variously called apolipoprotein J, clusterin, hs40, or testosterone-repressed prostate mRNA2 [TRPM-2]. It is a disulfide-linked heterodimeric sulfated glycoprotein,[35] which is increased in some epithelial cells by endocrine stimulation[36] or in settings of apoptosis.[37] It is induced by inflammation or degenerative disease[38] and is an acute-phase protein systemically.[39] Apolipoprotein J may protect against apoptosis. In one study where different methods of programmed cell death were used in various cell lines, clusterin was expressed only in morphologically normal surviving cells and not in apoptotic cells.[40] Overexpression prevents apoptosis in LNCaP cells[41] and may act by stabilizing cellular and nuclear membranes, inhibiting autophagic lysis.[42] Apoliprotein J is highly expressed at tissue barriers or fluid-tissue interfaces and may play a role in barrier cytoprotection.[42] It is induced by radiation in surviving but not apoptotic cells in intestinal mucosa, suggesting that it may also protect against apoptosis in gut mucosa.[43] Although SCFA induction of clusterin has not been described before, differentiation by 1,25,dihydroxyvitamin D_3 upregulates clusterin in some breast cancer cell lines.[44] Thus clusterin induction by SCFAs may be another mechanism by which fiber maintains the colonic mucosa in its normal physiologic state.

APLP2 is human amyloid precursor–like protein-2.[45] This member of the amyloid precursor family is increased when oxidative metabolism is impaired[46] and may be a zinc-modulated heparin-binding protein with proteolytic inhibitory activity.[47]

Homeobox genes code for DNA-binding homeodomains and broadly regulate gene transcription. Alterations in human caudal homeobox 2 (Cdx-2), in particular, may influence human colonocytic differentiation and carcinogenesis. Cdx-2 is abundant in intestinal mucosa, and regional variation through the small and large bowel may regulate regional differentiation.[48,49] Cdx-2 seems absent or downregulated in most sporadic colon cancers,[50] whereas mice het-

erozygous for wild-type Cdx-2 develop colonic polyps.[51] Cdx-2 overexpression in intestinal epithelial cell lines also induces intestinal differentiation[52,53] and is involved in the colon-specific regulation of carbonic anhydrase.[54] Decreased colonocytic differentiation after heterotopic transplantation of fetal colonic tissue was associated with decreased Cdx-2.[55]

In Caco-2 cells the expression of Cdx-2 and other homeobox proteins varies in a sequential fashion as these cells differentiate over time.[49,56] Cdx-2 overexpression stimulates conventional intestinal epithelial differentiation markers such as sucrase and lactase and expression of several molecules associated with cell-cell and cell-matrix interaction, whereas antisense inhibition of Cdx-2 inhibits cell adhesion.[57] Regulation of cell-matrix adhesion and adhesion molecules by Cdx-2 parallels our previous observation that SCFAs (which we now report to inhibit Cdx-2 expression) also inhibit Caco-2 adhesion to matrix proteins[10] and inhibit expression of some integrin subunits.[11] However, we[10,11] and others[12-14] have reported stimulation of conventional differentiation markers by SCFA, whereas butyrate downregulation of Cdx-2 would be expected to inhibit these markers. Thus, although Cdx-2 might mediate some effects of SCFA on human colonocyte cell-matrix or cell-cell interaction, other pathways are probably also involved. Butyrate downregulation of Cdx-2 could also be part of an as yet uncharacterized negative-feedback loop that controls butyrate-associated differentiation.

Cell phenotype reflects both original genotype and modulation of gene expression by environmental factors. These data are consistent with previous suggestions that luminal SCFAs dramatically modulate expression of the genotype and may therefore be highly relevant for the blockade of colonocyte malignant transformation. This study strongly suggests both the complexity of SCFA effects and the potential for differences among the effects of common SCFAs on colonocyte biology. As these effects are further elucidated, we may not only advocate high-fiber diets but also recommend specific fibers with desirable SCFA fermentation patterns.

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Chronic Diversion of Bile to the Urinary Bladder Induces Pancreatic Growth in Dogs

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The aim of this study was to elucidate the mechanism of chronic biliary diversion and its effect on pancreatic growth. In the first part of the study, nine mongrel dogs underwent diversion of bile from the gastrointestinal tract by ligating the common bile duct and interposing a segment of jejunum between the gallbladder and the urinary bladder (cholecystojejunocystostomy [CJC]). Despite the loss of 7% of their body weight at 12 weeks after bilioenteric diversion, CJC dogs had significantly greater pancreatic wet weight than control dogs (51.2 ± 2.2 g vs. 37.1 ± 2.2 g). In the second part of the study, six other dogs underwent CJC. Twelve weeks later, bilioenteric continuity was restored by creating a cholecystojejunoduodenostomy (CJD). The dogs were given butter (3 g/kg) by mouth (prior to surgery, 12 weeks after CJC, and 4 weeks after CJD). Pancreatic excisional biopsy specimens were obtained at each operation and at autopsy. CJC induced more pancreatic RNA per milligram of weight (743 ± 52, CJC; 579 ± 44, prior to surgery, $P < 0.05$ vs. CJC; 520 ± 26 μg/100 mg • tissue, CJD, $P < 0.01$ vs. CJC), but not more DNA, and significantly higher basal plasma cholecystokinin levels and butter-stimulated cholecystokinin responses when compared with values prior to surgery or following CJD. We conclude that chronic biliary diversion induces pancreatic growth associated with hypersecretion of cholecystokinin in dogs. (J GASTROINTEST SURG 2000;4:513-519.)

KEY WORDS: Dog, biliary diversion, pancreatic growth, cholecystokinin

Temporary or chronic diversion of bile from the gastrointestinal tract augments the release of cholecystokinin (CCK) in rats and dogs.[1-4] Patients with external bile drainage have an increased basal and postprandial plasma CCK concentration as well.[5] Furthermore, CCK appears to act as a growth factor for the pancreas. Continuous administration of CCK or oral administration of protease inhibitor, which is associated with increased plasma CCK concentrations, also induces pancreatic growth in rats.[6,7] Although CCK is an accepted stimulant for pancreatic growth in rats, the phenomenon has not been proved in dogs and humans. We hypothesized that if chronic biliary diversion enhances plasma CCK, then chronic diversion of bile from the gastrointestinal tract in dogs would also lead to pancreatic growth if CCK was a growth factor. The aim of this study, therefore, was to determine the effect of chronic biliary diversion on pancreatic growth in dogs.

MATERIAL AND METHODS

Animal Preparation: Part 1

Nine mongrel dogs, weighing 14 to 21 kg, were used. After an 18-hour fast, animals were anesthetized with intravenous pentobarbital (induction dose, 25 mg/kg) and then underwent laparotomy. After the common bile duct was ligated just distal to the cystic duct, cholecystojejunocystostomy (CJC) was accomplished by interposing a 20 cm segment of distal jejunum between the gallbladder and the urinary bladder as previously described.[4] The dogs were fed ordinary dry dog food (DS, Oriental Yeast Co., Tokyo, Japan) with a carbohydrate-rich meal consisting of Japanese steamed rice and miso soup. Dogs were sacrificed at 12 to 16 weeks postoperatively. The experimental protocol and procedures used in this study were approved by the Animal Care Committee of the School of Medicine of Tohoku University. Ten intact dogs, weighing 11 to 21 kg, served as controls.

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Electron Microscopic Study of the Pancreas. At the time of sacrifice, the entire pancreas was removed and the wet weight of the organ was obtained. After the wet weight of the organ was determined, a small amount of pancreatic tissue was minced into fragments and fixed for histologic examination using a mixture of 2.5% glutaraldehyde and 2.5% formaldehyde in cacodylate buffer (pH 7.4). The samples were stained with uranyl acetate, followed by staining with lead citrate. The sections were examined using an H-600 electron microscope (Hitachi Ltd., Tokyo, Japan).

Animal Preparation: Part 2

Six mongrel dogs, weighing 15 to 22 kg, underwent CJC as described in part 1. A tail of the dorsal portion of the pancreas (2 cm × 2 cm) was excised during the operation for determination of tissue RNA and DNA concentrations. The excised pancreatic tissue was stored at −80° C for later determination. Twelve weeks after CJC, the dogs were reoperated; the jejunocystostomy was transected, the bladder was closed, and the jejunum was anastomosed to the second portion of the duodenum (cholecystojejunoduodenostomy [CJD]) to restore entry of bile into the duodenum. A tail of the ventral portion of the pancreas (2 cm × 2 cm) was excised for determination of tissue RNA and DNA concentrations. Eight weeks later, the dogs were sacrificed and a biopsy of the pancreas was done.

Butter Feeding Test. Each dog was studied prior to surgery, 12 weeks after CJC, and 4 weeks after CJD. After an 18-hour fast, animals were offered 3 g butter/kg body weight dissolved in 30 ml lukewarm water, which was readily ingested. Blood samples (5 ml) were collected from the foreleg vein at times −5, 15, 30, 60, 90, 120, 150, 180, 210, and 240 minutes after butter ingestion. The samples were collected into chilled tubes containing 500 KIU aprotinin and 1 mg EDTA/ml blood, and later centrifuged at 3000 rpm for 10 minutes. The plasma was collected and stored at −30° C for later determination of the CCK concentration.

RNA and DNA Concentrations. RNA and DNA concentrations were measured by the Schmidt-Thannhauser-Schneider method.[8] After extracting the pancreatic tissue with 5% perchloric acid, the RNA concentration was determined by the orcinol reaction, using yeast RNA as a standard. The DNA concentration was determined by the diphenylamine reaction using calf thymus DNA as a standard.

CCK Concentration. The plasma CCK concentration was measured in duplicate by radioimmunoassay using CCK-8 N-terminal specific antibody OAL-656 (Otsuka Assay Laboratories, Tokushima, Japan[9]). The antiserum reacted specifically to the N-terminal region of CCK-8 and bound 100% of both CCK-8 and CCK-33, and 85% of CCK-39. There was no cross-reaction to the nonsulfated form of CCK-8, the sulfated or nonsulfated form of gastrin-17, or cerulein. Synthetic CCK-39 coupled to ^{125}I-hydroxyphenyl propionic acid–succinimide ester (Bolton-Hunter reagent) was used as a tracer, and synthetic pure porcine sulfated CCK-33 (Peptide Institute Inc., Osaka, Japan) served as a standard. Plasma samples were extracted with 96% ethanol (1/2 volume/volume).[10] Details of this method have been described elsewhere.[†]

Fecal Chymotrypsin Activity. Fresh fecal samples were collected before and after each operation, and kept frozen at −20° C until determination of fecal chymotrypsin activity. Fecal chymotrypsin activity was determined enzymatically with Monotest Chymotrypsin (Boehringer-Mannheim Corp., Mannheim, Germany).

Statistical Analysis

All values are expressed as mean ± standard error of the mean (SEM). Integrated responses of CCK after butter ingestion were represented by the area under the curve. Statistical analysis was carried out using an unpaired Student's t test for body weight and pancreatic wet weight, and analysis of variance (ANOVA) following Fisher's PSLD or Sheffe's test was used for other data. Differences were considered statistically significant when the P value was <0.05.

RESULTS

Condition of CJC Dogs

All dogs remained healthy with a good appetite until they were sacrificed. All, however, had acholic stools that were of foamy consistency, and they had lost 7% of their body weight at the time of sacrifice (17.1 ± 0.8 kg vs. 15.9 ± 0.8 kg).

Pancreatic Wet Weight

At autopsy, CJC dogs had a greater pancreatic wet weight and a higher ratio of pancreatic wet weight to body weight than control dogs (51.0 ± 2.2 g vs. 37.1 ± 2.2 g; ratio: 3.26 ± 0.17 g/kg vs. 2.37 ± 0.08 g/kg, $P<0.01$, respectively). Even when the body weight of the CJC dogs was adjusted for weight loss, the ratio remained higher (3.03 ± 0.19 g/kg, $P<0.05$) than that of the control dogs.

Electron Microscopic Study of the Pancreas

Most of the pancreatic acinar cells in the control dogs showed narrow rough endoplasmic reticulum (Fig. 1), but a high proportion of acinar cells in the CJC dogs were extremely swollen in association with

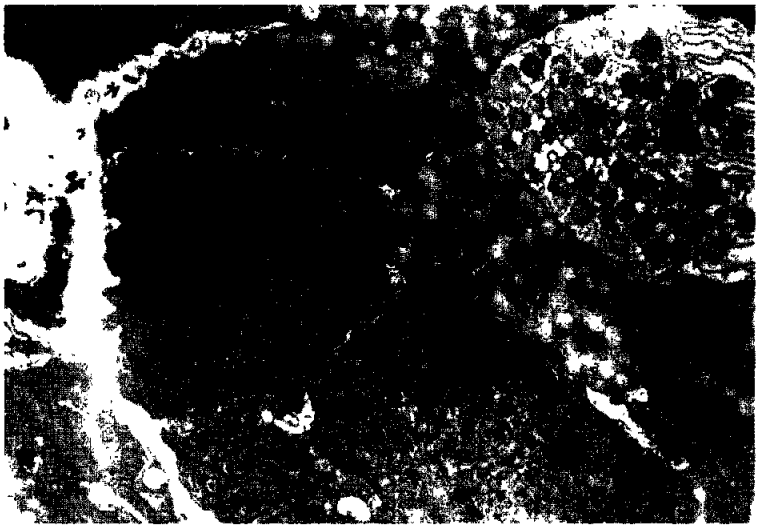

Fig. 1. Electron micrograph of the pancreas of a control dog. Most of the pancreatic acinar cells show narrow rough endoplasmic reticulum, but some of them reveal mildly dilated rough endoplasmic reticulum with plenty of zymogen granules. (×6000.)

Fig. 2. A and **B,** Electron micrograph of the pancreas of a CJC dog (×6000). A high proportion of acinar cells in CJC dogs were extremely swollen, associated with profuse and dilated rough endoplasmic reticulum. Nuclear proliferation of acinar cells could not be seen at all.

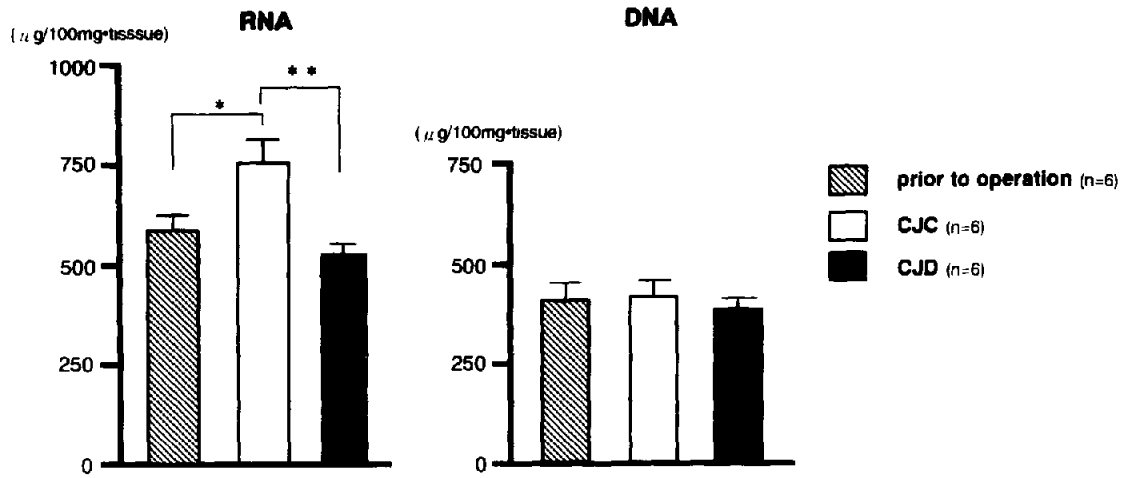

Fig. 3. Effect of bile diversion on pancreatic tissue RNA and DNA concentrations. A dorsal or ventral portion of the pancreas was excised at CJC (indicating prior to surgery) and CJD (indicating CJC) and at the autopsy (indicating CJD), respectively. Pancreatic RNA concentrations of CJC were significantly elevated compared to values prior to surgery or CJD, whereas DNA concentrations did not change after each operation. Values are expressed as mean ± SEM; asterisks indicate significant differences (* = $P < 0.05$ and ** = $P < 0.01$ vs. CJC).

profuse and dilated rough endoplasmic reticulum (Fig. 2). In addition, nuclear proliferation of the acinar cell could not be seen in the CJC dogs.

RNA and DNA Concentrations in Pancreatic Biopsies

RNA and DNA concentrations are shown in Fig. 3. The RNA concentration in pancreatic tissue after CJC was greater compared to the values in the dogs prior to surgery (743 ± 52 vs. 579 ± 44 μg/100 mg • tissue, $P < 0.05$). After CJD, this increase in the RNA concentration reverted to normal (520 ± 26 g/100 mg • tissue, $P < 0.01$ vs. CJC). The DNA concentration did not differ after each operation (before surgery, 370 ± 31; CJC, 416 ± 41; and CJD, 396 ± 35 μg/100 mg • tissue).

Plasma Cholecystokinin

After CJC, the basal plasma CCK concentration was greater than before surgery (33.8 ± 4.8 vs. 11.4 ± 1.1 pmol/L, $P < 0.01$). Basal concentrations of CCK after CJC were greater than the peak concentration of fat-stimulated CCK release in dogs before surgery (26.1 ± 4.3 pmol/L at 180 minutes after butter ingestion). Dogs after CJC also had greater fat-stimulated plasma CCK concentrations than prior to surgery at every time point; the peak value of 78.8 ± 10.2 pmol/L occurred at 240 minutes after butter ingestion (Fig. 4). CJD returned the increased basal and fat-stimulated plasma CCK concentrations to the same levels as before surgery (basal, 10.0 ± 1.4 pmol/L, $P < 0.01$ vs. CJC).

Integrated Incremental Cholecystokinin Responses

After CJC, there was a threefold greater integrated CCK response to butter ingestion than before surgery (8.7 ± 0.8 vs. 2.2 ± 0.6 nmol • 240 min/L, $P < 0.01$). After CJD, integrated CCK responses returned to values comparable to those prior to surgery (2.6 ± 0.2 nmol • 240 min/L, $P < 0.01$ vs. CJC).

Fecal Chymotrypsin Levels

Fecal chymotrypsin levels are presented in Table I. After CJC, fecal chymotrypsin activity decreased significantly and remained at one fifth or less compared to preoperative values ($P < 0.05$). After CJD, fecal chymotrypsin levels increased and were restored to preoperative levels.

DISCUSSION

Temporary or chronic bile diversion from the gut augments the plasma CCK concentration in dogs and humans,[1,3,5] whereas oral bile or bile acid administration inhibits fat-stimulated CCK release in dogs.[1,4] These observations suggest that bile has an inhibitory effect on CCK release in dogs and humans. Protease inhibitors such as camostat mesilate (camostate) and soy bean flour are well known to increase the basal plasma CCK concentration associated with pancreatic hyperplasia in rats when administered long term.[6,11] These findings, however, have not been confirmed in dogs[12] or humans.[13] In these species, protease inhibitors impede intraluminal trypsin activity

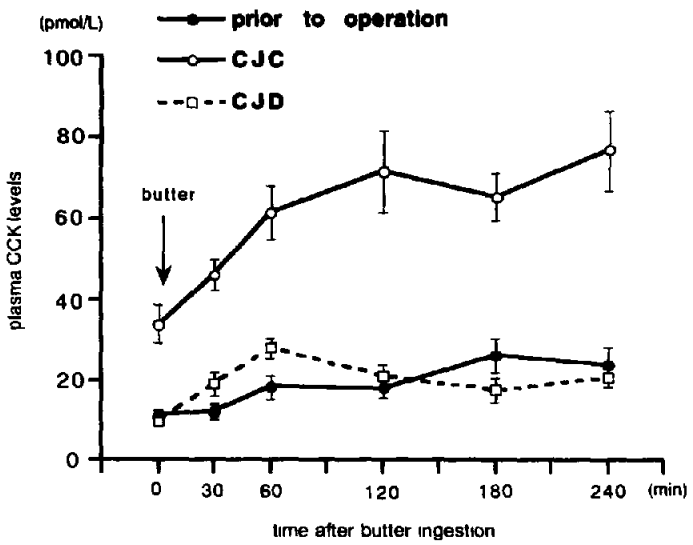

Fig. 4. Effect of bile diversion on butter-stimulated plasma CCK levels. Each dog was fed 3 g/kg body weight of butter dissolved in 30 ml of lukewarm water. The dogs were fed the butter solution prior to surgery, 12 weeks after CJC, and 4 weeks after CJD. CJC indicated higher CCK levels at any point compared with values prior to surgery or CJD. Values are expressed as mean ± SEM.

Table I. Effect of bile diversion on fecal chymotrypsin levels during the experiment

	Prior to operation (n = 6)	CJC (n = 6)			CJD (n = 6)
		5 days	4 wk	12 wk	7 days
Fecal chymotrypsin levels (U/g)	27.9 ± 5.7	6.0 ± 1.7*	3.2 ± 0.7*†	5.0 ± 6.1*	24.8 ± 6.1

CJC = cholecystojejunocystostomy; CJD = cholecystojejunoduodenostomy.
Values are mean ± SEM; asterisks indicate significant differences.
*P <0.05 vs. prior to operation.
†P <0.05 vs. CJD.

but have a relatively weak inhibitory effect on luminal chymotrypsin activity.[13,14] Because intraluminal bile protects proteases from autodigestion,[15] we hypothesized that both trypsin and chymotrypsin are involved in the regulation of CCK secretion, and enhanced CCK release induces pancreatic growth after biliary diversion from the gut. When bile was diverted from the gut by CJC, fecal chymotrypsin activity decreased. The reduced activity of luminal chymotrypsin (and presumably trypsin as well, although we did not measure luminal trypsin activity) is believed to cause hypersecretion of CCK with a mechanism similar to that seen in rats given camostate. With the return of bile to the duodenum after CJD, protease activity was restored and basal and fat-stimulated CCK secretion returned to control levels. These results are supported by Koide et al.[5] who showed that patients with external bile drainage had augmented fat-stimulated plasma CCK release. Allowing internal bile drainage into the gut caused a reduction in the augmented CCK release to normal levels. In addition, intraluminal bile may have a direct inhibitory effect on CCK release independent of the protease activity reported by Nakamura et al.[2] We suggest that biliary diversion from the gut led to augmented basal and fat-stimulated CCK release through inhibition of intraluminal protease activity or via loss of a direct inhibitory effect of bile on CCK release.

Pancreatic growth occurred after CJC as quantified both by wet weight of the pancreas and by the ratio of pancreas weight to body weight. Furthermore, CJC led to a greater RNA concentration per 100 mg weight of pancreas, and CJD reversed this effect of biliary diversion. In contrast, no difference was noted in the DNA concentration when it was measured in a similar fashion. These results are in partial agreement with findings in rats administered gut hormones continuously,[16,17] rats given oral protease inhibitors,[6,11] and rats with diverted bile from the intestine[18] in which DNA increased suggesting hyperplasia. We did

not measure total pancreatic DNA content in the pancreas so we could not confirm whether the pancreatic growth after CJC is hyperplasia or not. However, we would suggest the pancreatic hypertrophy may occur after CJC, because the DNA concentration per 100 mg weight of pancreas did not change after CJC and nuclear proliferation of pancreatic acinar cells in CJC was not observed at all on electron microscopic morphologic studies.

In terms of hormonal trophic factors, not only CCK but also secretin is considered to be a potent trophic factor for pancreatic growth.[16,17,19] Secretin release after biliary diversion has yet not been elucidated; however, it has been confirmed that secretin is released by intraduodenal bile or bile acid infusion.[20,21] Therefore a decrease in luminal bile resulting from CJC would cause a reduction rather than an increase in secretin release. Although other hormones such as neurotensin,[22,23] bombesin,[7,24] and gastrin-releasing peptide[25] remain as candidates for pancreatic growth, we believe that the augmented CCK release, not only in basal but in fat-stimulated concentrations, might have led to the pancreatic growth in this experiment.

We conclude that chronic diversion of bile to the urinary bladder in dogs induces pancreatic growth associated with increased basal and fat-stimulated plasma CCK concentrations. All abnormalities in CCK concentrations were restored to normal values by return of bile to the duodenum via CJD. These findings suggest that intraluminal bile plays an important role in pancreatic growth by partially regulating CCK release in dogs.

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Analysis of 154 Actual Five-Year Survivors of Gastric Cancer

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Gastric cancer patients in the United States have a poor prognosis with a collective 5-year survival rate of less than 15%. We identified a subset of actual 5-year survivors (long-term survivors) and analyzed clinicopathologic variables predictive of recurrence and survival beyond the 5-year mark. A review of our prospective database from July 1985 to February 1993 revealed 154 patients who were long-term survivors and 280 patients who died of disease prior to 5 years (short-term survivors) following curative resection (R0). Tumor (T) stage, nodal (N) status, tumor location, and median number of positive nodes were compared between the two groups. Univariate and multivariate analysis of disease-free and greater than 5-year disease-specific survival was performed within the long-term survivors. Among the long-term survivors, 29% were classified as "early gastric cancers" (T1NX). The median number of positive nodes (0 vs. 5; $P < 0.001$) and percentage of lesions that were T1/T2 (60% vs. 19%; $P < 0.001$), node negative (58% vs. 15%; $P < 0.001$), or proximal (40% vs. 65%; $P < 0.001$) was significantly different in long-term survivors vs. short-term survivors, respectively. Of the 154 five-year survivors, gastric cancer recurred in 23, and 13 patients (8%) died of the disease at a median of 84 months from the original diagnosis. On univariate and multivariate analysis of prognostic factors in the long-term survivors, only the Lauren histologic classification predicted disease-specific and disease-free survival with diffuse histologic types faring significantly less well. T stage and N status are powerful prognostic factors of outcome within the first 5 years after curative resection of gastric carcinoma. However, the Lauren histologic type emerges as the dominant predictor of outcome once a patient with gastric cancer has survived for 5 years or more. (J GASTROINTEST SURG 2000;4:520-525.)

KEY WORDS: Gastric cancer, Lauren histologic type, survival

There are approximately 23,000 new cases of gastric carcinoma per year in the United States. The prognosis is poor with an estimated 5% to 15% 5-year survival.[1] Standard indicators that have been used to predict prognosis include the tumor (T), node (N), and metastasis (M) stage of the tumor. Several studies have shown that with increasing depth of tumor invasion (T stage), the prognosis is progressively poorer.[2-6] Likewise, with increasing nodal involvement, survival diminishes.[4-6] Recently the number of lymph nodes involved with tumor has been found to be a powerful predictor of survival.[7-11]

Other prognostic factors have been evaluated in gastric cancer with inconsistent results as to their value in predicting outcome. These include, among others, tumor location in the stomach (proximal vs. distal), type of lymph node dissection performed (D1 vs. D2), and Lauren histologic classification of the tumor. These factors have, on univariate analysis, been found to be determinants of outcome but have not frequently been found to be independent predictors of survival.[3,12,13]

Because of the poor prognosis of patients with gastric cancer, individual institutions do not have a large experience with actual survivors following resection of gastric cancer. In addition, data are sparse concerning the incidence and factors affecting late recurrence and survival 5 years after treatment for gastric cancer. Our objectives were to review our experience with actual 5-year survivors of gastric cancer and to

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Table I. Comparison of pathologic factors for short-term and long-term survivors

Factor	STS (<yr)	LTS (>5 yr)	*P* value
No. of patients	280	154	
T1/T2	54 (19%)	92 (60%)	0.001
Node negative	41 (15%)	90 (58%)	0.001
Median number of positive nodes	5	0	0.001
Tumor location (proximal)	177 (63%)	62 (40%)	0.001
Lauren histologic type (diffuse)	108 (39%)	56 (36%)	0.68

STS = short-term survivors (less than 5 years); LTS = long-term survivors (more than 5 years).

determine factors predicting late recurrence and survival beyond 5 years.

PATIENTS AND METHODS

We retrospectively reviewed our prospective database for patients undergoing treatment for gastric cancer from July 1985 to February 1993 at Memorial Sloan-Kettering Cancer Center. A total of 793 patients underwent surgical exploration for gastric cancer during this time period. Of these, 154 patients lived at least 5 years (long-term survivors) and 280 patients died of disease prior to 5 years (short-term survivors) following curative resection (R0).[14] These two groups were used for study, whereas 359 patients who were found to be unresectable or who underwent palliative or R1 resections were eliminated from analysis.

Standard demographic and treatment factors were compared between the two groups including age, sex, race, type of gastric resection, and type of lymphadenectomy performed. In addition, pathologic variables analyzed and compared included the T and N stages of the tumors, the number of positive nodes, tumor location in the stomach, and distribution of Lauren histologic types. Factors affecting disease-specific survival in the short-term survivors and those influencing disease-free and disease-specific survival in the long-term survivors were analyzed.

Follow-up data were obtained from patient interviews and review of hospital charts. Comparisons between groups were performed using chi-square analysis. Survival within groups was analyzed by means of the Kaplan-Meier method with differences compared using the log-rank test. Multivariate analysis of survival was performed using the Cox proportional hazards model. Significance was defined as $P < 0.05$.

RESULTS

Analysis of demographic factors revealed the median age of patients in both short-term and long-term survivors to be 63 years. There was a significantly higher percentage of males among the short-term survivors as compared to the long-term survivors (74% vs. 60%, respectively; $P = 0.003$). In addition, although most patients in this study were white (short-term survivors, 90%; long-term survivors, 84%), there was a significantly higher proportion of Asians among the long-term survivors (8%) compared to the short-term survivors (3%; $P = 0.02$).

Analysis of treatment-related factors revealed that significantly more of the short-term survivors underwent an esophagogastrectomy or proximal gastrectomy as compared to the long-term survivors (50% vs. 23%, respectively; $P < 0.001$). At the same time significantly more of the long-term survivors underwent a distal subtotal gastrectomy as compared to the short-term survivors (46% vs. 19%, respectively; $P < 0.001$). Total gastrectomy was performed in a similar percentage of patients in both groups (short-term survivors, 19%; long-term survivors, 18%; $P = 0.80$). In addition, there was no difference in the percentage of patients who underwent a D2 lymph node dissection (short-term survivors, 63%; long-term survivors, 70%; $P = 0.17$).

Pathologic review revealed that the majority of tumors were stage T1 or T2 in the long-term survivors, whereas these made up the minority of tumors in the short-term survivors (60% vs. 19%, respectively; $P < 0.001$) (Table I). Of note, 40% of tumors in the long-term survivors were stage T3 or T4. In addition, there was a significantly higher percentage of node-negative tumors in the long-term survivors as compared to the short-term survivors (58% vs. 15%, respectively; $P < 0.001$). However, 42% of long-term survivors had node-positive tumors with 9% (n = 14) having N2 disease. The median number of positive nodes was 0 in the long-term survivors as compared to five in the short-term survivors ($P < 0.001$). Short-term survivors had tumors located in the proximal stomach more often than did long-term survivors (63% vs. 40%, respectively; $P < 0.001$). Finally, the percentage of patients having Lauren intestinal or diffuse histologic types was the same in the two groups.

Table II. Factors associated with disease-specific survival in short-term survivors

Factors	*P* value
Univariate analysis	
Nodal disease	0.002
No. of positive lymph nodes	0.001
T stage	0.11
Lauren histologic type	0.03
Tumor location	0.75
Lymph node dissection	0.83
Multivariate analysis	
Nodal disease	0.03
No. of positive nodes	0.002
T stage	0.50
Lauren histologic type	0.10
Tumor location	0.91

Table III. Factors associated with disease-free survival in long-term survivors

Factors	*P* value
Univariate analysis	
Nodal disease	0.62
No. of positive nodes	0.47
T stage	0.15
Lymph node dissection	0.84
Lauren histologic type	0.004
Multivariate analysis	
Nodal disease	0.77
No. of positive nodes	0.53
T stage	0.14
Lymph node dissection	0.47
Lauren histologic type	0.02

Lauren diffuse-type tumors were found in 39% of the short-term survivors as compared to 36% of the long-term survivors ($P = 0.68$).

Prognostic factors among the short-term survivors associated with disease-specific survival are shown in Table II. In this group the median survival was 17 months, and all patients died of disease within 5 years. On univariate analysis, nodal disease had a significant impact on survival, with node-negative patients (n = 41, median 27 months) faring better than node-positive patients (n = 237, median 14 months; $P = 0.002$). In addition, the number of positive nodes was an important determinant of survival ($P < 0.001$). The only other factor on univariate analysis that predicted survival was the Lauren classification, with intestinal and mixed types having a significantly longer survival (n = 161, median 19 months) compared to the diffuse histologic type (n = 108, median 13 months; $P = 0.03$). However, there was no significant impact of T stage on survival. Patients with T1 and T2 tumors (n = 54) had a median survival of 25 months as compared to 15 months for patients with T3 and T4 lesions (n = 224; $P = 0.11$). In addition, there was no significant impact of tumor location or type of lymph node dissection on survival. On multivariate analysis, nodal involvement and the number of positive nodes were independent predictors of outcome ($P = 0.03$ and $P = 0.002$, respectively).

There were 23 recurrences among the long-term survivors. The median time to recurrence was 49 months with an actuarial disease-free survival at 10 years of 81%. Analysis of factors affecting disease-free survival (Table III) revealed no impact of nodal disease ($P = 0.62$), tumor stage ($P = 0.15$), number of positive nodes ($P = 0.47$), or type of lymph node dissection ($P = 0.84$). Only the Lauren histologic type affected disease-free survival with diffuse types having a 10-year actuarial survival of 71% as compared to 89% for intestinal and mixed types ($P = 0.004$). On multivariate analysis, only Lauren histologic type significantly predicted disease-free survival.

Of the 23 recurrences, 12 were locoregional (T2, n = 3; T3, n = 8; and T4, n = 1), involved the stomach, lymph nodes, and peritoneum, whereas 11 were distant (T1, n = 2; T2, n = 4; and T3, n = 5). Among the patients with tumors of the Lauren diffuse histologic type, 10 recurrences (67%) were locoregional and five (33%) were distant. However, recurrences were characterized as locoregional in two patients (25%) and distant in six patients (75%) with tumors of the Lauren intestinal and mixed types (intestinal and mixed vs. diffuse, $P = 0.07$).

When evaluating all long-term survivors, it was noted that 13 patients died of disease after surviving for 5 years. With a median follow-up of 97 months in this group, the latest death from recurrent disease was recorded at 114 months of follow-up. It was identified that on analysis of disease-specific survival, on both univariate and multivariate analysis, only Lauren histologic type significantly predicted survival ($P = 0.02$ and $P < 0.05$, respectively) (Table IV). Actuarial disease-specific survival was 90% at 10 years for Lauren intestinal and mixed types, whereas it was 78% for Lauren diffuse histologic type ($P = 0.02$; Fig. 1).

When excluding those patients who had a recurrence prior to 5 years of follow-up and evaluating only

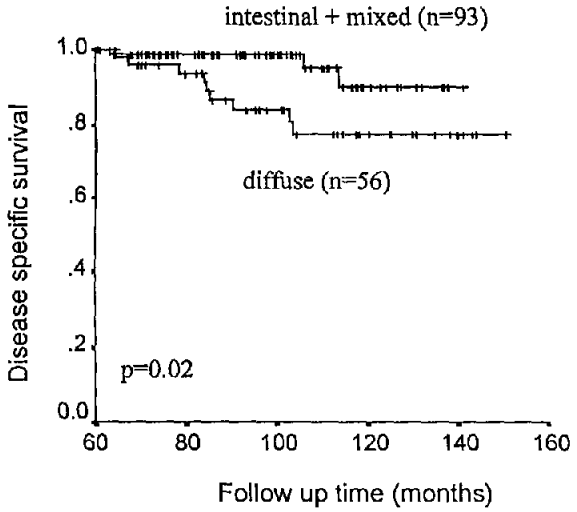

Fig. 1. Influence of Lauren histologic type on disease-specific survival in long-term survivors.

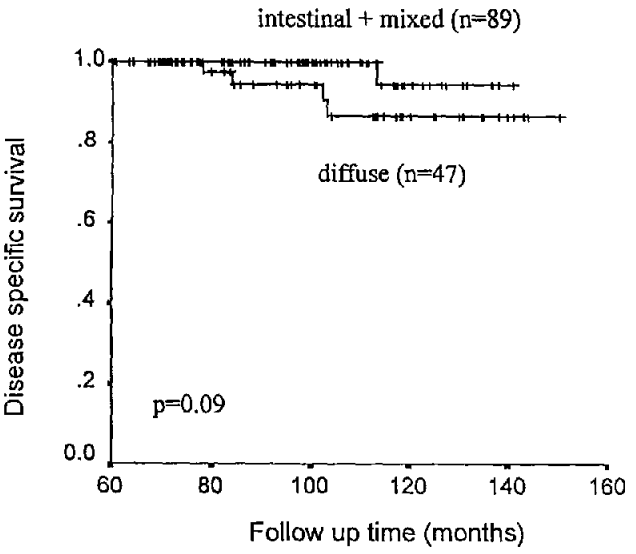

Fig. 2. Influence of Lauren histologic type on disease-specific survival in long-term survivors who had no evidence of disease at 5 years of follow-up.

Table IV. Factors associated with disease-specific survival in long-term survivors

Factors	*P* value
Univariate analysis	
Nodal disease	0.54
No. of positive nodes	0.19
T stage	0.13
Tumor location	0.53
Lymph node dissection	0.38
Lauren histologic type	0.02
Multivariate analysis	
Nodal disease	0.19
No. of positive nodes	0.43
T stage	0.31
Tumor location	0.65
Lymph node dissection	0.29
Lauren histologic type	0.05

those patients who had a recurrence after being without evidence of disease for at least 5 years, the significant association between Lauren classification and survival was lost. Actuarial 10-year disease-free survival was 93% for intestinal and mixed types (n = 89), whereas it was 84% for Lauren diffuse types (n = 47) ($P = 0.13$). In addition, with respect to disease-specific survival, actuarial 10-year survival was 95% for intestinal and mixed types, whereas it was 87% for the diffuse types ($P = 0.09$; Fig. 2).

DISCUSSION

Gastric cancer patients in Western countries have a poor prognosis with a 5% to 15% 5-year survival. Because of this limited survival, few Western centers have accumulated a large experience with actual 5-year survivors. Over a 13-year period, we identified 154 patients who underwent an R0 resection and survived for 5 years and analyzed factors associated with late recurrence and death. For purposes of comparison, we contrasted our findings with prognostic factors in short-term survivors who had undergone an R0 resection.

We found that among patients who died within less than 5 years, 81% had T3 or T4 tumors and 85% were node positive, reflecting the powerful influence of these factors on outcome. In this subset of patients undergoing surgery for gastric carcinoma, there was no influence of T stage on outcome. Nodal disease, including nodal positivity and the number of positive nodes, was a predictor of outcome on univariate and multivariate analysis. These findings are supported by other reports which indicate that nodal involvement is a powerful determinant of survival.[2,4,12,13] In addition, recent studies indicate that the number of involved lymph nodes is the most significant prognostic factor in gastric cancer.[7,8,10,11] In the present study the observation that T stage did not predict survival among the short-term survivors may be due to the subset of patients evaluated. This group does not represent the entire group of patients undergoing curative surgery for gastric cancer but examines a subset who die of

disease before 5 years following an R0 resection. The large percentage of patients with T3 and T4 tumors in this group may lead to failure to reveal a difference in survival based on T stage. In addition, with more advanced lesions (T3 or T4) it may be that nodal positivity and the number of involved nodes most strongly reflect outcome.

The only other factor predicting survival in the short-term survivors on univariate analysis was the Lauren histologic type. This factor was not an independent determinant or outcome as it lost significance on multivariate analysis. In this study this may be explained by the fact that tumors of the intestinal type were, on average, less advanced than carcinomas of the diffuse type. Among the T1 and T2 tumors in the short-term survivors, we found Lauren intestinal and mixed-type histologies in 27% of cases and the diffuse type in 7% of cases ($P < 0.001$). However, nodal positivity was found in 84% of intestinal and mixed-type histologies vs. 88% of the diffuse type ($P = 0.37$). Other investigators have found a correlation between the Lauren histologic type and both the extent of tumor penetration into the gastric wall and nodal disease.[3]

In contrast to our findings concerning the importance of nodal positivity and number of positive nodes on outcome among the short-term survivors, these factors did not correlate with outcome beyond 5 years in the long-term survivors. Similar findings have been described in other malignancies where standard prognostic features do not predict late recurrence and death. In a study of resected pancreatic adenocarcinoma, 42% of actual 5-year survivors were node positive. Death from recurrent disease occurred in 42% of patients after 5 years of follow-up and was identified in node-positive and node-negative patients with equal frequency.[15] In a study evaluating long-term survival in retroperitoneal sarcoma, 25% of patients (12 of 48) died of disease after 5 years of follow-up. Grade was not significant for tumor mortality, and the only factor found to increase the risk of late tumor mortality was an incomplete gross resection at the time of definitive operation.[16] In the present study of gastric carcinoma, nodal disease had a significant impact on early mortality, as demonstrated in the analysis of our short-term survivors. These patients are removed from any analysis of long-term survivors and therefore one would expect different variables to influence survival in our group of long-term survivors.

In evaluating all long-term survivors, only the Lauren histologic type was of independent prognostic significance in predicting recurrence and late death from disease. Most studies that use univariate analysis show a survival advantage for patients with intestinal-type tumors.[3,4,17,18] In one study it was shown to be an independent prognostic variable,[19] but most studies that employ multivariate analysis fail to demonstrate the Lauren gastric cancer classification to be an independent prognostic factor.[4,13] In this study, in the long-term survivors, although the Lauren diffuse histologic type was associated with significantly more node-positive ($P = 0.04$) and advanced T-stage (T3/T4) ($P = 0.04$) tumors than the intestinal and mixed types, T and N stage had no significant impact on long-term survival. We believe that this reflects the fairly homogeneous distribution of favorable T and N stages in our long-term survivors.

It appears that the Lauren classification describes gastric carcinoma subtypes with uniform structural and pathogenetic properties.[20] This histologic distinction may be characterized by tumors with different biologic behavior that need further detailing at the molecular level. The impact of these behavioral differences does not appear to outweigh lymphatic involvement, but as we have shown, once long-term survival has been achieved the Lauren histologic type becomes important.

However, for patients who survive without evidence of disease at the 5-year point, there is a very low likelihood (6%) of further recurrence. Although there was a trend for Lauren diffuse histologic type to predict worse outcome in this group, none of the prognostic factors evaluated in this study significantly predicted further disease-free or disease-specific survival.

These findings support the observation that diffuse and intestinal-type tumors differ in their biologic behavior. The clinical impact of these differences appears to be masked by the profound influence of T and N stage early in the progression of this disease.

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Antral Compensation After Proximal Gastric Vagotomy

Mehran Anvari, Jenny Myers, Charles Malbert, Michael Horowitz, John Dent, Glyn Jamieson

Proximal gastric vagotomy (PGV) has little impact on the normal pattern of solid gastric emptying, despite denervation of the proximal two thirds of the stomach and loss of the proximal gastric pump. In four healthy volunteers and four patients with PGV, we investigated the possible compensatory mechanisms that may come into play after proximal denervation of the stomach. We measured antropyloroduodenal motility with a 10-lumen sleeve/side-hole catheter for 180 minutes after ingestion of a dual-isotope radiolabeled mixed liquid/solid meal. Patients with PGV exhibited faster liquid emptying, but the rate of solid emptying was similar to that in healthy volunteers. The frequency of propagated antropyloric pressure waves was similar between the two groups, but patients with PGV exhibited less isolated pressure waves in the proximal antrum. The amplitude and duration of pressure waves recorded in the distal antrum were significantly increased in the PGV patients as compared to healthy volunteers. Although the pattern of propagated antral contractions and solid gastric emptying remains unchanged after PGV, there is an increase in the amplitude and duration of distal antral contractions, which may compensate for loss of proximal gastric pumping mechanisms. (J GASTROINTEST SURG 2000;4:526-530.)

KEY WORDS: Proximal gastric vagotomy, antral motility, gastric emptying

Complete or partial denervation of the stomach is associated with alteration in gastric motility and emptying[1] and is often followed by undesirable symptoms and significant long-term morbidity in 25% of patients.[2] These include symptoms such as early satiety, bloating, dumping, and diarrhea, which have attributed to the alteration in the normal control of gastric motility and emptying subsequent to the denervation and the drainage procedures that are often added to facilitate gastric emptying.[3-6] Proximal gastric vagotomy (PGV), in which the innervation of the distal antrum and the pylorus is preserved, is associated with a near-normal pattern of solid gastric emptying.[1,7,8] Although this procedure leads to vagal denervation of the fundus and body, including the gastric pacemaker region, it does not significantly alter the cyclical generation of pacesetter potentials by the pacemaker and their distal propagation.[9,10] It does, however, impair receptive relaxation and accommodation of the fundus[9,11,12] and leads to more rapid liquid emptying,[8,9,13] but hyperosmolar and nutrient liquids continue to empty slower than non-nutrient isotonic solutions.[9]

PGV is, however, associated with near-normal patterns of trituration,[9] sieving,[14] and solid emptying.[1,7,8] This is believed to be due to the importance of the antrum and the pylorus, in regulation of solid emptying,[15] whose vagal innervation is preserved. However, the possible role of compensatory motor mechanisms that can overcome the loss of pumping function of the corpus and proximal antrum, in maintaining the normal pattern of solid gastric emptying after proximal gastric vagotomy, is unknown. Increased fundic tone due to the loss of vagal innervation may be one mechanism. Another could be a change in antral motor function.

In this study, we investigated whether patients after PGV have changes in distal antral motility, which could compensate for the loss of proximal motor mechanisms.

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METHODS

Subjects

Studies were carried out in four healthy volunteers (2 females and 2 males) between the ages of 21 and 30 years (mean age 24 years) and four patients (2 females and 2 males) between the ages of 20 and 67 years (mean age 42 years) who had undergone PGV for ulcer disease at least 1 year prior to study (mean 3.8 years, range 2 to 6 years).

Study Protocol

The volunteers were fasted overnight and were requested not to drink alcohol the night before the study. Antropyloroduodenal motility was measured with a 10-lumen sleeve/side-hole catheter, for 3 hours, during emptying of 100 g of a ^{99m}technetium (Tc)-labeled beef burger and 150 ml ^{113m}indium-labeled dextrose (10%) drink in a sitting position.

Recordings and Analysis

Antropyloroduodenal Manometry. A 10-lumen sleeve/side-hole manometric assembly incorporating a 4.5 cm sleeve sensor was passed transnasally and positioned with the sleeve astride the pylorus.[16] The pyloric pressures were measured with the sleeve sensor, and its position was continuously verified by measurement of the transmucosal potential difference (TMPD) by side holes at either end of the sleeve.[17] These side holes, together with side holes at 1.5 cm intervals along the sleeve, in the distal antrum 1.5, 3, 4.5, and 6 cm proximal to the sleeve, and in the proximal duodenum 3 cm distal to the sleeve, allowed the extent of a pressure wave in relation to the pylorus to be determined.[17]

The data from all studies were recorded on polygraph paper. In addition, the output of the polygraphs was digitized with an A/D card at a frequency of 10 Hz (NB Mio 16, National Instruments Corp., Austin, Texas), and the signals were stored on disk in a Macintosh II ci, Apple Computer, with a purpose-developed program based on a Labview software program (National Instruments Corp.).

Recordings were only analyzed when transmucosal potential difference criteria confirmed that the manometric assembly was correctly positioned across the pylorus.[17,18] Any resolvable pressure increase of less than 25 seconds in duration, which was not attributable to respiration, straining, or changes in posture, was scored. These appeared as identical pressure rises in all antral, pyloric, and duodenal channels.

The computer program was used to analyze the amplitude and duration of pressure waves observed in the two antral side holes (2 and 6 cm proximal to the sleeve) and the sleeve sensor (pyloric). We analyzed all pressure waves recorded by these channels irrespective of whether they were isolated pressure waves or part of a sequence, that is, an antropyloric or pyloroduodenal pressure wave.

Patterns of Pressure Waves. Pressure waves seen in more than one side hole or in the sleeve were scored according to previously published criteria[19] as antropyloric pressure waves, pyloroduodenal pressure waves, or isolated pyloric pressure waves.

Dual-Isotope Mixed Liquid/Solid Gastric Emptying. The test meal used was a 100 g cooked ground beef labeled with 40 MBq (1 mCi) ^{99m}Tc-sulphur colloid and 150 ml of 10% dextrose labeled with 20 to 24 MBq ^{113m}In.[20,21] The subjects were asked to eat the beef burger over 5 minutes and were then asked to drink the labeled dextrose over 30 seconds.

The subjects were seated on a stool with their arms on a table.[21] Data were collected continuously by a gamma camera (Nuclear Chicago Pho-Gamma 111 Hp, Digital Equipment Corporation, Chicago, Ill.), frames being formed for 30-second periods for the first 30 minutes and then every 3 minutes for another 150 minutes. At the conclusion of the study, a lateral image was again obtained, as previously described, and was used to determine the necessary corrections. The time interval for 50% of the liquid (liquid T_{50}) and solid meal (solid T_{50}) to leave the whole stomach was measured.

Statistical Analysis

Parametric data are presented as mean ± standard error of the mean. A one-way analysis of variance was used to compare the parametric data between the two groups. Nonparametric data are given as median and interquartile ranges. A Mann-Whitney U test was used to compare nonparametric data between the two groups.

RESULTS

Gastric Emptying

The patients with PGV demonstrated a more rapid emptying of liquids ($P < 0.05$) than the healthy control subjects but solid emptying was similar in the two groups (Fig. 1).

Frequency of Pressure Waves

In the PGV patients, the number of antral pressure waves in the proximal and distal antrum was significantly ($P < 0.05$) less than that in the control group (Table I). The frequency of pyloric pressure waves was higher ($P < 0.01$) than proximal or distal antral pres-

Table I. Frequency, amplitude, and duration of proximal antral, distal antral, and pyloric pressure waves over the 180-minute duration of study

	Group	Proximal antrum	Distal antrum	Pylorus
Frequency (per hour)	Control	1.9 ± 1.0*	16.8 ± 2.6*	39.6 ± 3.4
	PGV	0.6 ± 0.4*†	9.0 ± 1.5*†	43.8 ± 4.0
Amplitude (mm Hg)	Control	30.1 ± 3.0*	19.2 ± 1.1	13.3 ± 1.7
	PGV	22.2 ± 1.5*†	24.8 ± 3.1*†	14.7 ± 0.7
Duration (sec)	Control	2.0 ± 0.3*	1.0 ± 0.02*	2.7 ± 0.13
	PGV	1.1 ± 0.06*†	2.4 ± 0.3†	2.8 ± 0.2

PGV = proximal gastric vagotomy.
Values shown are mean ± standard error.
*P <0.05 compared with pylorus.
†P <0.05 compared to control value.

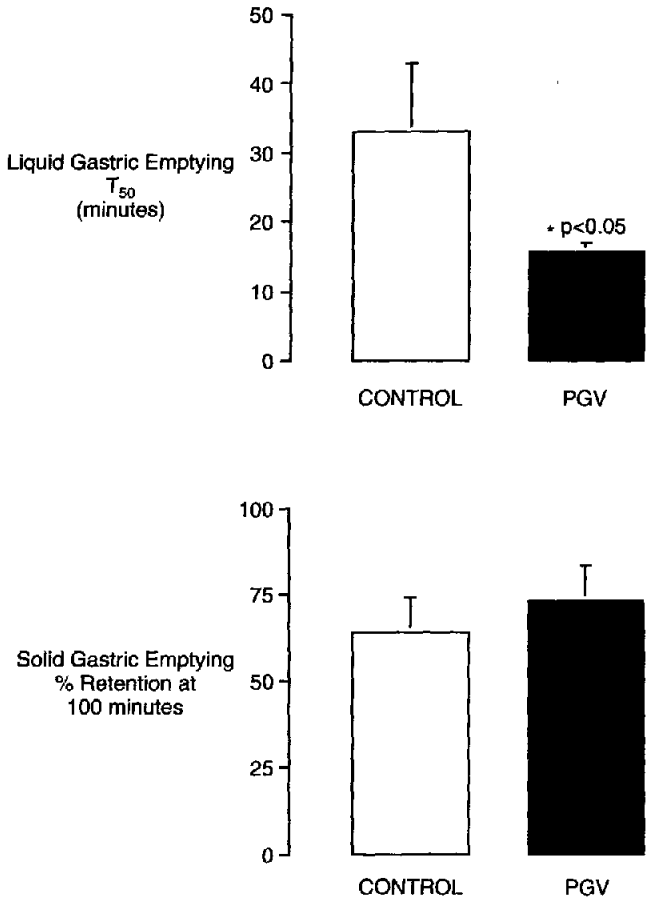

Fig. 1. Liquid and solid emptying in patients with proximal gastric vagotomy (PGV), and control group. Liquid T_{50} = time for 50% of liquid meal to empty from the stomach.

sure waves in both groups, but was not significantly different between the two groups.

Amplitude of Pressure Waves

In the PGV group, in comparison to the control group, the amplitude of pressure waves was significantly (P <0.05) *lower* in the proximal antrum but significantly (P <0.05) *higher* in the distal antrum (see Table I). The mean amplitude of the pressure waves recorded in the proximal antrum was higher than the mean amplitude of pyloric pressure waves recorded by the sleeve in both groups.

Duration of Pressure Waves

The duration of pressure waves followed the same trend as the amplitude (see Table I), with the PGV

Table II. Number of pressure waves per minute for 180-minute duration of study in the two groups

Group	IPPW per min	APPW per min	PDPW per min
Control	0.47 (0.18-0.68)	0.79 (0.47-1.01)	0.15 (0.08-0.64)
PGV	0.34 (0.21-0.64)	0.78 (0.29-0.76)	0.33 (0.11-0.59)

IPPW = isolated pyloric pressure waves; APPW = antropyloric pressure waves; PDPW = pyloroduodenal pressure waves; PGV = proximal gastric vagotomy.
Values shown are median (interquartile ranges) for 180 minutes.

patients exhibiting a shorter duration proximal antral and longer duration distal antral pressure waves, in comparison to the control group. The duration of pyloric (sleeve-detected) pressure waves was longer than the duration of antral pressure waves in either group.

Patterns of Pressure Waves

There was no significant difference in the frequency of antropyloric, pyloroduodenal, or isolated pyloric pressure waves between the two groups (Table II).

DISCUSSION

This study is the first to demonstrate that after PGV, compensatory contractile changes occur in the antrum, which is the only segment of the stomach with intact innervation. These changes may contribute, in part, to the near-normal patterns of solid emptying observed in these patients.

The accelerated liquid emptying observed is consistent with earlier results.[8,9,13] This is believed to be the result of increased fundic tone after loss of vagal innervation to the proximal stomach.[22] The solid gastric emptying was, however, maintained within normal limits. This was associated with normal patterns of propagated contractions in the distal stomach,[23] which have been previously shown to be important in expulsion of food particles through the pyloric ring.[19,24]

Previous studies have shown that the speed of gastric emptying of solids and liquids may diminish with age.[25,26] These studies compared healthy adults between the ages of 20 and 30 with adults over the age of 60 years. Even with such a significant age difference, the change in the rate of gastric emptying was small and not clinically significant.[25] In our study there was a mean age difference of 18 years between the two groups. There is no evidence to suggest that this would have had a bearing on our results. If anything the older surgical group showed a more rapid liquid emptying than the younger healthy control group, and there was no difference in the solid emptying rate between the two groups.

There were more frequent pressure waves recorded by the sleeve than by any of the antral side holes. This is not surprising as the sleeve would record any increase in pressure related to antipyloric pressure waves, isolated pyloric pressure waves, or pyloroduodenal pressure waves.[17,19] The lower amplitude of pressure waves recorded by the sleeve in comparison to the proximal antral side hole may be related to the following two factors: (1) a difference between the sleeve and side holes in recording the pressure wave amplitude and (2) the fact that the number of pressure waves recorded by the sleeve was more than the proximal antral side hole by a factor of 20 to 40 times; thus the few pressure waves recorded in the proximal antrum may have had to have been of considerable amplitude to achieve lumen occlusion and be recorded. It is widely recognized that pyloric pressure waves tend to be of longer duration than antral or duodenal pressure waves.[17] The mechanical significance of this is not yet known, but it is postulated that this will allow for a more effective arrest of transpyloric flow when the pylorus contracts.

The decrease in amplitude and duration of proximal antral pressure waves in comparison to control values supports the hypothesis that proximal vagal denervation disturbs proximal antral motility in PGV patients. The reduced number of isolated distal antral pressure waves in the PGV group was, however, unexpected and may suggest that distal antral motility is also disturbed after this procedure, but the exact clinical implications of these pressure waves are still poorly understood.

The effect of an increase in amplitude and duration of distal antral contractions is not known. It is possible that such changes may be compensatory, and associated with more effective "pumping" of ingesta across the pylorus by the distal antrum.

In this study we did not measure fundic tone, as it is technically not possible to conduct concurrent measurements of fundic tone with gastric emptying. Changes in fundic tone influence gastric distribution of solids and are expected to alter gastric emptying. It has been demonstrated that the fundic tone is chronically raised after gastric vagotomy,[9] and it is therefore possible that this change may also contribute to compensate the loss of phasic activity in the body of the stomach during solid gastric emptying.

CONCLUSION

Despite the relatively small number of subjects studied, our results suggest that after PGV, the pattern of propagated antral contractions remains unchanged, and there are changes in the amplitude and

duration of distal antral contractions that may partially compensate for the loss of proximal phasic contractions of the stomach. These factors help to maintain near-normal patterns of solid gastric emptying. In the era of *Helicobacter pylori* eradication, the frequency of gastric denervation procedures for peptic ulcer disease has been reduced significantly. However, in a small group of *H. pylori*–negative patients with chronic peptic ulcers, surgery may be necessary. The evidence presented here and in other studies confirms that PGV, in which innervation to the distal antrum is preserved, should be the operation of choice. This will ensure that the patient will enjoy a near-normal pattern of solid gastric emptying and experience fewer unwanted side effects.[3] This procedure can be performed by both open and laparoscopic techniques.

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Luminal Osmolarity Downregulates Gene Expression of Na^+/H^+ Exchanger (NHE3) in Rat Colon Mucosa

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Water-coupled Na^+ absorption in the colon is mediated principally by Na^+/H^+ exchange (isoforms NHE2 and NHE3). To determine whether luminal ion composition or osmolarity influences NHE expression in colon mucosa, two groups (n = 6 in each) of adult male Sprague-Dawley rats underwent sham laparotomy or loop ileostomy. In these studies, diversion did not markedly alter mRNA levels for NHE2, NHE3, or Na^+/K^+, at 8 or 21 days, indicating that loss of luminal volume does not alter NHE gene expression. To evaluate the effects of specific luminal components, we infused equal volumes of half-normal (154 mOsm) or iso-osmolar (308 mOsm) solutions of saline and mannitol into the diverted colon. All solutions elicited significant (45% to 60%; $P < 0.05$) decreases in mRNA levels for NHE3, with iso-osmolar mannitol eliciting the greatest changes. Decreases in NHE2 and Na^+/K^+ mRNA levels were observed following these infusions but were not as marked as the changes for NHE3. These findings suggest that (1) loss of luminal Na^+ is not, in itself, a signal that regulates NHE expression and (2) infusion of any solute, including Na^+ itself, provides a signal to downregulate expression of NHE3 in colon mucosa. (J GASTROINTEST SURG 2000;4:531-535.)

KEY WORDS: Na^+/H^+ exchanger, ileostomy, colon mucosa, ion transport, osmolarity

The role of Na^+/H^+ exchangers in epithelial sodium absorption has been extensively studied. In their normal mode of operation, these proteins mediate the exchange of intracellular H^+ for extracellular Na^+ in a one-to-one ratio. Na^+/H^+ exchangers function to maintain intracellular pH and aid in cell volume regulation and cell proliferation. To date, five different isoforms of the NHE have been found.[1-11] NHE1 has been found, with few exceptions, in all cells, and has been localized to the basolateral membrane of epithelial cells.[3]

In contrast, NHE2 and NHE3 have been localized to the apical membranes of diverse epithelial cells. With the use of Northern blot analysis, NHE2 has been found in the kidney medulla and cortex, liver, stomach, duodenum, ileum, jejunum, colon, and adrenal gland.[4,5,12] The functional role of NHE2 in the intestine has yet to be determined. With respect to the NHE3 isoform, high levels of mRNA have been found in the kidney medulla and cortex, stomach, jejunum, ileum, and colon.[7,9,12] Functional studies using transgenic knockout models have shown serious absorptive defects in mice lacking the NHE3 exchanger.[13] It has also been revealed that aldosterone, secreted during periods of salt deprivation, increases Na^+ uptake by increasing cellular NHE3 expression in the colon.[14] From these and other studies, it is believed that NHE3 is the dominant Na^+/H^+ exchanger in the intestine and colon.[6-9,12,15,16]

NHE4 has been localized to the basolateral membranes of epithelial cells in the stomach and kidney, as well as nonpolar cells in the brain, uterus, and skeletal muscle.[3] Its exact function has not yet been determined. With the exception of NHE4, all Na^+/H^+ exchangers are inhibited by the diuretic amiloride and are selectively inhibited by several new amiloride derivatives.[17]

Despite considerable investigation of the neurohumoral and cellular mechanisms that regulate expression and function of NHE isoforms, there is lit-

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tle information regarding the role of the luminal environment in regulating their expression. Thus this study was undertaken (1) to determine whether the presence or absence of the fecal stream influences levels of mRNA encoding the proabsorptive NHE2 and NHE3 isoforms in the rat colon and (2) to explore the effects of different luminal components on these levels.

MATERIAL AND METHODS

Animal Model

The animal protocol was approved by the Harvard Medical Area Standing Committee on Animals at Harvard Medical School, Boston, Massachusetts. Twenty-four adult male Sprague-Dawley rats (450 to 500 g) were used for the study. All animals underwent a loop ileostomy surgical procedure as previously described.[18] Briefly, animals were fasted for 24 hours before and after the operation. All groups had free access to liquids, water and 0.25 normal saline solution, and had similar preoperative and postoperative care. Animals were fed *ad libitum* 24 hours after surgery. Animals were anesthetized using a combination of ketamine (40 mg/kg) and xylazine (5 mg/kg) injected intramuscularly. A warming pad was used to maintain body temperature during the procedure. Animal weights were monitored daily. A daily washing was performed to keep the skin free of the caustic luminal effluent.

All operations were performed under sterile conditions. Once the animals were anesthetized, their abdomens were shaved and washed with 95% ethanol. Loop ileostomies were performed in all animals using the following procedure. A 4 cm incision was made in the abdominal wall to gain access to the gut. A 4 to 6 mm circular stoma was made in the abdominal musculature and skin approximately 1.0 cm to the right of the midline incision. A loop of distal ileum 5 cm proximal to the ileocecal valve was passed through the stoma. The ileum was opened along the antimesenteric border for a distance of 1.0 cm. The free edges of the ileum were sutured to the abdominal musculature and skin with interrupted 4-0 silk sutures. Afferent and efferent ileal limbs were examined for patency by infusion of warm saline solution through a 22-gauge intravenous catheter. Prior to closure, 3.0 ml of saline solution was placed into the abdominal cavity to compensate for dehydration during the procedure. Midline incisions were closed with a running 4-0 Vicryl suture. The skin edges of the midline incision were closed with a running 4-0 nylon monofilament suture. A group of sham laparotomy animals, receiving no diversion, was created concurrently with the ileostomy animals for preliminary comparison.

Animals were allowed 48 hours to recover from surgery. They were then divided into four groups (n = 6 in each). Infusions were made via pediatric feeding tubes inserted through the stoma approximately 3 cm into the distal (defunctionalized) ileum. The defunctionalized segment was identified as the stoma, which had no output of fecal material. One group, designated as the control group, had feeding tubes inserted but received no fluid infusion. The remaining three groups received infusions of either normal saline (308 mOsm), half-normal saline (154 mOsm), or iso-osmotic mannitol (308 mOsm). Animals were temporarily rendered unconscious using a carbon dioxide chamber. Three to 4 ml of infusate was injected into the lumen. Infusions continued twice daily for 5 days. Animals were killed on the sixth day of infusion, 8 days after surgery, 18 hours after the final infusion.

Mucosal Harvesting and RNA Isolation

Animals were anesthetized again using a mixture of ketamine (40 mg/kg) and xylazine (5 mg/kg). Colonic tissue was harvested and mucosa was quickly separated from the underlying muscularis by sharp dissection. The mucosal scrapings were snap-frozen and stored in liquid nitrogen to be processed later. Total RNA was isolated from the mucosal scrapings using a commercially available RNA isolation and purification kit (RNeasy, Qiagen, Inc., Valencia, Calif.). Total RNA samples were stored at −80° C until use.

Northern Blot Analysis

Total RNA was run on a 1% agarose-formaldehyde gel. RNA was transferred to a nylon membrane (Stratagene, La Jolla, Calif.) by capillary action. The RNA was linked to the membrane using a UV-crosslinker (Stratalinker 2400, Stratagene). Membranes were washed at 50° C in a NaCl, sodium dodecyl sulfate (SDS) solution for 30 minutes, then prehybridized for 2 hours at 42° C in a formamide, dextran sulfate, and NaCl solution. cDNA probes were labeled with [α-^{32}P]dCTP and a random primer labeling kit (Oligolabeling kit, Amersham Pharmacia, Piscataway, N.J.). Membranes were hybridized overnight with 10^6 counts × min^{-1} × ml^{-1} probes for NHE2, NHE3, Na^+/K^+, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The membranes were washed twice at low stringency (room temperature, 2× standard saline citrate [SSC] + 0.1% SDS) for 20 minutes and once at higher stringency (55° C, 0.2× SSC + 0.1% SDS). Membranes were then exposed to autoradiography film (Kodak X-Omat Blue XB-1, Eastman Kodak Co., Rochester,

N.Y.) at −80° C. Film was developed at various times. Quantification of relative mRNA abundance was performed via scanning densitometry (HP Scanjet IIc; NIH Image 1.61, National Institutes of Health, Bethesda, Md.). Membranes were stripped before being reprobed. All blots were normalized to GAPDH, a housekeeping enzyme, to account for any variance in gel loading.

Statistical Analysis

Data were recorded and analyzed using a statistical software package (Excel, Microsoft Corp., Redmond, Wash.). Results are reported as mean ± standard deviation. A paired *t* test was performed between two data groups to determine statistical significance. When more than two groups were compared, a one-way analysis of variance was performed. Statistical significance was taken at a *P* value of <0.05.

RESULTS

Gross Appearance of Diverted Segments

As previously reported, the body weight of the ileostomy animals decreased by approximately 30% (from 445 ± 14 g to 336 ± 17 g; $P < 0.000001$), whereas weight loss in the animals undergoing infusion was approximately 20% (saline 503 ± 21 g to 410 ± 24 g, $P < 0.001$; half-saline 476 ± 34 g to 383 ± 32 g, $P < 0.001$; and mannitol 493 ± 56 g to 383 ± 53 g, $P < 0.01$). All animals maintained normal behavior regardless of their experimental group. External appearance of the stoma was similar in all groups. As also reported earlier,[18] the stomachs of the ileostomy animals appeared distended and full of solid food. This was also seen in the infusion animals.

The lumen of the bowel distal to the stoma in the ileostomy animals appeared to be contracted in contrast to the animals not undergoing diversion. The distal bowel of the infusion animals, regardless of infusate, appeared more dilated than that of the animals undergoing ileostomy alone. The cecum of the animals in the ileostomy, saline, and half-saline groups appeared contracted. However, the cecum of the animals undergoing infusion consistently contained between 1 and 2 ml of white cloudy fluid, even though the last infusions were 18 hours prior to sacrifice.

Transporter mRNA Levels

Eight-day ileostomy did not markedly alter the levels of NHE3 (from 100 ± 6 to 100 ± 18), NHE2 (from 100 ± 10 to 95 ± 21), or Na^+/K^+ ATPase (from 100 ± 6 to 128 ± 18; $P < 0.05$). Also, 21-day ileostomy did not markedly alter the levels of mRNA encoding NHE3 (from 100 ± 14 to 96 ± 29), NHE2 (from 100 ± 9 to 83 ± 18; $P < 0.05$), or Na^+/K^+ ATPase (from 100 ± 10 to 98 ± 26).

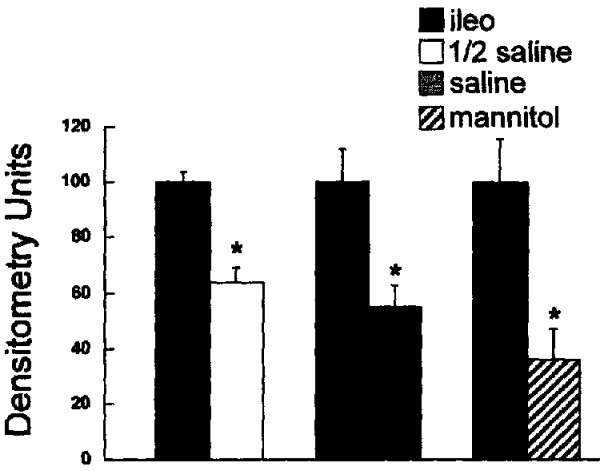

Fig. 1. Relative levels of mRNA encoding NHE3. Levels of mRNA were standardized to GAPDH and normalized to an arbitrary value of 100%. Results are expressed as mean ± standard error of the mean. * = $P < 0.001$.

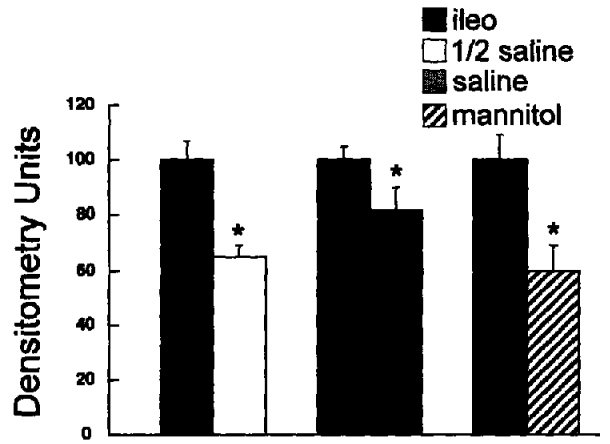

Fig. 2. Relative levels of mRNA encoding NHE2. Levels of mRNA were standardized to GAPDH and normalized to an arbitrary value of 100%. Results are expressed as mean ± standard error of the mean. * = $P < 0.001$.

As shown in Fig. 1, the levels of mRNA encoding the Na^+/H^+ exchanger isoform NHE3 decreased in the middle colon of infusion animals in comparison to the midcolon of ileostomy control animals. The greatest decrease was in the mannitol-infused colons (from 100 ± 16 to 36 ± 11; $P < 0.001$), followed by saline infusion (100 ± 12 to 55 ± 8; $P < 0.001$) and half-saline infusion (100 ± 4 to 64 ± 5; $P < 0.001$).

The levels of mRNA encoding NHE2 are shown in Fig. 2. Animals receiving mannitol infusions had the greatest decrease in levels of mRNA for NHE2 (from 100 ± 9 to 60 ± 9; $P < 0.05$) followed by half-

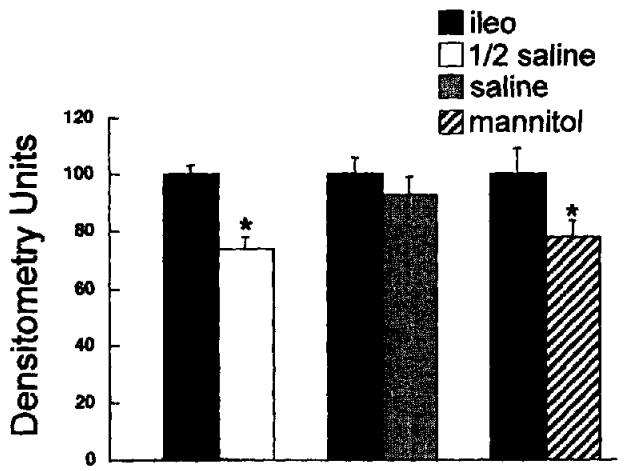

Fig. 3. Relative levels of mRNA encoding Na^+/K^+ ATPase. Levels of mRNA were standardized to GAPDH and normalized to an arbitrary value of 100%. Results are expressed as mean ± standard error of the mean. * = $P < 0.001$.

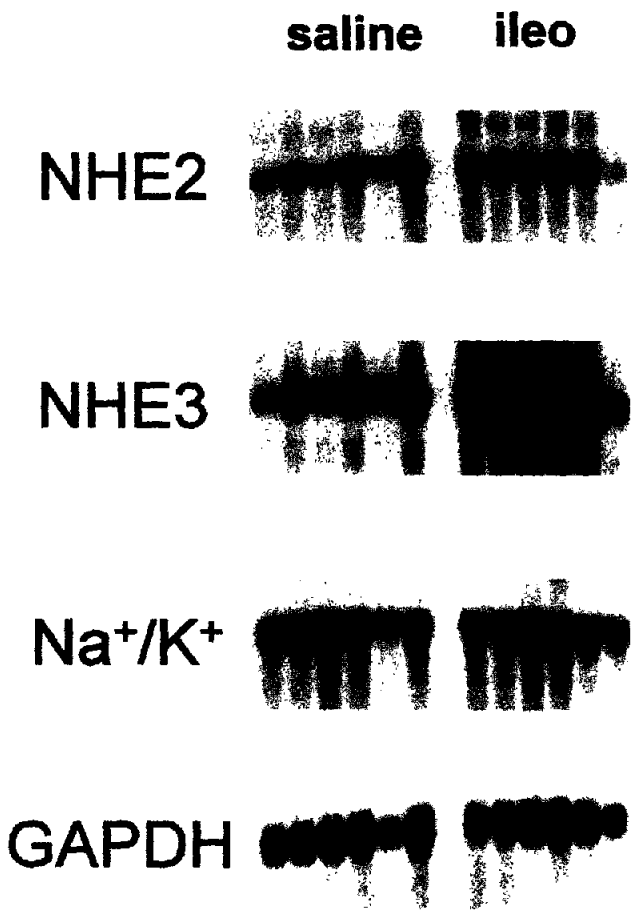

Fig. 4. Northern blot analysis of NHE2, NHE3, Na^+/K^+ ATPase, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) message levels in middle colon of animals with saline infusions into defunctionalized stoma. Hybridization conditions and probe are described in Material and Methods.

saline (100 ± 7 to 65 ± 4; $P < 0.05$) and normal saline infusions (100 ± 5 to 82 ± 8; $P < 0.05$).

The levels of mRNA encoding Na^+/K^+ ATPase are shown in Fig. 3. Animals receiving infusions of mannitol and half-saline experienced a slight decrease (mannitol from 100 ± 9 to 78 ± 6, $P < 0.05$; half-saline 100 ± 3 to 74 ± 4, $P < 0.05$), whereas saline infusion alone did not significantly alter the levels of Na^+/K^+ mRNA (100 ± 6 to 93 ± 6). A representative Northern blot is shown in Fig. 4.

DISCUSSION

The role of Na^+/H^+ exchangers in cellular ion homeostasis has been widely studied in recent years. However, little is known about the role of individual luminal constituents in regulating expression of mRNA encoding these Na^+/H^+ exchangers. In this study we created a loop ileostomy model to examine the effects of ileal diversion on levels of mRNA in the colon encoding several Na^+ transporters, namely, NHE2, NHE3, and Na^+/K^+ ATPase. Surprisingly there were no significant alterations in the mRNA levels in the diverted mucosa, indicating that total loss of luminal content, per se, does not alter expression of these transporters.

To determine whether individual luminal constituents might influence NHE expression under some conditions, three different solutions were infused into the distal (defunctionalized) limb of the intestine. The levels of mRNA for the Na^+/H^+ exchanger NHE3 were found to decrease quite markedly in all infusions. The NHE3 mRNA levels in the mannitol- and normal saline–infused animals decreased the most, followed by the half-normal saline–infused animals. The trend of decreasing levels of mRNA followed that of the relative osmotic strength of the solutions (mannitol ≥ saline > half-normal saline). These findings suggest that luminal osmolarity, rather than Na^+ content specifically, may play a dominant role in the regulation of the Na^+/H^+ exchangers at the transcriptional level.

Accompanying luminal constituent changes, we observed large changes in the mRNA levels of NHE3. We then examined the response of two different epithelial Na^+ transporters. The levels of mRNA encoding NHE2 and Na^+/K^+ ATPase were not altered as much as the mRNA level encoding the NHE3. The absence of marked changes in the NHE2 and Na^+/K^+ ATPase supports the idea that NHE3 is the dominant Na^+/H^+ exchanger involved in adaptation to a changing luminal environment in the colon.[6-9,12,16]

In summary, we have shown that luminal diversion alone, via a loop ileostomy, does not alter mRNA lev-

els for NHE2, NHE3, or Na^+/K^+ ATPase in the rat colon. We further demonstrated that luminal osmolarity affects the levels of mRNA encoding NHE3 and, to a lesser extent, NHE2 and Na^+/K^+ ATPase. The animal model described here may be a useful means by which to study the effects of ileostomy on the colon, particularly in determining the effects of individual luminal constituents on the expression of different colonic ion transporters.

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Matrix Metalloproteinase Inhibition Protects Hepatic Integrity in Hemorrhagic Shock

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Hemorrhagic shock increases cytokines, such as tumor necrosis factor-alpha (TNF-α), and interleukin-6 (IL-6), and compromises hepatic function and integrity. The production of TNF-α involves a cascade reaction regulated by the enzyme TNF-α convertase. The purpose of this study was to examine the effects of matrix metalloproteinase inhibitor (MMPI) (British Biotech 1101) in vivo on hepatic integrity in a rat model of hemorrhagic shock. Sprague-Dawley rats (n = 26) were divided as follows: hemorrhagic shock (group 1) and hemorrhagic shock plus MMPI (group 2). TNF-α, IL-6, and hepatic membrane potentials (Em) were obtained. The administration of MMPI significantly decreased TNF-α levels ($P < 0.001$) and stabilized the membrane potential at −30 mV as compared to the depolarized membrane potential at −20 mV for hemorrhagic shock without MMPI. IL-6 levels were not affected by the MMPI. This study demonstrates that MMPI decreases TNF-α levels and protects hepatic integrity in hemorrhagic shock, as evidenced by the stabilization of the membrane potential, independent of the mean arterial pressure. The hepatic protection is closely related to the decrease in TNF-α levels seen in the portal circulation. (J GASTROINTEST SURG 2000;4:536-541.)

KEY WORDS: Shock, hemorrhage, liver, cytokines

The use of early fluid resuscitation and expedient health care delivery has resulted in an improvement in the immediate survival of trauma patients. The improved immediate survival has allowed for severely injured patients to succumb to the systemic inflammatory response and develop multiple organ failure (MOF).[1,2] It is thus not surprising that MOF is an important cause of death in critically ill patients, despite advances in critical care.[3,4]

The organs most commonly involved in the systemic inflammatory response are the bowel-liver[5] and liver-lung axis.[6] The liver plays an important role in metabolism, immunomodulation, and acute-phase response after shock injury. The hepatic parenchyma is subject to hypoperfusion and watershed infarct leading to hepatic impairment. Not surprising, ischemia/reperfusion injury of the liver remains a limiting factor in liver transplantation.[7,8]

The exact underlying cellular mechanism leading to MOF has remained elusive. Nonetheless, significant involvement of the systemic inflammatory response that precedes MOF involves the participation of monocytes, macrophages, neutrophils, and the endothelium. The activation of these cells leads to the expression of cytotoxic oxygen free radicals, cytokines (tumor necrosis factor-alpha [TNF-α] and interleukin-6 [IL-6]), matrix metalloproteases, and the complement system.[9-15]

The cytokines, TNF-α and IL-6, have been seen to play an important role in ischemia/reperfusion tissue injury.[15-20] Hemorrhagic shock increases cytokines, such as tumor necrosis factor (TNF-α), and compromises hepatic function and integrity.[16] The production of TNF-α involves a cascade reaction regulated by the matrix metalloproteinase enzyme TNF-α convertase.[15,21-23] The purpose of this study was to examine the effects of matrix metalloproteinase inhibitor (MMPI) (British Biotech 1101) in vivo on hepatic integrity in a hemorrhagic shock model.

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MATERIAL AND METHODS

Twenty-six male Sprague-Dawley rats (Benton and Kingman, Fremont, Calif.) weighing 200 to 270 grams were subjected to controlled hemorrhagic shock. Animals were kept under controlled temperature, humidity, and lighting, with free access to standard laboratory chow and water ad libitum, in accordance with animal care guidelines.

Surgical Procedure

Sodium pentobarbital (50 mg/kg) was used to anesthetize animals via an intraperitoneal injection, and animals were maintained on small doses (5 mg) of pentobarbital throughout the experiment as needed. Endotracheal intubation through a midline tracheostomy approach was performed. Animals were allowed to breathe spontaneously.

The right carotid artery was isolated and cannulated with a polyethylene (PE-50) catheter filled with 10 units/ml heparin. The catheter was used for the controlled hemorrhage, for blood sampling, and for monitoring mean arterial pressure. The catheter was connected to a physiologic pressure transducer (model 1280, Hewlett-Packard) attached to an oscillographic recording system (model 7702B, Hewlett-Packard) via a three-way stopcock for monitoring purposes.

A minilaparotomy was performed and the liver parenchyma exposed for hepatic resting membrane potentials utilizing a modified Ling-Gerard microelectrode.[23,24] The animals were kept on a temperature-controlled surgical board (37° ± 1° C) for the duration of the experiment.

Experimental Model

Controlled hemorrhage was produced by removing 35% of the total blood volume over 10 to 15 minutes following systemic heparinization (250 units/kg). The systolic blood pressure was maintained at 30 to 40 mm Hg for the duration of the 180-minute experiment, by either removing additional blood or adding small aliquots of shed blood. After hemorrhage, animals were randomly divided into two groups: Group 1 was subjected to hemorrhagic shock but received no treatment, whereas group 2 was given 2.5 mg/kg of MMPI (British Biotech 1101) into the peritoneum 15 minutes after the induction of hemorrhagic shock. Measurements of mean arterial pressure, resting membrane potential (Em), and cytokine levels were obtained at baseline (−15 minutes) and 90 and 180 minutes after hemorrhage. Blood samples (0.5 ml aliquots) were collected from the carotid artery and portal vein. All samples were centrifuged and stored at −70° C until TNF-α and IL-6 assays were performed (Biosource International Inc., Camarillo, Calif.).

STATISTICS

Student's t test was performed and statistical significance determined at $P < 0.05$.

RESULTS

Hemorrhagic shock (HS) resulted in a decrease in mean arterial pressure from 121 ± 11 mm Hg and 117 ± 16 mm Hg to 15 ± 7 and 20 ± 7 mm Hg for the HS group and the HS + MMPI group, respectively (Fig. 1). The total shed blood was 48% of the

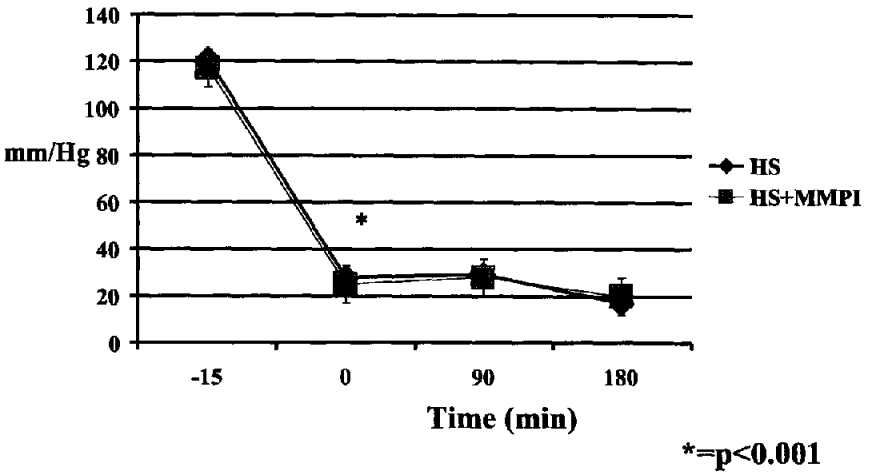

Fig. 1. Mean arterial blood pressure at baseline (−15 minutes), during hemorrhagic shock (*HS;* time 0), and at 90 and 180 minutes. After HS, there is a significant and persistent decrease in mean arterial pressure ($P < 0.001$).

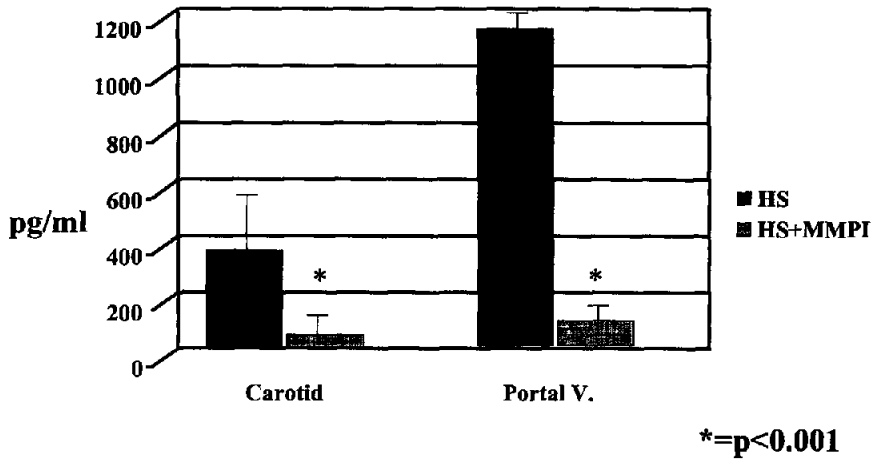

Fig. 2. TNF-α levels following hemorrhagic shock. MMPI significantly decreased TNF-α levels ($P < 0.001$).

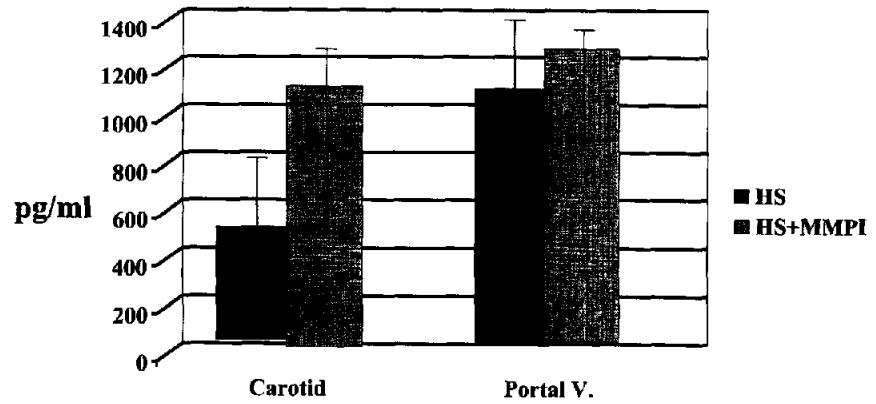

Fig. 3. IL-6 levels following hemorrhagic shock. MMPI did not attenuate IL-6 levels.

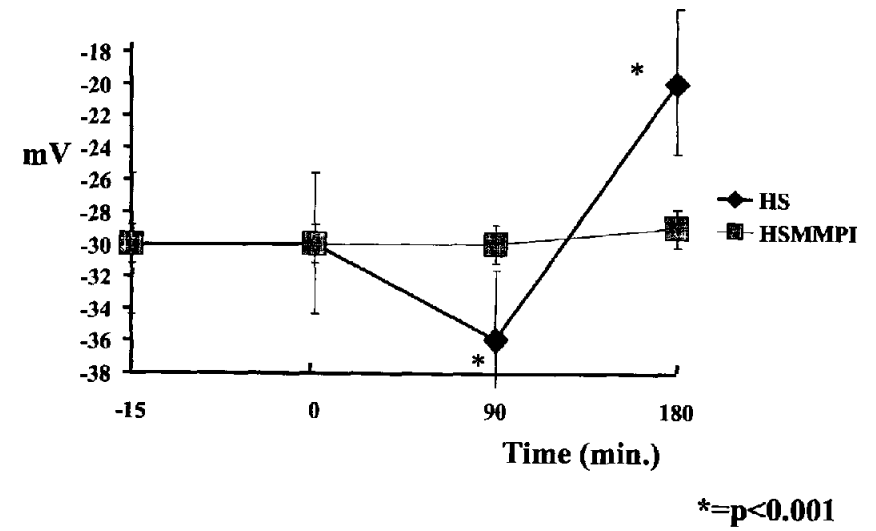

Fig. 4. Hepatic resting membrane potential *(Em)* in millivolts *(mV)*. Following hemorrhagic shock, MMPI maintained a stable hepatic resting membrane potential. The HS group developed hyperpolarization at 90 minutes followed by depolarization at 180 minutes, which is indicative of cellular damage ($P < 0.001$).

total blood volume for both the HS and HS + MMPI groups.

TNF-α levels from the carotid artery and portal vein were obtained at 90 minutes. The carotid TNF-α level was 338 ± 500 pg/ml for the HS group, which was in sharp contrast to the MMPI-treated group with a level of 43 ± 30 pg/ml ($P < 0.0001$) (Fig. 2).

IL-6 levels were obtained at 180 minutes also from the carotid artery and portal vein. The IL-6 levels were both elevated regardless of the use of MMPI (Fig. 3).

The baseline value for the resting membrane potential (Em) was −30 mV for both groups. However, the resting membrane potentials for the 90-minute and 180-minute measurements were significantly different in the HS and HS + MMPI groups. The 90-minute Em value for the HS group was hyperpolarized to −36 mV, whereas the Em value for the HS + MMPI group was maintained at −30 mV. At 180 minutes the Em value in the HS group had decreased significantly to −20 mV, which is in sharp contrast to the −30 mV in the HS + MMPI group (Fig. 4).

DISCUSSION

Progressive multiple organ system dysfunction develops in more than 15% of critically ill patients and culminates in the syndrome of multiple organ failure.[25] MOF has become the leading cause of death in critically ill and injured patients.[1-4,25,26] The basic pathologic mechanisms of MOF involve an inflammatory tissue injury response that proceeds unchecked.[1-3,27-29]

MOF represents a systemic disorder of immunoregulation, endothelial dysfunction, and hypermetabolism with varying manifestations in individual organs.[1-3,27-30] A persistent nonseptic inflammatory response commonly initiates and sustains MOF. Inappropriate regulation of the production of cytokines, and other inflammatory response mediators such as eicosanoids, reactive oxygen species, and nitric oxide, is thought to be of causal significance in MOF.[9-14] Furthermore, activated neutrophils, and the complement system, also participate in this inflammatory cascade.[9] TNF-α and interleukins such as IL-1 and IL-6 are pivotal as early mediators in the host response to shock.[17-19]

Derangement of organ interaction in the bowel-liver axis may result in endogenous endotoxemia and bacterial translocation in animals.[5,31] However, selective bowel sterilization trials have not consistently prevented organ dysfunction or improved outcome in patients.[32-34] The neutrophil and activated cytokines and reactive oxygen species are potential mechanisms of remote organ damage.

The liver plays a pivotal role in the response to systemic injury through several mechanisms. Mononuclear phagocytic cells (Kupffer) control the magnitude of circulating endotoxins, bacteria, vasoactive substances, and cytokines.[5,17] Production and secretion of cytokines such as TNF-α and other mediators directly modulate lung function.[6] Hepatobiliary clearance is important in the metabolic inactivation and detoxification of inflammatory mediators.[6] Synthesis of acute-phase reactants regulates several key aspects of metabolism and inflammation.[35]

Matrix metalloproteinases are a major group of enzymes that regulate extra cell-matrix composition, which is critical for the normal development and function of the organism. By regulating the integrity and composition of the extra cell-matrix structure, these enzyme systems play a pivotal role in the control of signals elicited by matrix molecules, which regulate cell proliferation, differentiation, and cell death. Furthermore, matrix metalloproteinases appear to be critical components of TNF-α release.[15] Processing of the TNF-α precursor is dependent on at least one matrix metalloproteinase–like enzyme, inhibition of which represents a therapeutic modality to prevent MOF.[15,21] TNF-α exists as a 26 kDa cell-associated form and a 17 kDa soluble form. the 17 kDa TNF-α is released by proteolytic cleavage of the 26 kDa form by TNF-α convertase that is a member of the matrix metalloproteinase family.[22] MMPIs prevent the release of the soluble 17 kDa form of TNF-α without affecting the 26 kDa cell-associated activity.[15,21,22]

The use of an MMPI was reported by Murakami et al.[36] to inhibit TNF-α and reduce biochemical indices of hepatic injury to normal levels in endotoxemic mice. Histopathologic findings further indicated prevention of centrilobular necrosis in the liver following the use of the MMPI.

Our results demonstrate that after hemorrhagic shock, the cytokine levels of TNF-α and IL-6 were elevated. The elevation of TNF-α levels, however, was significantly attenuated with the use of the MMPI ($P < 0.001$). IL-6 levels, however, were not reduced with the MMPI. These data are consistent with those of Gearing et al.,[22] who showed that the use of a TNF-α convertase MMPI decreased the TNF-α without affecting other cytokines. The importance of reducing the TNF-α level is significant in that attenuation of TNF-α with a monoclonal antibody significantly improved the survival rate.[19] Furthermore, in the HS group without MMPI, the cytokine levels were highest in the portal circulation. These findings have led to the hypothesis that the bowel is the "motor" of the systemic inflammatory response that follows hemorrhagic shock. Consistent with this hy-

pothesis, Chang[37] demonstrated improvement of survival after hemorrhagic shock by enterectomy, implicating the role of the gut for irreversible cell injury and hepatic protection with adenosine triphosphate stabilization.

The resting membrane potential (Em) of the liver is very sensitive to cellular dysfunction and metabolic abnormalities[38] and was therefore used to assess the hepatic integrity. Hemorrhagic shock produced elevated TNF-α levels, which correlated with hepatic membrane destabilization and cellular injury from hemorrhagic shock. The present study coincides with previous studies demonstrating that the difference in resting membrane potential is a reliable and accurate indicator of cellular function.[39-44] The initial hyperpolarization at 90 minutes in hepatic Em following hemorrhagic shock in the untreated group has also been reported by Peitzman et al.[38] The hyperpolarization may be due to increased serum catecholamine levels in response to hemorrhage.[43,44] The maintenance of the hepatic Em relies on energy-dependent ATPase transport. Hemorrhagic shock decreases hepatic blood flow and decreases hepatic adenosine triphosphate and may result in the hyperpolarization seen at 90 minutes followed by the complete cell depolarization from cellular injury at 180 minutes. The hepatic injury correlated with TNF-α elevation in our study. However, despite hemorrhagic shock, the MMPI-treated group maintained hepatic cellular integrity as evidenced by maintenance of the baseline Em value of −30 mV. The TNF-α levels were depressed even though MMPI was given 15 minutes after hemorrhagic shock.

CONCLUSION

This study demonstrates that MMPI decreases TNF-α levels and protects hepatic integrity in hemorrhagic shock, as evidenced by the stabilization of the membrane potential, independent of the mean arterial pressure. The hepatic protection is closely related to the decrease in TNF-α levels seen in the portal circulation produced from the bowel. The effect of MMPI might be on the bowel.

The mechanism of action of the protective effect of MMPI may be due to the inhibition of the TNF-α convertase enzyme. This inhibition may prevent the escalation of the cytokine cascade induced by hemorrhagic shock.

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Staging Laparoscopy Promotes Increased Utilization of Postoperative Therapy for Unresectable Intra-Abdominal Malignancies

Vic Velanovich, M.D., Ira Wollner, M.D., Munther Ajlouni, M.D.

Staging laparoscopy avoids unnecessary laparotomies in patients with unresectable intra-abdominal malignancies. However, the postoperative oncologic treatment of these patients has not been documented. This study compares rates and timing of postoperative chemotherapy (ChT) and/or radiation therapy (XRT) in patients with unresectable intra-abdominal malignancies initially evaluated by staging laparoscopy (SL) or exploratory laparotomy (EL). The records of patients surgically evaluated for esophageal, gastric, hepatobiliary, and pancreatic cancers or abdominal lymphoma were retrospectively reviewed. Data gathered included type of exploration (SL or EL), resectability, whether postoperative cancer treatment was given (ChT, XRT, or both), and the time from surgery to the beginning of such treatment. This study includes only patients with unresectable malignancies. Twenty-one patients underwent SL and 58 EL. Sixteen of the SL patients (76%) and 25 of the EL patients (43%) received postoperative cancer treatment ($P = 0.009$). The median number of days from surgery to postoperative cancer treatment was 13 days (range 5 to 41 days) for the SL group and 35 days (range 16 to 89 days) for the EL group ($P = 0.0004$). We conclude that patients with unresectable intra-abdominal malignancies discovered by SL are more likely to receive postoperative ChT and/or XRT than patients surgically evaluated by EL. Further studies to determine whether this better utilization of postoperative treatment results in better outcomes in these patients are needed. (J GASTROINTEST SURG 2000;4:542-546.)

KEY WORDS: Staging laparoscopy, exploratory laparotomy, esophageal cancer, gastric cancer, pancreatic cancer, hepatobiliary cancer, chemotherapy, radiation therapy

Patients with unresectable intra-abdominal malignancies have short life expectancies. Alternative therapies such as chemotherapy (ChT) and radiation therapy (XRT) are usually recommended in good-risk patients to possibly extend life expectancy and palliate symptoms. Therefore these patients are best served by avoiding extensive intra-abdominal procedures in order to minimize recovery periods without sacrificing good palliation.

Preoperative staging includes a complete history, physical examination, chest radiographs, and CT scans in most patients. Other modalities such as endoscopy, endoscopic ultrasonography, and angiography can be used on a selective basis. In addition to these modalities, staging laparoscopy (SL) has also been advocated to increase resectability rates in a variety of intra-abdominal malignancies including esophageal, gastric, hepatobiliary, and pancreatic cancers.[1-6] Staging laparoscopy can not only increase resectability rates, but can also decrease the length of hospital stay,[1] select patients for neoadjuvant protocols,[3] and some palliative procedures can be accomplished laparoscopically.[7]

Nevertheless, opponents of SL argue that this procedure only identifies a minority of unresectable patients thought to be resectable by conventional means.[8] In addition, for some malignancies, such as gastric cancer, palliative resections are warranted,[3] and for others, such as pancreatic cancer, some claim that operative palliation is superior to any nonoperative method, thereby always justifying open exploration.[9]

Although these arguments may have merit in individual cases, the fact remains that for most patients

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with unresectable malignancies, ChT and/or XRT will be the primary anticancer treatments offered.[10-16] Therefore promoting such postoperative therapy would, at least theoretically, benefit this group of patients. We asked the question: Is there a difference in the postoperative oncologic treatment of patients found to be unresectable by SL vs. EL.

MATERIAL AND METHODS

Patient Data

The records of patients surgically treated for esophageal, gastric, hepatobiliary, and pancreatic cancers and abdominal lymphoma between January 1, 1996, and June 30, 1998, were retrospectively reviewed. Only cases that were unresectable were further reviewed for the following information: exploration which determined unresectability (EL or SL), whether any palliative procedures were done, whether postoperative cancer treatment was given (ChT, XRT or both), and the time from surgery to the beginning of such treatment. All patients who underwent EL or SL were thought by the operating surgeon to be resectable prior to surgery.

Staging Laparoscopy

Staging laparoscopy was done by entering the abdomen through an infraumbilical approach, using either the Veress needle or Hasson cannula technique, as previously described.[1] Briefly, the abdomen was insufflated with CO_2 gas to a pressure of 15 mm Hg. A 30-degree scope was used. The abdomen was inspected for signs of carcinomatosis, ascites, or liver metastasis. Additional ports were placed as needed, usually only one but occasionally two. Biopsies were obtained of all suspicious lesions to confirm the presence of malignancy. The lesser omentum was divided to expose and inspect the celiac axis, whereas the hepatoduodenal ligament and the root of the mesentery were inspected and suspicious lymph nodes biopsied. The primary tumor was assessed to determine the extent of direct extension into the adjacent organs, and whether this precluded resection. No extensive laparoscopic dissections, such as Kocher maneuvers, were attempted. Most staging laparoscopies were completed within 15 minutes, but none took longer than 30 minutes.

Exploratory Laparotomy

Exploratory laparotomy was done through either an upper midline incision or unilateral or bilateral subcostal incisions as per surgeon preference. Patients with esophageal or gastroesophageal junction carcinomas underwent surgical exploration through a single incision, a left thoracoabdominal incision, or a two-incision abdominal and right thoracic (Ivor-Lewis) approach. Determination of resectability was done in the customary manner for each malignancy.

Table I. Distribution of cancers evaluated by staging laparoscopy and exploratory laparotomy

	Exploratory laparotomy	Staging laparoscopy
Esophageal cancer	2	2
Gastric cancer	4	3
Biliopancreatic cancers	34	7
Duodenal/ampullary cancers	2	0
Hepatic cancers	13	5
Abdominal lymphoma	1	4

Statistical Analysis

All data were analyzed using the *True Epistat* statistical computer program. Nominal data were analyzed using chi-square analysis. Time to treatment data were initially analyzed for normality using the Wilk-Shapiro test and found not to fit a normal distribution pattern. These data were subsequently analyzed nonparametrically using the Mann-Whitney U test. A p value of 0.05 was considered significant.

RESULTS

Table I presents the distribution of cancer types between the EL and SL groups. The preponderance of biliopancreatic malignancies in the EL group reflects the difficulty in establishing unresectability due to direct vascular invasion by bile duct or pancreatic cancers using SL. A total of 58 patients were evaluated by EL and 21 by SL. Nineteen patients were initially evaluated by SL, but were converted to EL because of a false negative SL (usually due to unappreciated vascular invasion). These patients were analyzed in the EL group.

Sixteen of the SL patients (76.2%) and 25 of the EL patients (43.1%) received postoperative anticancer treatment with ChT, XRT, or both ($P = 0.009$; Fig. 1). The median number of days from the time of the operation to the commencement of this therapy was 13 days (range 5 to 41 days) in the SL group and 35 days (range 16 to 89 days) in the EL group ($P = 0.0004$; Fig. 2).

Table II shows the distribution of the palliative procedures performed in the EL and SL patients.

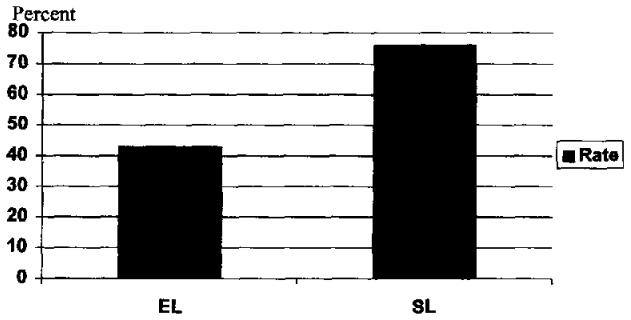

Fig. 1. Frequency of patients receiving postoperative anticancer treatment based on the type of exploration.

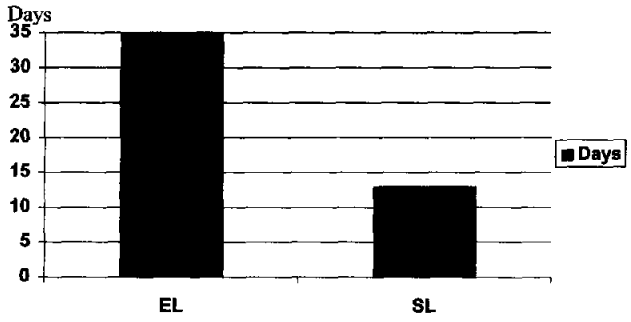

Fig. 2. Median time from type of exploration to beginning of postoperative anticancer treatment.

Table II. Distribution of palliative procedures

	Exploratory laparotomy	Staging laparoscopy
Celiac plexus block	12	4
Tube gastrostomy	1	2
Tube jejunostomy	3	4
Cholecystectomy	3	
Gastric bypass	9	
Biliary bypass	2	
"Double" bypass*	21	

*Choledochojejunostomy and gastrojejunostomy.

DISCUSSION

One of the purposes of staging is to identify which treatment modality is best suited for each individual patient. In many patients with unresectable disease, the only feasible treatment is ChT, XRT, or some combination of both. However, it is also clear that many patients are simply not fit enough for these treatment options. In patients found to be unresectable at surgical exploration, one of the reasons why these patients may not receive postoperative therapy is the surgery itself, which may have led to a worsening of their performance status. For example, in a study comparing laparoscopic surgery to open cholecystectomy, splenectomy, and esophageal surgery, patients undergoing laparoscopic surgery had better quality-of-life outcomes in the domains of physical functioning, vitality, and bodily pain.[17] If these results can be translated to operative staging of cancer patients, it would be reasonable to assume that patients staged by SL would be physiologically better able to undergo postoperative ChT or XRT.

What we have shown here is that more patients found to be unresectable by SL will undergo postoperative anticancer therapy than EL patients. Moreover, the median time from discovery of their unresectability to treatment is significantly shorter in the SL group compared to the EL group. This implies that unresectable patients who were found so by SL recover more quickly than do EL patients; they are most likely to have better performance status and therefore are more likely to tolerate and accept postoperative treatment. This notion is not without precedent. One of the arguments favoring preoperative chemoradiation in patients with pancreatic cancer is that more patients will complete the entire treatment course than if the chemoradiation is offered postoperatively.[18,19] Therefore, although SL is not without its stresses, patients seem to recover rapidly from this intrusion.

One of the potential criticisms of this study is that it appears that the EL group has a disproportionate number of patients with biliopancreatic malignancies (see Table I). However, most of these patients had local disease found unresectable by vascular invasion only, whereas in the SL group, patients with biliopancreatic malignancies were found to be unresectable because of liver metastases or carcinomatosis. Another criticism is that 19 patients initially evaluated by SL but converted to EL were analyzed in the EL group. Once again, these patients had disease that was deemed unresectable because of vascular invasion rather than distant spread. In this sense, there was a shift of patients with less advanced disease to the EL group. Therefore it is the SL group that had the more advanced malignancies, and because of this, one can argue that they should have had a poorer performance status compared to the EL group. Nevertheless, the SL group still had a higher rate of postoperative therapy compared to the EL group.

Some have argued that SL offers no great advantage over EL in some intra-abdominal malignancies. For example, Rumstadt et al.[8] claimed that only 7% of patients with pancreatic cancer would have benefited from SL. In addition, some cancers, such as esophageal or gastric cancers, cause bleeding or obstruction and are best palliated by resection.[3] On the other hand, Luque-de León et al.[9] asserted that even if SL is not beneficial in maximizing the resectability

rate of pancreatic cancers, it still can be used to select the best method of palliation. Burke et al.[3] argued that for nonbleeding, nonobstructed patients with stage IV gastric cancer, SL can be used to identify patients who may benefit from newer induction chemotherapeutic techniques. Therefore, even if one accepts the argument that SL does not avoid "unnecessary" laparotomies in the majority of patients, an argument can be made that a significant number of patients would still benefit from it.

It is important that patients with unresectable disease be treated with other modalities. We have previously shown that postoperative chemoradiation does in fact prolong survival in patients with unresectable pancreatic cancer.[10] Others have also demonstrated survival advantages of chemoradiation in unresectable pancreatic cancer.[11,12] Studies of unresectable or locally advanced gastric cancer have demonstrated survival benefits in patients treated with chemoradiation.[13,14] With respect to stage IV colorectal cancer with liver metastases, the Nordic Gastrointestinal Tumor Adjuvant Therapy Group demonstrated a survival advantage with prompt treatment with systemic chemotherapy compared to supportive care only.[15] In addition, another study of hepatic metastases from colorectal cancer demonstrated improved quality of life in patients treated with chemotherapy.[16] Therefore it seems reasonable that patients can benefit from ChT and XRT despite having unresectable disease.

With respect to pancreatic cancer, SL has other advantages. Fernandez-del Castillo et al.[20] performed peritoneal washings in conjunction with SL. They found a significant correlation between visible liver or peritoneal metastases and unresectable disease due to local invasion with positive peritoneal washings. In addition, this group reported that patients with positive peritoneal washings with no visible metastatic disease had the same poor prognosis as patients with visible metastatic disease discovered by SL.[21] This information, which can only be identified by SL, demonstrates the advantage of SL over spiral CT scanning alone.

Another argument against SL is that many patients still require open surgery for the best palliation.[9] In fact, in our series the majority of open palliative procedures were some type of bypass (see Table II). In the case of pancreatic cancer, these bypasses were performed after the discovery of vascular invasion, which precluded resection. If such vascular invasion were discovered prior to conversion to EL, such as with endoscopic ultrasonography, high-quality spiral CT scanning,[23] or laparoscopic[4] ultrasonographic techniques, many of these patients could have been spared EL. In these patients, palliation of obstructive jaundice can be accomplished by endoscopic placement of metallic wall stents.[24] Another limiting factor is developing expertise in laparoscopic gastrointestinal anastomoses. As experience is gained with both techniques, more patients should be palliated without the need for open laparotomy.

CONCLUSION

SL can identify patients with unresectable intra-abdominal malignancies. Nevertheless, it must be emphasized that SL should only be performed in patients who appear resectable by spiral CT scan or MRI (for liver lesions). These patients have a higher rate of receiving postoperative anticancer treatments, and receive these treatments sooner. Presumably this will translate into better survival for this group of patients.

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Pigment Gallstone Pathogenesis: Slime Production by Biliary Bacteria Is More Important Than Beta-Glucuronidase Production

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Pigment stones are thought to form as a result of deconjugation of bilirubin by bacterial β-glucuronidase, which results in precipitation of calcium bilirubinate. Calcium bilirubinate is then aggregated into stones by an anionic glycoprotein. Slime (glycocalyx), an anionic glycoprotein produced by bacteria causing foreign body infections, has been implicated in the formation of the precipitate that blocks biliary stents. We previously showed that bacteria are present within the pigment portions of gallstones and postulated a bacterial role in pigment stone formation through β-glucuronidase or slime production. Ninety-one biliary bacterial isolates from 61 patients and 12 control stool organisms were tested for their production of β-glucuronidase and slime. The average slime production was 42 for biliary bacteria and 2.5 for stool bacteria ($P < 0.001$). Overall, 73% of biliary bacteria and 8% of stool bacteria produced slime (optical density >3). In contrast, only 38% of biliary bacteria produced β-glucuronidase. Eighty-two percent of all patients, 90% of patients with common bile duct (CBD) stones, 100% of patients with primary CBD stones, and 93% of patients with biliary tubes had one or more bacterial species in their stones that produced slime. By comparison, only 47% of all patients, 60% of patients with CBD stones, 62% of patients with primary CBD stones, and 50% of patients with biliary tubes had one or more bacteria that produced β-glucuronidase. Most biliary bacteria produced slime, and slime production correlated better than β-glucuronidase production did with stone formation and the presence of biliary tubes or stents. Patients with primary CBD stones and biliary tubes had the highest incidence of slime production. These findings suggest that bacterial slime is important in gallstone formation and the blockage of biliary tubes. (J GASTROINTEST SURG 2000;4:547-553.)

KEY WORDS: Gallstones, bacterial slime, glycocalyx, β-glucuronidase

Our group was the first to report the presence of bacteria in gallstones.[1] In those studies, bacterial microcolonies were demonstrated within the gallstone matrix using scanning electron microscopy. Bacteria were found in most (78%) pigment gallstones and most (78%) pigmented portions of mixed stones, but rarely in pure cholesterol stones.[1] This suggested a causal role for bacteria in the formation of pigment gallstones and the pigment portions of mixed stones. The most commonly accepted mechanism for this process involves the deconjugation of bilirubin by β-glucuronidase (produced by biliary bacteria), leading to precipitation of calcium bilirubinate.[2] β-Glucuronidase levels have been reported to be increased in the bile of patients with primary common bile duct (CBD) stones and brown pigment stones.[3-10] It has also been reported in noninfected bile.[11-14]

The formation of macroscopic stones from precipitate requires the action of an anionic glycoprotein. In the original experiments by Maki,[2] sodium alginate was used as a conjugating agent to agglomerate calcium bilirubinate crystals into stones. We previously suggested that bacterial slime (glycocalyx, an anionic glycoprotein) might be the conjugating agent that builds pigment stones from calcium bilirubinate crystals.[1] Others have suggested that bacterial glycocalyx

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is important in cementing together the material that blocks biliary stents.[15-17] The present study was designed to examine the production of β-glucuronidase and bacterial slime by biliary bacteria.

MATERIAL AND METHODS

Between October 1989 and December 1997, gallstone cultures from 171 consecutive patients with gallstone disease were studied: 26 patients from University of California San Francisco-Moffitt Hospital and 145 from the San Francisco Veterans Hospital. Under sterile conditions, gallstones were obtained at surgery, washed with normal saline solution, crushed, and cultured in tryptic soy broth for 24 to 48 hours. Bacteria obtained from these stones constituted the study population. For comparison, 12 commonly occurring stool isolates, obtained from 12 healthy volunteers (age range 35 to 73 years, with equal numbers of men and women), were studied as well. One bacterial species was obtained from each volunteer. The ages of the volunteers were not significantly different from the ages of the patient population. There were proportionately more women in the volunteer group than in the patient group, but sex did not influence the incidence of bacterial slime production.

Gallstones were classified into the four following groups based on their visual appearance: (1) cholesterol = stones that appeared to be pure cholesterol; (2) mixed = stones that appeared to be composed of both cholesterol and pigment solids; (3) black pigment = totally black stones; and (4) brown pigment = brown, or brown *and* black pigmented stones. The location of the stones (gallbladder only, both gallbladder and CBD, or primary CBD stones) and history of biliary tract instrumentation were noted and correlated with the findings. Cultures were always obtained from gallstones, not from biliary tubes or stents.

Bacterial β-glucuronidase production was assessed using the method of Jackson et al.[18] Bacteria were pour-plated on MacConkey agar containing 100 mg of 4-methylumbelliferyl-β-D-glucuronide/liter. They were then incubated at 37° C for 24 hours, and colonies were examined under ultraviolet light at 360 nm. Bacterial colonies producing β-glucuronidase are fluorescent when examined under ultraviolet light.

Bacterial slime production was quantitatively assessed using the method of Tsai et al.[19] Glass test tubes (American Scientific Products, McGaw Park, Ill.) containing 1 ml tryptic soy broth supplemented with 10% (vol/vol) glucose were inoculated with a single colony of bacteria and incubated stationary at 37° C for 24 hours. Each tube was decanted and washed twice with 1 ml H_2O and reacted with Carnoy's solution (absolute ethanol, chloroform, glacial acetic acid, 6:3:1). Next, 1 ml safranin was added to the tubes, and the tubes were gently rotated to uniformly coat the adherent material (slime). Excess stain was removed by washing twice with 3 ml H_2O, 1 ml 0.2 M NaOH was added, and the sample was heated for 1 hour at 85° C. The samples were then vortexed, cooled to room temperature, and optical density was determined at 530 nm. Optical density (OD) correlates with the amount of slime produced.[19]

RESULTS

Patient Demographics

There were 143 men and 28 women whose average age was 60 years (women, 54 years; men, 61 years). Three percent of the patients were Asian, 11% were Hispanic, and the remainder were Caucasian, or African-American. Patients whose gallstones contained bacteria were older (63 years) than those with sterile stones (58 years) ($P < 0.02$; t test), and were more commonly male. Forty-six percent of gallstones from men contained bacteria compared with 25% of those from women ($P = 0.065$; chi-square analysis). The location of the stones and incidence of positive gallstone culture, in relation to stone type, is shown in Table I. As noted, bacteria were recovered from the stones in 61 (36%) of 171 patients. Of these 61 pa-

Table I. Gallstone location and culture results by visual stone classification

	GB only		GB/CBD		Primary CBD stones		Total	
	Bacteria No. (%)	Sterile No. (%)	Bacteria No. (%)	Sterile No. (%)	Bacteria No. (%)	Sterile No. (%)	Bacteria No. (%)	Sterile No. (%)
Cholesterol	2 (1)	39 (23)	1 (0.5)	1 (0.5)	0	0	3 (2)	40 (23)
Mixed stones	18 (11)	31 (18)	6 (3.5)	4 (2)	0	1*†	24 (14)	36 (21)
Brown pigment	8 (5)	3 (2)†	9 (5)	2 (1)†	12 (7)	0	29 (17)	5 (3)
Black pigment	4 (2)	24 (14)	1 (0.5)	5 (3)	0	0	5 (3)	29 (17)
TOTAL	32 (19)	97 (57)	17 (10)	12 (7)	12 (7)	1 (0.5)	61 (36)	110 (64)

GB = gallbladder; CBD = common bile duct.

*Patient with agenesis of the gallbladder.

†Scanning electron microscopic examination of these stones revealed bacterial colonies.

tients whose gallstones contained bacteria, 32 (52%) had stones in the gallbladder only, 17 (28%) had stones in the gallbladder and common duct, and 12 (20%) had primary common duct stones. The stones containing bacteria were predominantly brown pigment stones (48%) or mixed stones (39%).

Fifteen patients had undergone instrumentation of the biliary tree. CBD stones from 13 of these patients contained bacteria (11 brown pigment stones, one mixed stone, and one black pigment stone). Two patients with gallbladder stones had biliary instrumentation. The first had sterile black pigment stones and underwent percutaneous transhepatic cholangiography when he presented with Mirizzi's syndrome. The other, a transplant patient with infected brown pigment stones, had a cholecystostomy tube placed preoperatively when he presented with sepsis.

Slime and β-Glucuronidase Production by Bacterial Species

Ninety-one biliary bacterial isolates were cultured from 61 patients. Two or more organisms were cultured from gallstones from 41% of patients and three or more organisms from 13% of patients. The distribution of bacterial species recovered is shown in Table II. Bacterial slime production according to bacterial species is given in Fig. 1, where the OD measurement for each biliary and stool bacterial isolate is given. On this logarithmic plot, slime production by biliary bacteria is greater than that by stool bacteria. Fig. 2 demonstrates the average slime production for individual bacterial species, and Fig. 3 demonstrates the average slime production for the two groups (biliary and stool bacteria). Average slime production (OD) was 42 for biliary bacteria and 2.5 for stool bacteria ($P < 0.001$; t test).

Slime production varied among the species (Fig. 4 and Table III). *Pseudomonas* species made the most slime (average OD 113). Overall, 73% of biliary bacteria and 8% of stool bacteria produced slime (OD >3). All *Pseudomonas* species produced slime, whereas 60% to 80% of the other species produced slime.

Since *Pseudomonas* is not a stool organism (it is found in water and soil), it was not represented in our stool specimens. For this reason, and because it always produced abundant slime, we also compared stool and gallstone bacteria excluding the contribution from *Pseudomonas* species. This did not alter the result. The average slime production by gallstone bacteria, other than *Pseudomonas*, was 35.4 compared with 2.4 by stool bacteria ($P < 0.002$; t test).

β-Glucuronidase production was not as common as slime production: 38% of biliary bacteria produced β-glucuronidase. Most *E. coli*, no *Enterococcus* species,

Table II. Bacterial species recovered from gallstones

Bacterial species	No.
E. coli	17
Enterococcus	17
Klebsiella	15
Enterobacter	13
Pseudomonas	11
Citrobacter	5
Other (*Aeromonas, Serratia, Staphylococcus, Streptococcus, Xanthomonas*)	13

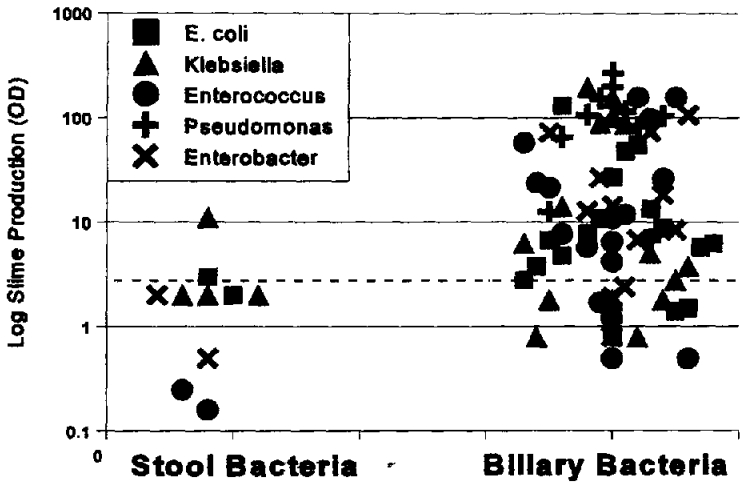

Fig. 1. Quantitative bacterial slime production by individual bacterial species recovered from gallstones and stool. Slime production, reflected by optical density *(OD)*, is shown for all stool bacteria and the most prevalent gallstone bacterial species. Note that the Y-axis is a logarithmic plot, and that slime production by biliary bacteria is greater than slime production by stool bacteria.

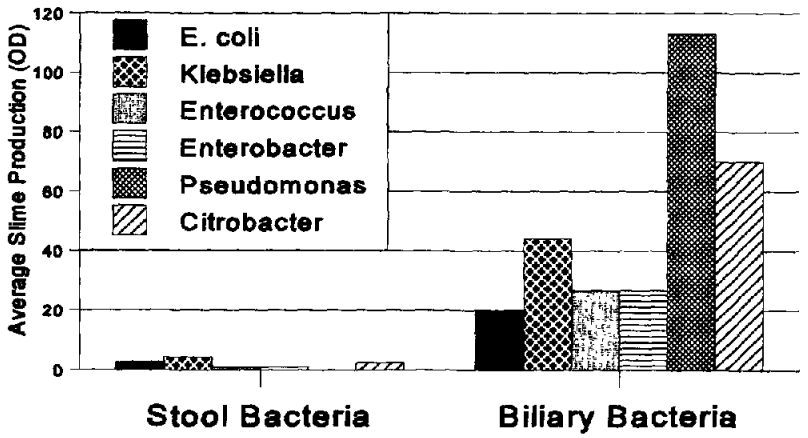

Fig. 2. Average slime production of the individual bacterial species recovered from gallstones and stool. Optical density *(OD)* measurements for each species were averaged. Note that the average slime production by gallstone bacteria is greater than slime production of stool bacteria. *Pseudomonas* produced the most slime.

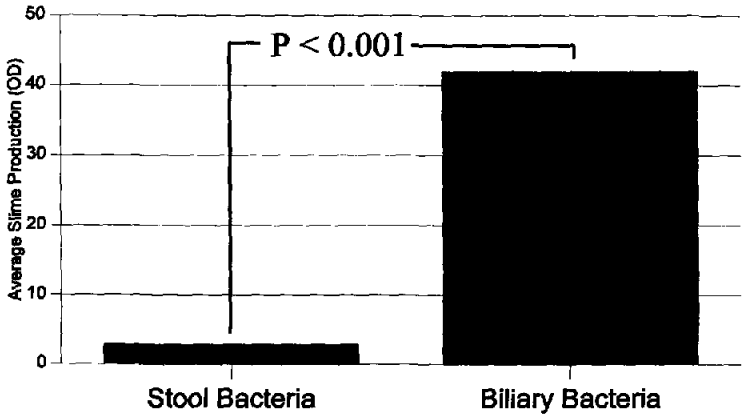

Fig. 3. Overall average slime production for the two groups (biliary and stool bacteria). Average slime production was 42 for biliary bacteria and 2.9 for stool bacteria ($P < 0.001$; t test).

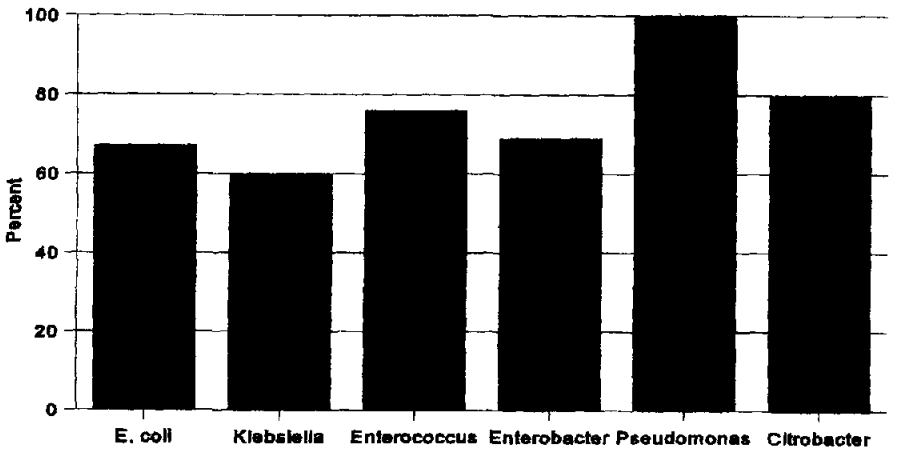

Fig. 4. Incidence of bacterial slime production, defined as OD >3, for the most prevalent gallstone bacteria. The Y-axis demonstrates the percentage of bacteria that produced slime.

and few of the other species produced β-glucuronidase. Two (17%) of 12 stool bacteria produced β-glucuronidase (both were *E. coli* species). The incidence of β-glucuronidase production by stool bacteria was totally controlled by the number of *E. coli* isolates, making any comparisons with the gallstone bacteria meaningless.

Slime Production, β-Glucuronidase Production, and Gallstone Formation

The data were analyzed by patient to look for correlations with stone location or the presence of a stent or tube, and because more than one organism was cultured from many patients' stones. Eighty-two percent of all patients with colonized stones had one or more bacterial species that produced slime (OD >3), whereas only 47% had one or more bacterial species that produced β-glucuronidase. There were no differences in the age ($p = 0.27$; t test) or sex ($P = 0.64$; chi-square analysis) of the patients with slime-producing bacteria compared to patients without slime-producing bacteria. The location of the gallstone and a history of biliary instrumentation correlated with bacterial slime production. Among patients with positive cultures, one or more bacterial species that produced slime were cultured from 75% of patients with stones in the gallbladder, 82% of patients with stones in the gallbladder and CBD, 100% of patients with primary CBD stones, and 93% of patients with biliary stents or tubes. By comparison, bacteria that produced β-glucuronidase (Fig. 5) were cultured

Table III. Slime production by gallstone bacteria

Bacterial species	Average slime production (OD)	Range slime production (OD)
Aeromonas hydrophilia	1	0
Citrobacter freundii	86	2-184
E. coli	20	1-132
Enterobacter	27	1-107
Enterococcus	27	0.5-158
Klebsiella	34	1-156
Pseudomonas aeruginosa	113	7-270
Serratia	1.4	1.1-1.7
Staphylococcus	68	20-115
Streptococcus	3	2.9-3.4
Xanthomonas malto	69	2.8-162

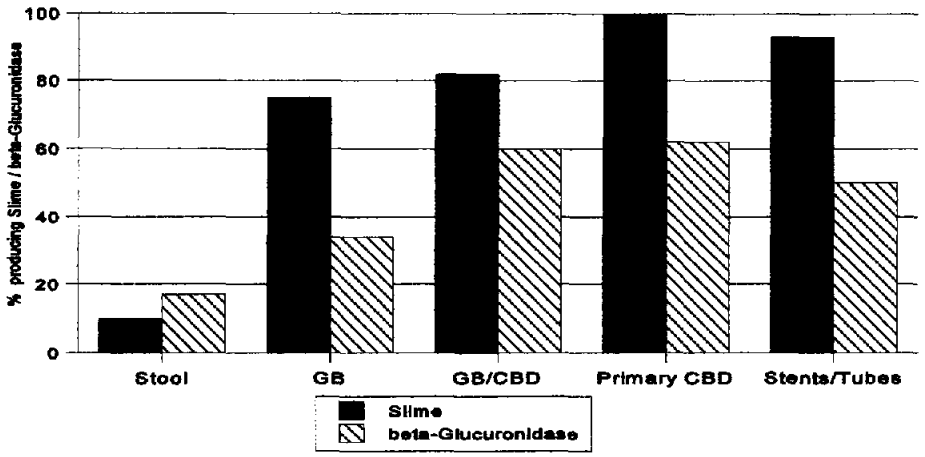

Fig. 5. Incidence of bacterial slime and β-glucuronidase production correlated with the location of the bacteria or gallstone and/or presence of biliary stents or tubes. Data are shown only for patients with positive cultures. They demonstrate the percentages of patients who had one or more bacteria that produced slime and β-glucuronidase when their stones were located in the gallbladder only *(GB)* or in both the gallbladder and common bile duct *(GB/CBD)*, and whether they had primary common bile duct stones or biliary stents or tubes. The incidence of slime and β-glucuronidase production by stool bacteria is shown for comparison.

from only 34% of patients with gallbladder stones, 60% of patients with gallbladder and CBD stones, 62% of patients with primary CBD stones, and 50% of patients with biliary stents or tubes.

DISCUSSION

Ever since Maki[2] first described the formation of calcium bilirubinate crystals by the action of bacterial β-glucuronidase deconjugating bilirubin, β-glucuronidase has been considered to have a dominant role in pigment gallstone formation. Several studies have noted an increased incidence of bacterial β-glucuronidase in the bile of patients with brown pigment CBD stones compared with patients without common duct stones.[3-10] Maki's observations, however, related specifically to *E. coli*, a species that usually produces β-glucuronidase. Other enteric bacteria usually do not produce β-glucuronidase.[7] In fact, the production of β-glucuronidase is so specific that its presence has been used as a test for *E. coli* contamination.[20] *Clostridium perfringens* and *Bacteroides* species usually produce β-glucuronidase,[7,9,21] but other bacterial species commonly found in gallstones and bile, such as *Klebsiella*, *Enterococcus*, *Enterobacter*, and *Pseudomonas*, often do not. Thus, because *E. coli* is not the only organism associated with biliary infections, the role of *bacterial* β-glucuronidase may be less important in pigment stone formation than originally thought.

In this study only 38% of bacteria recovered from gallstones produced β-glucuronidase, and only 47% of patients with bacteria in gallstones had one or more species that produced β-glucuronidase. Bacterial β-glucuronidase production was more common in patients with primary CBD stones (62%), but it still did not seem to be common enough to fulfill the important place in pigment stone formation that had originally been postulated.

β-Glucuronidase activity has also been found in bile.[11-14] The pH optimum (5.2) of the human enzyme, however, is lower than that of bacterial β-glucuronidase (6.6 to 7.0).[10,12] Since bile has a pH range of 7 to 9, the human enzyme would not be expected to be as active as bacterial β-glucuronidase. In fact, the human enzyme has a 23% activity in bile. The activity is enhanced by bile salts at pH 7, however, which tends to restore the activity of human β-glucuronidase.[12] Given the inconsistent presence of bacteria that produced β-glucuronidase in gallstones, both the human and bacterial enzyme probably contribute to the formation of calcium bilirubinate.

Active β-glucuronidase is insufficient by itself to cause stone formation. Something must cement the precipitate into discrete stones. In Maki's original study,[2] sodium alginate was used to agglomerate calcium bilirubinate crystals. Before aliginate was added, only calcium bilirubinate crystals were formed. Bacterial slime is chemically similar to sodium alginate. In fact, alginate production is characteristic of *Pseudomonas* species that cause chronic infections associated with biofilms.[22] We initially thought that bacterial slime or glycocalyx was the material that agglomerated bacteria and calcium bilirubinate crystals into stones.[1] The present study examined that hypothesis. Slime production by a large cohort of gallstone bacteria was examined to determine whether slime was associated with gallstone formation. Unlike β-glucuronidase, slime production was highly prevalent among gallstone bacteria, and it correlated closely with primary CBD stone formation (100%). Furthermore, slime production was almost unique to bacterial species obtained from gallstones, since stool isolates of similar species rarely produced slime ($P < 0.001$; t test).

The most prevalent bacterial species obtained from gallstones were: *E. coli*, *Klebsiella*, *Enterococcus*, *Enterobacter*, and *Pseudomonas*. All of these species were represented in our stool isolates except for *Pseudomonas*, which is not a stool organism. It is found in water and soil. Excluding *Pseudomonas* species from the comparison of slime production between stool and gallstone bacteria, however, did not alter the result. The average slime production by gallstone bacteria, other than *Pseudomonas*, was 35.4 compared with 2.4 by stool bacteria ($P < 0.002$; t test). The age of the comparison volunteer group (donating the stool isolates) was similar to that of the patient group, but there were more women in the volunteer group than in the patient population. Patient age and/or sex, however, did not influence bacterial slime production. There were no differences in the age or sex of patients whose bacteria produced slime compared with those whose bacteria did not.

Slime production allows bacteria to grow in a protected environment. It enables the organisms to resist phagocytosis, antibodies, surfactants, and antibiotics. It has been shown to be associated with chronic infections, especially those involving foreign bodies.[23-25] After bacteria were identified in gallstones, the association between bacterial biofilm formation and the blockage of biliary stents was examined.[14-16, 26-28] These studies noted that bacterial biofilms were important in the blockage of biliary stents. In a study of bacterial β-glucuronidase production and clogging of endoprostheses, Dowidar et al.[26] noted that although contaminated bile was associated with more sludge formation than was sterile bile, bacteria that produced β-glucuronidase *(E. coli)* did not form more sludge than bacteria not producing β-glucuronidase. In fact, *Pseudomonas* and *Staphylococcus* species pro-

duced the most sludge. This is interesting because in the current study (see Table III) these species produced large amounts of slime, which further implicates bacteria and bacterial slime in this process irrespective of β-glucuronidase production. Furthermore, whereas only 50% of patients with biliary stents or tubes had bacteria that produced β-glucuronidase, 93% of them had one or more bacteria that produced slime, underscoring the importance of bacterial slime production.

In these experiments, we examined bacterial slime and β-glucuronidase production by a large number of bacteria isolated from gallstones. Because the bacteria were obtained from gallstones, it allowed the importance of these two factors in gallstone pathogenesis to be examined. Most biliary bacteria produced slime, and slime production correlated better than β-glucuronidase production with stone formation. Patients with primary CBD stones and biliary tubes had a high incidence (93% to 100%) of slime production, whereas β-glucuronidase production was less consistent (50% to 62%). This suggests that bacterial slime may have a necessary role in gallstone formation and the blockage of biliary tubes, and *bacterial* β-glucuronidase production may not be essential for the formation of sludge and pigment stones.

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The Art of Surgery: Exceptional Cases—Unique Solutions: 100 Case Studies

Michael Trede. New York, N.Y.: Thieme-Stuttgart, 1999. Pages: 212. Illustrations: 460. Price: $125.

As the preface of this superb illustrative text freely states, "It is not a surgical textbook, nor an art volume, but rather a collection of interesting cases acquired over 40 years as a general surgeon." The author makes no apologies for the absence of references in this enlightening surgical text.

The *Art of Surgery* covers the wide range of general surgery through 100 case presentations divided into four chapters: Visceral surgery (esophagus, stomach, intestine, retroperitoneum, liver, biliary tract, and pancreas), vascular surgery, thoracic surgery, and cardiac surgery. Each and every case is presented in a straightforward and understandable manner. A brief history of each illness is given along with pertinent x-ray films, drawings, photographs, electrocardiograms, and operative photographs. A diagnostic and/or operative dilemma is then presented. The superb operative illustrations along with the pathologic photographs are extraordinarily educational. Documentation regarding complications and patient follow-up including personal letters and pictures provides the reader with a complete understanding of what "surgical intervention" entails in these extraordinarily complex 100 patients.

Although biased by my previous exposure to this sage surgeon and accomplished artist, I found the text educational, useful, and timely. The author's candor, honesty, and humility regarding both good and bad outcomes of surgical intervention are refreshing. Although the text covers the multitude of surgical fields of Professor Trede's 40 years of diverse clinical experience, a heavy weighting (35 cases) is given to the section on the pancreas. Department chairs and program directors would do well to have this text in their surgical libraries to give each and every medical student and surgical trainee a unique opportunity to learn of an experienced surgeon's attempts at solving complex problems. Pancreatic surgeons will find 35 of the most difficult cases involving the pancreas presented in a concise manner, and they will find the author's management and technique helpful to their own practices. Surgical trainees preparing for oral board examinations will find this text a valuable review for treating complex surgical conditions, as is typical of the American certification examination.

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