Does Prophylactic Octreotide Benefit Patients Undergoing Elective Pancreatic Resection?

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Pancreatic fistula and its potential sequelae (fluid collection, abscess, hemorrhage, sepsis, etc.) remain troublesome and at times life-threatening complications following elective pancreatic resection. Elsewhere in this issue (see p. 225), Rosenberg from McGill University Health Center and MacNeil and Turcotte from Novartis Pharmaceuticals Canada (makers of octreotide and financial supporters of the study) use statistical meta-analysis techniques to generate a cost-effectiveness analysis of four previously published randomized, controlled, double-blind studies.[1-4] These studies all evaluated subcutaneous octreotide as prophylaxis against complications in patients undergoing elective pancreatic resection. The authors are to be congratulated for applying a sophisticated pharmacoeconomic evaluation and modeling technique to generate a cost-effectiveness analysis for pancreatic resection.

The rationale for using octreotide, the synthetic octapeptide analogue of native somatostatin, to reduce complications is sound. This concept originated in 1979, when a continuous perioperative infusion of somatostatin, at a dose of 250 μg/hr, was reported to reduce complications after pancreaticoduodenectomy.[5] Octreotide is more potent in inhibiting pancreatic enzyme secretion than somatostatin, and it has a much longer half-life.[6,7]

Although the rationale for studying octreotide is sound, the four studies that serve as the foundation for this meta-analysis are not without criticism.[1-4] Each of the trials was carried out in multiple centers in Europe (between 18 and 33 centers per trial), used a dose of octreotide of 100 μg every 8 hours for 7 to 8 days, and analyzed more than 200 patients. Each trial reported statistically significant decreases in overall morbidity, often not solely related to pancreatic complications. It is unclear how octreotide can influence complications not pancreas related. Each trial reported a lower incidence of pancreatic fistula in the octreotide group. However, in none of the trials was there a significant decrease in overall mortality, and in two trials the mortality rate in the octreotide group exceeded that of the placebo group. Furthermore, these trials all included many types of pancreatic resections (pancreaticoduodenectomy, distal pancreatectomy, enucleations, etc.), which serves to confound the interpretations as fistula rates vary based on the type of resection. In fact, only the study by Montorsi et al.[3] specifically distinguished between resectional procedures, noting that octreotide did not decrease fistula rates for pancreaticoduodenectomy (15% placebo vs. 11% octreotide), but rather only for distal resections (21% placebo vs. 6% octreotide) and enucleations (57% placebo vs. 0% octreotide). These fistula rates of 21% for distal pancreatectomy and 57% for enucleations in the placebo groups are quite high. In fact, although discounted by Rosenberg and colleagues, the four European trials had an exceedingly high overall rate of pancreatic fistula in each of their placebo groups (ranging from 19% to 37%). These rates are much higher than current pancreatic fistula rates at major institutions in the United States[8,9] and would certainly magnify any benefit to the use of octreotide. For example, if the rate of pancreatic fistula were only 10% without octreotide and 8% with octreotide, the results of cost-benefit analysis would change dramatically.

It is important to note that Rosenberg and colleagues base their results and conclusions solely on these four European trials,[1-4] generating their two models of cost savings from the European data. Their cost-effectiveness analyses indicate that, for model 1, octreotide use would save $853 per patient and spare 16% of patients complications; for model 2 the figures would be $1642 savings per patient and again, 16% of patients would be without complications. In their discussion they state "a pharmacoeconomic analysis will *always* be as good as the information used

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to produce it." Perhaps it would be better to substitute "only" for "always."

Since this meta-analysis was performed and submitted for publication, another prospective, randomized trial has been published.[10] This trial, by Lowy et al.,[10] reports a single-institution experience from a high-volume institution, the University of Texas M.D. Anderson Cancer Center in Houston, Texas. Over a 4½-year period, 120 patients undergoing only pancreaticoduodenectomy (one operation, not many types of resection) for malignancy were evaluated. The rate of pancreatic fistula was 6% in the control group and twofold *higher* at 12% in the octreotide group. Furthermore, perioperative morbidity was 25% in the control group and 30% in the octreotide group. Althought these data could be criticized for the inclusion of only resections for malignancy, they stand in direct opposition to the European multicenter trials and the meta-analysis results. The study by Lowy et al. does, however, fulfill the acceptance criteria stated by Rosenberg and colleagues in their Methods section for inclusion in the meta-analysis. One wonders what would result from adding the Lowy data to the meta-analysis.

In conclusion, many pancreatic surgeons (myself included) remain unconvinced that prophylactic octreotide benefits all patients undergoing elective pancreatic resection. I interpret the data published to date regarding the use of prophylactic octreotide as failing to show a benefit in patients undergoing pancreaticoduodenectomy but suggesting a benefit in patients undergoing distal pancreatic resection or tumor enucleation. We have our own prospective, randomized placebo-controlled trial of octreotide ongoing at The Johns Hopkins Hospital, studying its use in all patients undergoing pancreaticoduodenectomy. The interim analysis with 77 evaluable patients reveals a pancreatic fistula rate of 10.3% in the control group and 15.8% in the octreotide group. Although we plan to continue accrual, these interim data do not lend support for the use of octreotide prophylaxis. Octreotide may not be the savior of the pancreatic surgeon. As Rosenberg and colleagues note in the final paragraph of their report, "It is difficult to justify the use of octreotide in all patients undergoing pancreatic surgery." Doubtless, we will need further studies of specific patient groups (malignant vs. benign disease; firm gland vs. soft gland; Whipple vs. distal resection; etc.) before we can determine whether there is truly a role for octreotide as prophylaxis in elective pancreatic surgery.

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Economic Evaluation of the Use of Octreotide for Prevention of Complications Following Pancreatic Resection

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Recent studies have concluded that octreotide can prevent complications in patients undergoing pancreatic resections. Given the acquisition cost of octreotide, a cost-effectiveness analysis was performed to establish whether if the additional cost associated with its use was justified by a decrease in the consumption of other resources. To evaluate success rates and complication rates, a meta-analysis of double-blind, randomized, controlled clinical trials was conducted. The rates for pancreatic fistula and fluid collection were 10.7% (95% confidence interval [CI] 7.9 to 13.4) and 3.6% (95% CI 1.9 to 5.2) for octreotide vs. 23.4% (95% CI 19.7 to 27.1) and 8.8% (95% CI 6.2 to 11.3) for placebo. In a second phase we evaluated the treatment cost for patients with and without complications using two different models of cost savings. In the first model the cost to treat a pancreatic fistula was calculated as the per diem rate (as determined by Statistics Canada) multiplied by the incremental length of stay associated with the complication. In the second model we used data from institutions participating in the Ontario Case Costing Project. In model 1 the estimated incremental length of hospital stay attributed to a pancreatic fistula was 7 days, based on a review of the literature, and the per diem was $552. In model 2 the average cost of care for patients with or without complication was $32,347 (n = 17; 95% CI $20,882 to $43,812) and $11,169 (n = 18; 95% CI $7558 to $14,779), respectively. The data suggest that when compared to placebo, octreotide is a dominant treatment strategy. In model 1, in a cohort of 100 patients, octreotide saved an average of $853 per patient while allowing 16 incremental patients to avoid complications. In model 2 use of octreotide resulted in an average savings of $1642 per patient while still allowing 16 patients to avoid complications. Detailed one-way and two-way sensitivity analyses suggest that both models were robust. The use of octreotide is a cost-effective strategy in patients undergoing elective pancreatic resection. Consideration should be given to extending its use to patients who are at high risk for development of complications following pancreatic surgery and who do not have any contraindications to the use of this drug. (J GASTROINTEST SURG 1999;3:225-232.)

KEY WORDS: Octreotide, pancreatic surgery, pharmacoeconomics, complications

Despite improvements in surgical techniques, the mortality rate for elective pancreatic surgery remains between 3% and 10%,[1] whereas morbidity is approximately 30% to 40%.[2,3] The major complications following elective pancreatic surgery are related to the exocrine pancreatic secretions and include intra-abdominal fluid collection, pancreatic fistulas, abscess formation, sepsis, and multisystem organ failure.[1] Octreotide (Sandostatin, Novartis Pharma, Basel, Switzerland), a synthetic long-acting analogue of somatostatin, can play an important role in the treatment of fistulas by decreasing the volume and enzyme content of the secretions.[4] This raises the question of whether octreotide would be effective in reducing complications related to pancreatic surgery. Buchler et al.[5] reported an overall rate of complications of 32% in a group of patients treated with octreotide compared to 55% in a group receiving placebo. In the same study

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the average length of stay was 22.1 days and 26.2 days in the octreotide and placebo groups, respectively. Although not statistically different, this trend may be of importance if the cost associated with an increased length of hospital stay is considered. Holbrook et al.[6] determined the cost incurred by their hospital for a single pancreaticoduodenectomy to be $17,252, 79% of which was for time spent on the ward. The cost increased to $21,574 when only patients with postoperative complications were considered.

However, it is important to consider the effect of octreotide on health care resource consumption in the context of current budget constraints. Therefore a cost-effectiveness study was conducted to establish the value of octreotide in the prevention of complications related to pancreatic surgery and to consider whether its use should be generalized to the majority of patients undergoing pancreatic resection.

METHODS

A cost-effectiveness analysis was conducted to compare subcutaneous octreotide with placebo in the prevention of complications related to pancreatic resection. A two-phase model was used to guide this research. Phase 1 consisted of an evaluation of clinical outcomes using meta-analysis of clinical trials. Phase 2 consisted of a pharmacoeconomic analysis.

Phase 1: Meta-Analysis

A stepwise meta-analytic approach was taken, as suggested by Sacks et al.[7] and L'abbé et al.[8] The purpose was to determine the efficacy rate of octreotide in preventing complications, and the rate of occurrence of each complication, as defined in the literature.[5,9-11] The statistical method used for data pooling was the one developed by DerSimonian and Laird,[12] as modified for single-group analysis by Velanovich.[13] This method produces a sample-size weighted average value for each comparator, along with a standard error so that a 95% confidence interval may be constructed.

Data sources included computerized searches of MEDLINE® for the years 1985 to 1996. We also used the ancestry approach where references from retrieved articles and reviews located by the search are inspected for additional articles. Once studies were identified, each article was reviewed by an independent reviewer and relevant information was compiled using a data collection sheet.

To be included in the analysis, studies had to meet the following criteria: (1) studies had to be clinical trials involving adult men and women undergoing elective pancreatic resection, whether for chronic pancreatitis or neoplastic diseases of the pancreas and the periampullary region; (2) studies had to be randomized, controlled, double blind, and involve the use of subcutaneous octreotide at the recommended dosage for this indication (i.e., 100 mg 3 times a day for 7 days, beginning at least 1 hour before surgery); (3) studies had to provide primary or secondary outcome measures of success and failure by complication, as well as definitions of the complication; (4) results of the studies had to be interpretable (i.e., a global proportion of success or complication could be extracted from the data presented); and (5) studies had to be published in the form of an original research article.

Phase 2: Pharmacoeconomic Analysis

The first step in developing the economic model was to draft a decision tree using the software DATA version 3.03 (TreeAge Software, Inc., Williamstown, Mass.). The tree was based on an analytic time horizon of 6 months. Each of the possible outcomes is represented by a branch (Fig. 1). For the two groups, branch 1 represents success (i.e., no complications postoperatively) and branches 2, 3, and 4 represent failure (i.e., occurrence of complications). The same software was used to calculate the expected costs of therapy for each of the two options, as well as the cost-effectiveness ratio.

A questionnaire distributed to gastroenterology and general surgery specialists was used to verify and validate the model and the related assumptions discussed below, as well as to provide estimates of a number of variables that could not be found in the scientific literature or in the database consulted.

Effectiveness of Treatment. A treatment was considered a success if no complications occurred during the 60-day period following the surgery. A treatment success was assigned an effectiveness value of 1. A treatment failure was considered to be the occurrence of one of the complications defined in the literature[5,9-11] during the 60-day follow-up period. A treatment failure was assigned an effectiveness value of 0. The complications are not mutually exclusive. In the decision tree the sum of the probabilities associated with each node has to equal 1 (i.e., the sum of the probability of each individual complication does not add up to the global rate of complications). Therefore, for the purpose of using them in the model, the rate for each individual complication was transposed using the following formula:

$$p_1 = r_1 / Sr_1 \ldots r_n,$$

where p = proportion included in the tree; r = rate of complication from the meta-analysis; 1 = complication number 1; and n = number of complications.

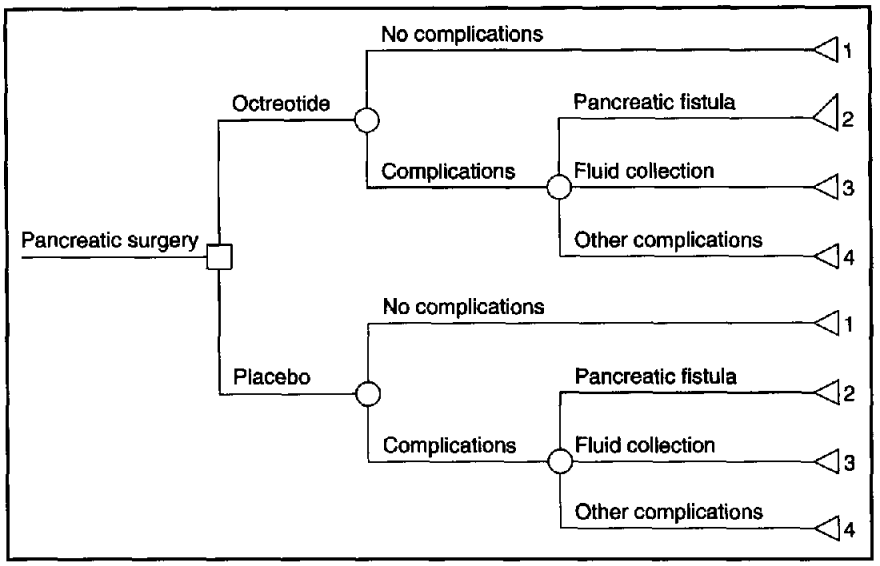

Fig. 1. Decision tree.

$C1 = DPC$
$C2 = DPC + (PD \times LS_F)$
$C3 = DPC + (PD \times LS_C)$
$C4 = DPC + (PD \times LS_O)$

Fig. 2. Equations used to calculate the cost associated with each branch of the decision tree for costing model 1. C1 = cost associated with branch 1; DPC = drug prophylaxis cost; C2 = cost associated with branch 2; PD = per diem; LS_F = incremental length of stay for patients with pancreatic fistula; C3 = cost associated with branch 3; LS_C = incremental length of stay for patients with fluid collection; C4 = cost associated with branch 4; LS_O = incremental length of stay for patients with other complications.

C1 = DPC
C2 = DPC + CTF
C3 = DPC + CTC
C4 = DPC + CTO

Fig. 3. Equations used to calculate the cost associated with each branch of the decision tree for costing model 2. C1 = cost associated with branch 1; DPC = drug prophylaxis cost; C2 = cost associated with branch 2; CTF = cost to treat a pancreatic fistula; C3 = cost associated with branch 3; CTC = cost to treat a fluid collection; C4 = cost associated with branch 4; CTO = cost to treat other complications.

Costs. Two different costing approaches were used to determine the cost of treating the complications.[14,15] For these two models, the cost associated with each branch of the decision tree is illustrated in Figs. 2 and 3.

Model 1. The cost of treating the complications was based on the Statistics Canada average per diem hospitalization cost[16] and on the estimated incremental length of hospital stay associated with the complication. The length of stay was assumed to be equal regardless of whether the patient did or did not receive prophylaxis and was estimated from the available literature. The per diem cost includes all direct medical charges and is assumed to be equal for both treatment groups. To the per diem cost, we therefore had to add the cost of the octreotide prophylaxis (when appropriate). It was assumed that the prophylaxis was of a fixed duration of 7 days. Since the use of total parenteral nutrition cannot be predicted or generalized, it was not included in this model.

Model 2. Treatment cost was estimated using data from two hospitals participating in the Ontario Case Costing Project (OCCP).[17] The data obtained reported the detailed costs (hospitalization cost, direct nursing time, laboratory, radiologic, and diagnostic tests, drugs used, and medical supplies), diagnosis, and procedures for each patient. To the total cost we had to add the cost of the octreotide prophylaxis (when appropriate). It was assumed that the prophylaxis was of a fixed duration of 7 days. The cost of treating a single complication was assumed to be equal, regardless of whether or not the patient received octreotide prophylaxis.

Calculation of Expected Cost of Therapy

For the two models we calculated an expected cost of therapy for each group using the decision tree and the following formulas:

$$EC = \hat{A}\ (P_1 \times C_1) + (P_2 \times C_2) + (P_3 \times C_3) + (P_4 \times C_4),$$

where EC = expected cost of therapy; C_i = costs associated with each individual branch as defined in Figs. 2 and 3; P_i = outcome probabilities at a specific branch; and indices = branch numbers (1 = success; 2,3,4 = failure).

Calculation of Cost-Effectiveness Ratio. To obtain the values required to calculate the incremental cost-effectiveness ratio, we determined the proportion of patients treated successfully (no complication) (E) and the expected cost of each therapy (EC).

Sensitivity Analysis. To verify the robustness of the conclusions reached, we performed a sensitivity analysis on all variables influencing cost and on the complication rates. The one-way sensitivity analysis enabled us to test the sensitivity of the results to changes in the value of a single variable. The analysis was judged to be sensitive to a variable when a small change in the magnitude of this variable resulted in a change in the conclusion or in the established rank ordering of the cost-effectiveness ratio. Since sometimes many assumptions are made at the same time to evaluate a scenario, we also assessed how a simultaneous change of two variables affected our conclusion. This is called a two-way sensitivity analysis. Similar to the one-way analysis, it consists of changing the value of two variables over a range of values to evaluate to what extent they affect the cost-effectiveness ratios.

Table I. Studies excluded from the meta-analysis

Reference	Reason for exclusion
Paran et al.[4] (1995)	Inappropriate outcome measure
Buchler et al.[18] (1993)	Not original study
Friess et al.[19] (1993)	Not original study
Buchler et al.[20] (1993)	Not original study
Bassi et al.[21] (1994)	Not original study
Friess et al.[22] (1994)	Not original study
Buchler et al.[23] (1992)	Not original study
Scott et al.[24] (1993)	Inappropriate outcome measure

RESULTS

Four studies met the criteria previously enumerated and were therefore included in the meta-analysis.[5,9-11] Table I shows the eight studies that were excluded from the meta-analysis either because they were not original papers or because the results were not presented as complication rates.[4,18-24] The results of the meta-analysis are presented in Table II.

Model 1

The per diem cost obtained from Statistics Canada for a regular hospital bed was $552 per day.[16] The cost of a 7-day prophylaxis of octreotide was $197,[25] whereas the cost of placebo was of course $0. Because the meta-analysis showed that the rate of occurrence of pancreatic fistula is the complication that is the most decreased by the use of octreotide, followed by fluid collection, we decided that only the cost to treat a fistula would be calculated and that the cost of fluid collection as well as the cost to treat the other complications would be arbitrarily set to $0 in order to keep this model as conservative as possible. Such an approach is consistent with the Canadian Guidelines for Economic Evaluation of pharmaceuticals[26] where it is recommended to bias the analysis against the pre-

Table II. Results of meta-analysis (±95% confidence interval)

Complication	Octreotide	95% CI	Placebo	95% CI
Pancreatic fistula	10.7	7.9-13.4	23.4	19.7-27.1
Fluid collection	3.6	1.9-5.2	8.8	6.2-11.3
Sepsis	1.8	0.4-3.2	3.6	0.1-7.2
Anastomosis leakage	2.4	0.6-4.2	4.0	1.1-6.9
Death	2.8	0.7-4.8	3.3	1.0-5.5
Renal failure	1.2	0.1-2.3	1.5	0.3-2.8
Shock	2.4	0.8-4.0	2.4	0.9-4.0
Postoperative pancreatitis	1.2	0.08-2.3	2.9	1.4-4.4
Abscess	3.7	2.0-5.4	3.1	1.6-4.7
Respiratory failure	4.2	1.0-7.4	3.3	1.7-4.9
Bleeding	5.8	2.6-8.9	4.3	1.7-6.9
No complications	78.9	71.9-85.9	62.8	56.5-69.0

NOTE: All values are percentages; CI = confidence interval.

ferred option when faced with variables that are difficult to measure. The additional length of stay for patients with a fistula was estimated to be 21 days based on expert opinion. This estimation was tested with sensitivity analysis.

The expected cost of therapy associated with this model was $884 in the octreotide group and $1737 in the placebo group. The cost-effectiveness analysis reveals that in a 100-patient cohort, the use of octreotide would save an average of $853 per patient while permitting 16 incremental patients to avoid complications. Octreotide was therefore a dominant strategy, that is, more effective and less costly.

One-way sensitivity analysis was performed on the per diem cost and on the length of stay (Fig. 4). The break-even points were $76 and 2.9 days, meaning that if the cost per patient per day is equal to $76, then the use of octreotide is cost neutral. Similarly, if the incremental length of stay in patients with complications is equal to 2.9 days, it is also cost neutral to use octreotide. The probability that these instances occur, however, are very low. Two-way sensitivity analysis was performed in a similar manner. A break-even point was reached when daily cost was decreased to $300 and length of stay was decreased to 5.3 days, simultaneously.

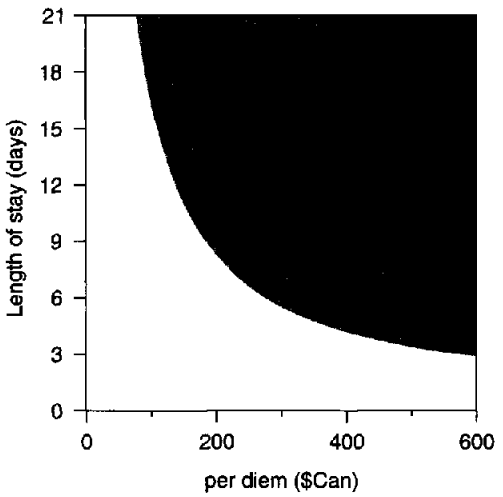

Fig. 4. Two-way sensitivity analysis of per diem and incremental length of stay.

Table III. Definition of the case costing sample

	No complications	Complications
No. of patients	18	17
Age (yr)	63.2	61.1
Sex		
M	13	11
F	5	6
Cost ($Can)	11,169	32,347
Lower limit	7558	20,882
Higher limit	14,779	43,812
Length of hospital stay (days)	17.0	28.3
Lower limit	9.4	20.9
Higher limit	24.6	35.7

Model 2

The case costing data were obtained from two Ontario hospitals that participated in the OCCP.[17] After screening all of the cases to verify all diagnoses and procedures, we collected the cost and demographic data for a total of 35 patients who had undergone pancreatic resection at these institutions between April 1, 1995, and March 31, 1996. The complication rate was 48.6%: 17 patients had complications, and 18 patients did not. These results are presented in Table III. The cost of a 7-day prophylaxis of octreotide was $197, whereas the cost of placebo was $0. Similar to model 1, it was decided that only the cost to treat a fistula would be considered a major cost driver. As in model 1, the analysis was biased against octreotide, and it was assumed that the cost to treat complications other than pancreatic fistula was the same as the cost for treating patients without complications.

The expected cost of therapy associated with model 2 was $12,568 in the octreotide group and $14,210 in the placebo group. The cost-effectiveness analysis demonstrated that in a 100-patient cohort, the use of octreotide would save an average of $1642 per patient while permitting 16 incremental patients to avoid complications. Once again, octreotide was a dominant strategy.

One-way and two-way sensitivity analyses were also performed. The cost to treat patients with and without complications as well as the rates of complication were varied through their respective confidence intervals to verify the robustness of our model. One-way and two-way analyses showed no break-even points when the cost to treat complications as well as the complication rates were varied in between their 95% confidence intervals. This suggests that the model is robust since the rate had to be varied outside of the confidence intervals in order to change the conclusions of the analysis.

DISCUSSION

Pharmacoeconomic evaluation often uses various modeling techniques that have been developed in disciplines such as epidemiology, statistics, operations research, and decision science. These techniques are

used mainly in two circumstances. First, where the relevant clinical trials have not been conducted or did not include economic data capture, then decision analytic models are used to synthesize the best available data.[27] The study presented herein is an example of such a model.

The techniques of decision analysis are commonly used by practitioners of health care economic evaluation. In summary, decision analysis for economic evaluation proceeds by careful structuring of the problem with the aid of a decision tree—a graphic schema where we begin with the decision (e.g., treatment A or treatment B) and trace out all probable pathways and consequences (e.g., health outcomes and costs) that can arise over time.[28] The model draws data from multiple sources—mainly clinical trials for probabilities such as rates of complication, administrative data for costs, and expert physician opinion concerning treatment algorithms. For a good discussion on the use of modeling in pharmacoeconomics, see Buxton et al.[27] and O'Brien.[29]

In the case of pancreatic surgery, there are many factors that can affect the outcome of the procedure. In a retrospective analysis, Lerut et al.[30] cited a significant influence of age, preoperative renal insufficiency, and urgency of surgery on the occurrence of pancreatic fistula. Montorsi et al.[10] analyzed several risk factors to conclude that preoperative comorbid disease and intraoperative requirements of blood components significantly increase the risk of overall postoperative complications. Miedema et al.[31] added to this list of risk factors the duration of the surgery. More recently, the rate of occurrence of complications has been shown to decrease with the prophylactic use of octreotide,[5,9-11] although this decrease has been difficult to quantify. This is partly due to important discrepancies between the studies. For example, Buchler et al.[5] cited a high rate of complications in the placebo group (55%),[9,10] whereas Pederzoli et al.[9] obtained a rate as low as 29.2% in their placebo group. The meta-analysis that we performed in our study yielded a complication rate of 37.2%. It is interesting to note that the complication rate in our case costing sample was 48.6%, almost as high as that reported in the study by Buchler et al.[5] However, it must be appreciated that the data are tabulated by an accounting office without the benefit of clinical input. This makes the database rather imprecise when relevant clinical information such as type of procedure, primary versus secondary diagnosis, or type of complication of surgery is involved. For the same reason it is difficult to determine whether octreotide or total parenteral nutrition, where applicable, was used prophylactically or therapeutically. This would have been interesting to analyze and to compare with our model. However, since these data were collected only to obtain their costing values for later use, these areas of bias can be minimized if one uses the right assumptions.

Comparing the costs for the two models is also of great interest. In the case costing example, patients without complications had an average cost of $11,169 and an average length of stay of 17.0 days, leading to a per diem cost of approximately $657 per patient. This demonstrates that the per diem amount used in model 1 ($552) was actually quite conservative considering that pancreatic operations generally are major procedures. For patients who did have complications, the costing data showed an average cost of $32,347 and an average length of stay of 28.3 days, for an average per diem cost of $1143. The difference between the two groups is easy to understand, since patients with complications not only take more time to be discharged, but also require more care than those without complications. This can be emphasized by searching the case costing database to retrieve patients with a fistula as a complication. Our search resulted in two patients with this specific complication. Their average cost and length of stay were, respectively, $38,007 and 39 days, for an average cost of $975 per day per patient. There were obviously no statistically significant differences between the patients with fistulas and the group with other complications and between the patients with fistulas and the group with no complications because of the small sample size. Schaefer[32] reported on 13 patients undergoing a Whipple procedure at a cost of $22,559.65 and a length of stay of 20.5 days, without considering the occurrence of complications. These figures are comparable to ours, since our average cost and length of stay were, when patient groups with and without complications were combined, $21,455 and 22.5 days. Gordon et al.,[33] in a similar study, obtained a cost of $24,478 and a length of stay 22.5 days for their patients discharged alive.

The two models used the same conservative assumptions: that pancreatic fistula is the most frequent complication and a major cost driver. The cost used in model 2 was the average cost of any complication. The other complications were presumed to cost the same as no complications. In model 1, because we were using per diem cost, we had to use data cited in the literature to estimate the additional length of stay for patients with complications. In model 2 this meant calculating the average cost for patients with and without complications. These estimations all proved to be robust after the sensitivity analysis demonstrated no change in outcomes, when values were varied through their 95% confidence intervals.

It was also assumed that the cost used in the models did not include the cost of octreotide or total par-

enteral nutrition. This assumption cannot be fully supported by case costing data because the detailed cost summary for each patient only showed which ones used octreotide and which used total parenteral nutrition, but did not indicate whether the use was prophylactic or therapeutic. Nevertheless, since octreotide and total parenteral nutrition were not considered major cost drivers, they were included in the total cost for patients who received them. Sensitivity analysis also showed that the cost-effectiveness ratio was not influenced by changes in costs, which supports this approach.

Pharmacoeconomic evaluations are meant to be a tool to assist in decision making more so from a public health or general population perspective than a bedside perspective. They are certainly not meant to be or replace the decision-making process, which should encompass a multitude of other considerations such as clinical expertise, ethics, justice, equity, or politics. By providing information derived from the best possible sources at the time of the analysis, pharmacoeconomics strives to make more explicit and evidence based the tough choices required in allocating health care resources. Ultimately, when put into practice, such an approach should allow us to achieve the best health outcomes for the most people at the lowest possible cost.

A pharmacoeconomic analysis will always be as good as the information used to produce it. If anything, the structured process of evaluating the evidence normally used in performing a pharmacoeconomic analysis, the explicit statement of assumptions, and analyzing their implications is helpful in reaching a decision. Decisions will be made regarding which drugs to include in the formulary or which procedure should be carried out. The idea here is to structure and use the available information to help in making these decisions.

CONCLUSION

The use of octreotide in prevention of complications following pancreatic surgery is a strategy that is both more effective and less costly than the use of a placebo. Since the rate of occurrence of pancreatic fistula is relatively low, it is difficult to justify the use of octreotide in all patients undergoing pancreatic surgery. However, in patients at risk for developing a complication, its administration is a cost-effective strategy. These patients would include those undergoing a pancreatic resection in whom there is a heightened expectation that a pancreatic leak will ensue, for example, a Whipple resection for a periampullary tumor with normal pancreatic parenchyma. Prinz et al.,[34] in a study of the treatment of pancreatic fistulas, even reported that some patients were discharged within 48 hours after beginning their treatment and continued their treatment as outpatients. This practice has not yet been approved nor is it current practice, but it demonstrates the potential for the use of octreotide, especially when financial issues are considered.

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Major Vascular Resection as Part of Pancreaticoduodenectomy for Cancer: Radiologic, Intraoperative, and Pathologic Analysis

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Intraoperative assessment is inaccurate in defining the relationship of a pancreatic head neoplasm to adjacent vascular structures. We evaluated the ability of preoperative contrast-enhanced CT to predict the need for vascular resection during pancreaticoduodenectomy and examined the resected vessels for histologic evidence of tumor invasion. During a 7-year period, 63 patients underwent pancreaticoduodenectomy with en bloc resection of adjacent vascular structures for a presumed pancreatic head malignancy. Clinical, radiologic, operative, and pathologic data were reviewed and analyzed. Fifty-six patients underwent resection of the superior mesenteric–portal vein confluence, three patients required inferior vena cava resection, and the hepatic artery was resected and reconstructed in eight patients. The operative mortality rate was 1.6%, and the overall complication rate was 22%. CT predicted the need for resection of the superior mesenteric or portal veins in 84% of patients. Pathologic analysis revealed tumor invasion of the vein wall in 71% of resected specimens. Tumor invasion of vascular structures adjacent to the pancreas can be predicted with preoperative CT and should alert the surgeon that vascular resection may be required. Histologic evidence of tumor cell infiltration of vessel walls was present in the majority of the resected specimens. (J GASTROINTEST SURG 1999;3:233-243.)

KEY WORDS: Pancreaticoduodenectomy, pancreatic cancer, vascular resection

Less than 50% of patients taken to surgery for planned pancreaticoduodenectomy for pancreatic cancer undergo successful tumor resection because of previously unsuspected extrapancreatic metastatic disease or the surgeon's perception that the primary tumor is unresectable because of local tumor extension to the mesenteric vessels.[1,2] The importance of avoiding laparotomy in patients with locally advanced or metastatic disease is obvious; their median survival is only 6 to 10 months, and a laparotomy without tumor resection provides no anticancer benefit but is associated with substantial morbidity, the potential for death, and a lengthy period of recovery.[3] Neoplasms in the pancreatic head may be deemed locally unresectable at laparotomy because they appear to encase the superior mesenteric artery (SMA), superior mesenteric vein (SMV), or portal vein. Palpation at the time of the Kocher maneuver is commonly done to assess the relationship of a pancreatic head tumor to the SMA.[4] When resecting small periampullary tumors, the surgeon can easily appreciate the pulse in the posterior wall of the SMA with his or her left hand. However, if the tumor is large, there is associated pancreatitis, or the patient is undergoing reoperation, palpation is an inaccurate way to assess this critical tumor-vessel relationship prior to gastric and

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pancreatic transection.[5] Similar to the SMA, intraoperative evaluation of the relationship of a pancreatic head tumor to the SMV or portal vein is also prone to inaccuracy.[6] As initially described by Moore et al.,[7] in 1951, a tumor may appear not to involve the superior mesenteric–portal vein (SMPV) confluence early in the operation yet, following pancreatic transection, may be found to be inseparable from the vein (lateral and/or posterior wall). The development of a plane of dissection between the anterior surface of the SMPV confluence and the neck of the pancreas early in the operation is a maneuver to be discarded; it provides no information relevant to the presence or absence of tumor invasion of the lateral or posterior walls of the SMV.

Preoperative high-quality contrast-enhanced CT accurately defines the relationship of a pancreatic head tumor to the SMA and celiac axis.[8,9] Because surgeons presently consider resection of the celiac axis and/or SMA inappropriate in patients with adenocarcinoma of pancreatic origin, patients in whom CT demonstrates involvement of these structures can be spared laparotomy.[1] In patients with no CT evidence of tumor extension to the celiac axis or SMA, tumor manipulation and retroperitoneal dissection in an effort to assess resectability are unnecessary prior to gastric and pancreatic transection, at which point the SMA can be directly visualized.[10] Although most surgeons outside referral centers still consider tumor extension to the SMV or portal vein also to be a contraindication to pancreaticoduodenectomy, recent data suggest that resection and reconstruction of the SMV or SMPV confluence can be performed safely by experienced surgeons; furthermore, patients who require venous resection have a survival duration no different from that of patients who undergo pancreaticoduodenectomy without vascular resection and reconstruction.[11-13] However, limited data exist on the ability of CT to accurately predict the need for venous resection at the time of pancreaticoduodenectomy.[14,15] To determine the ability of preoperative CT to predict the need for vascular resection, we reviewed our experience with vascular resection and reconstruction at the time of pancreaticoduodenectomy for malignancy. We also compared the surgeons' intraoperative assessments of venous involvement with the results of histologic assessment of tumor cell infiltration of the vessel wall. It is important to note that vascular resection was undertaken only when deemed necessary, in the opinion of the operative surgeon, to achieve a negative-margin pancreaticoduodenectomy. In contrast to the large published experience from Japan, vascular resection and reconstruction were not performed as part of a more extensive or regional pancreatectomy designed to achieve greater lymphatic clearance.

METHODS

Patients

Using the pancreatic tumor database, which prospectively records information on all patients with pancreatic tumors at our institution, we identified all patients who underwent pancreaticoduodenectomy with vascular resection and reconstruction for a presumed pancreatic head neoplasm from July 1990 to July 1997. Vascular resection was defined as segmental resection of the SMV, portal vein, or hepatic artery, or tangential resection of the SMV, portal vein, or inferior vena cava that required a saphenous vein patch. Patients who underwent tangential resection of a small section of the SMV or portal vein that was closed primarily, without a patch, were not included in this study. We also excluded from analysis all patients who underwent operations other than pancreaticoduodenectomy (i.e., total pancreatectomy or distal pancreatectomy). The majority of patients with adenocarcinoma received chemotherapy (5-fluorouracil) and radiation therapy (30 or 50.4 Gy) before (neoadjuvant) or after (adjuvant) pancreaticoduodenectomy, as previously described.[16] Following tumor resection and venous reconstruction, many patients received electron-beam intraoperative radiation therapy; 10 Gy was delivered to the bed of the resected pancreas in a dedicated radiation therapy–surgical suite.[17] For determining length of hospital stay, the day of surgery was counted as day 1.

Preoperative Imaging

Preoperative evaluation included physical examination, chest roentgenography, and contrast-enhanced CT. Angiography and laparoscopy were used selectively. To be considered for operation, patients were required to fulfill the following objective CT criteria for tumor resectability[18]: (1) absence of extrapancreatic disease; (2) no evidence of tumor extension to the SMA or celiac axis as defined by the presence of a normal fat plane between the tumor and these arterial structures; and (3) a patent SMPV confluence. Patients were defined as having vessel (SMV, portal vein, or hepatic artery) involvement by tumor when the hypodense tumor was inseparable from the vessel wall (Fig. 1). For assessment of vessel involvement, all CT scans were reviewed by a single diagnostic radiologist (C.C.) who was blinded to surgical findings and pathology results.

CT scanning was performed using the following protocol. Precontrast CT of the liver and pancreas was performed at 10 mm slice thickness after oral contrast administration of dilute Gastrografin, 2% barium sulfate, or water. Nonionic contrast material (300 mg/ml) was then delivered intravenously by an automatic injector at a rate of 2 to 3 ml/sec for a total

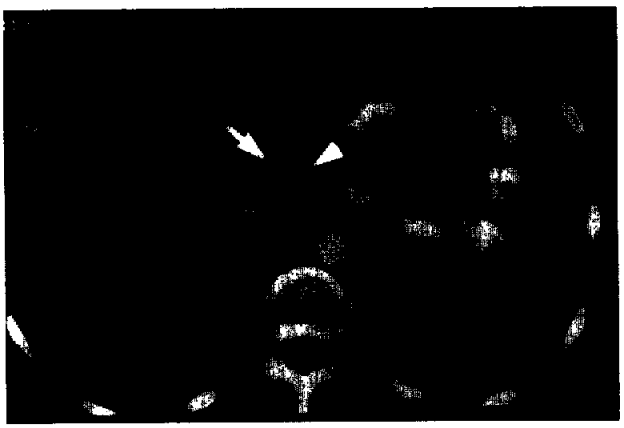

Fig. 1. Contrast-enhanced CT scan demonstrating a low-density tumor in the pancreatic head inseparable from the superior mesenteric vein *(arrow)*. A metallic clip is seen on the anterior wall of the superior mesenteric vein. The clip was placed at the patient's initial operation (prior to referral), at which time the tumor was judged unresectable because of mesenteric vessel involvement. However, the tumor does not extend to the superior mesenteric artery *(arrowhead)*. Pancreaticoduodenectomy required segmental resection of the superior mesenteric–portal vein confluence and internal jugular vein interposition grafting; pathologic evaluation of the retroperitoneal margin was negative. A biliary stent can be seen in the intrapancreatic portion of the common bile duct.

of 150 ml. When a conventional CT scanner was used, scans through the pancreas were obtained at 1.5 or 3 mm slice thickness and 5 mm scan intervals depending on the size of the patient. Scanning began 40 seconds after the start of contrast agent injection. When a helical or spiral CT scanner was used, scanning was performed at 3 or 5 mm slice thickness with a pitch factor of 1.5 to 2.0, depending on the anatomic extent of the tumor and the size of the patient. Scanning began 25 to 30 seconds after the start of contrast agent injection for the arterial phase and at 60 to 70 seconds for the portal phase. Each scan series was completed while the patient held respirations for 20 seconds. The rest of the abdomen was then scanned at 7 mm slice thickness.

Pancreaticoduodenectomy Technique

The surgical resection was divided into six clearly defined steps.[8]

1. A Cattell-Braasch maneuver was performed by mobilizing the right colon and incising the visceral peritoneum to the ligament of Treitz.[19] When complete, this maneuver allowed cephalad retraction of the right colon and small bowel, exposing the third and fourth portions of the duodenum. Mobilization of the retroperitoneal attachments of the mesentery was particularly important (in patients who required venous resection and reconstruction) to facilitate cephalad displacement of the SMV. The lesser sac was entered by taking the greater omentum off of the transverse colon. The middle colic vein was identified, ligated, and divided prior to its junction with the SMV. Routine division of the middle colic vein allowed greater exposure of the infrapancreatic SMV and prevented iatrogenic traction injury during exposure of the infrapancreatic SMV. We usually found it easier to isolate the infrapancreatic SMV prior to mobilizing ("Kocherizing") the duodenum. However, in difficult reoperative cases, we occasionally performed steps 2 and 3 prior to fully isolating the infrapancreatic SMV; in such cases we identified the SMV in a more distal location as initially described by Cameron.[20]
2. The Kocher maneuver was begun at the junction of the ureter and right gonadal vein. The right gonadal vein was ligated and divided, and all fibrofatty and lymphatic tissue overlying the inferior vena cava was elevated with the pancreatic head and duodenum to the left lateral edge of the aorta. Palpation was not used to assess the relationship of the tumor to the SMA to determine resectability. The proximity of the pancreatic head (tumor) to the SMA makes accurate assessment by palpation of this vital tumor-vessel relationship an unrealistic expectation. The relationship of the tumor to the SMA and celiac axis had been objectively defined by preoperative contrast-enhanced CT.
3. The portal dissection was initiated by exposing the common hepatic artery proximal and distal to the gastroduodenal artery, which was then ligated and divided. Encasement of a short segment of the hepatic artery was treated with segmental resection and either primary anastomosis or interposition grafting with reversed saphenous vein. Arterial resection was performed before proceeding to step 4 or at the completion of step 6 following specimen removal. The gallbladder was dissected out of the liver bed, and the common bile duct was transected just cephalad to the cystic duct–common duct junction. Following division of the common bile duct and medial retraction of the common hepatic artery, the anterior wall of the portal vein was exposed. We did not make any attempt (at this stage in the operation) to develop a plane of dissection between the anterior surface of the SMPV confluence and the posterior surface of the neck of the pancreas. Invasion of the lateral or posterior wall of the SMPV confluence by tumors of the pancreatic head or uncinate process can be directly detected only after gastric and pancreatic transection.
4. The stomach was transected at the level of the third or fourth transverse vein on the lesser curva-

ture and at the confluence of the gastroepiploic veins on the greater curvature.

5. The jejunum was transected approximately 10 cm distal to the ligament of Treitz, and its mesentery sequentially ligated and divided. The duodenal mesentery was similarly divided to the level of the aorta, and the duodenum and jejunum were reflected beneath the mesenteric vessels.
6. Following pancreatic transection, segmental resection of the SMPV confluence was performed when, in the opinion of the surgeon, the tumor was inseparable from the lateral wall of the SMV or portal vein.[11,21] It is important to note that routine resection of the SMPV confluence, as performed in regional pancreatectomy,[22] was not done; the SMV or SMPV confluence was resected only when deemed necessary to complete a negative-margin pancreaticoduodenectomy. When tumor invasion of the SMV or portal vein prevented mobilization and medial retraction of the SMPV confluence from the pancreatic head and uncinate process, access to the proximal SMA and completion of the retroperitoneal dissection (removal of the specimen from the right lateral wall of the SMA) were achieved in one of two ways: ligation and division of the splenic vein or venous resection and reconstruction. Early in our experience with segmental resection of the SMV or SMPV confluence, division of the splenic vein was routine. Division of the splenic vein at its junction with the SMPV confluence allowed access to the proximal SMA medial to the SMV and provided increased mobility of the portal vein, usually enabling a primary venous anastomosis to be constructed without tension. If the segment of SMPV confluence to be resected was 4 cm or greater, an internal jugular vein interposition graft was used. Splenic vein ligation occasionally resulted in gastrointestinal hemorrhage because of sinistral portal hypertension, and therefore we began trying to preserve the splenic vein–portal vein confluence when technically possible.[6,11] However, maintaining an intact splenic vein–portal vein junction significantly limited the mobilization of the portal vein and prevented primary anastomosis between the SMV and portal vein unless excision of the SMV was approximately 2 cm or less. Furthermore, an intact splenic vein prevented direct access to the proximal SMA, making completion of the retroperitoneal dissection impossible in most patients. This difficulty was circumvented by performing venous resection and reconstruction with autologous internal jugular vein prior to completion of the retroperitoneal dissection and removal of the specimen.[13] However, vascular reconstruction prior to specimen removal can be more difficult because of the limited exposure created by the specimen remaining in situ. We developed an alternative technique for dissection of the specimen from the right lateral border of the SMA prior to venous resection and reconstruction.[23] This allowed separation of the specimen from the SMA and retroperitoneal tissues, leaving the specimen attached only to the SMPV confluence.

Reconstruction of the Superior Mesenteric–Portal Vein Confluence

Reconstruction of the SMPV confluence was performed using one of four techniques. Patients requiring tangential resection of less than one third of the circumference of the SMPV confluence underwent repair with an autologous saphenous vein patch. Patients requiring segmental resection of the SMV or SMPV confluence had reconstruction with an autologous internal jugular vein interposition graft or with a primary anastomosis without interposition grafting; early in our experience a single patient received a Gore-Tex (W.L. Gore & Associates, Flagstaff, Ariz.) interposition graft. Inflow occlusion (SMA) during the time of venous resection and reconstruction was commonly performed to prevent small bowel edema; systemic heparinization was used at the discretion of the operating surgeon. Venous anastomoses were all completed using 6-0 Prolene (Ethicon Inc., Somerville, N.J.) sutures. Early in our experience, the posterior wall was completed with a running suture; however, most anastomoses are currently completed entirely with interrupted sutures. All patients who underwent venous resection were begun on aspirin within 24 hours of surgery and were requested to take low-dose aspirin (80 mg daily) indefinitely.

Pathologic Analysis of the Resected Specimen

For pathologic analysis, the operative specimen was oriented and dissected by the surgeon and pathologist in a pathology suite in the operating room complex.[24] The retroperitoneal margin was defined as the soft tissue margin directly adjacent to the proximal 3 to 4 cm of the SMA. A 2 to 3 mm, full-face (en face) section of the margin was evaluated by frozen-section microscopic examination, and the margin was interpreted as positive if tumor was seen on this section. Tumor size was calculated following surgical resection by measuring the greatest transverse diameter of the tumor. In patients who had received preoperative chemoradiation, this was often difficult; in some specimens, gross tumor could not be demarcated from uninvolved pancreatic parenchyma. In late

Fig. 2. Histologic section demonstrating tumor invasion of the tunica media of a segment of resected superior mesenteric vein. Malignant gland formation can be seen in the tunica media; the vessel lumen is to the right.

1993, a standardized system for the pathologic evaluation of pancreaticoduodenectomy specimens was established. It included histologic assessment of the segment of resected vein to determine the presence or absence of tumor cell infiltration of the vein wall, defined as the presence of neoplastic cells within the tunica adventitia and/or tunica media of the vein wall[24] (Fig. 2). On completion of pathologic analysis, all cases were reviewed by a single histopathologist (K.R.C.).

RESULTS

Pancreaticoduodenectomy was performed in 204 patients and 63 (31%) required en bloc resection of the SMV or SMPV confluence (n = 56), inferior vena cava (n = 3), and/or hepatic artery (n = 8). Patient demographics are presented in Table I. Most patients had adenocarcinoma of pancreatic origin, and 76% received preoperative or postoperative chemoradiation. Three patients were found to have chronic pancreatitis on final pathologic evaluation of the resected specimen. All three patients underwent pancreaticoduodenectomy for presumed periampullary carcinoma; intraoperative attempts at mobilizing the SMPV confluence were unsuccessful. In one patient, marked lymphocytic infiltration and fibrosis extended into the wall of the SMV.

Operative characteristics, perioperative complications, and pathologic findings are listed in Table II. There was one perioperative treatment-related death;

Table I. Patient demographics

Variable	No. of patients (%)
Total	63
Sex	
Male	36 (57)
Female	27 (43)
Median age	63 years
Histologic findings	
Adenocarcinoma	52 (83)
Pancreas	49
Bile duct	2
Duodenum	1
Neuroendocrine carcinoma	5 (9)
Benign disease	3 (4)
Other*	3 (4)
Reoperative pancreaticoduodenectomy	
Yes†	17 (27)
No	46 (73)
Adjuvant therapy	
Preoperative chemoradiation	36 (57)
Postoperative chemoradiation	12 (19)
No adjuvant therapy	15 (24)
Intraoperative radiation therapy	
Yes	24 (38)
No	39 (62)

*Serous (microcystic) neoplasm of the pancreas, retroperitoneal sarcoma, and lymphoma (one each).

†Patients who had undergone abdominal laparotomy for planned pancreatic resection prior to referral, excluding patients who had undergone recent abdominal or biliary surgery for reasons other than resection of a pancreatic head tumor and patients who had undergone minilaparotomy for biopsy alone.

Table II. Operative characteristics, perioperative complications, and pathologic findings

Median operative blood loss (ml)	1950
Median operative time (hr)	10.8
Median hospital stay (days)	14.5
Perioperative deaths*	1 (1.6%)
Perioperative complications	14 (22%)
Reoperation	1
Myocardial infarction	2
Pancreaticojejunal anastomotic leak†	2
Intra-abdominal abscess	2
Hepatic abscess	1
Pulmonary complications (pneumonia, aspiration)	6
Gastrointestinal bleeding (marginal ulcer)	3
Superficial wound infection	3
Median tumor size (cm)	3.8
Microscopically positive retroperitoneal margin	
Total	8 (13%)
Adenocarcinoma of pancreas	6/49 (12%)
Positive lymph nodes	
Total	26 (41%)
Adenocarcinoma of pancreas	25/49 (51%)

*In-hospital or within 30 days of surgery.
†A (clinical) pancreatic anastomotic leak was defined as drainage of amylase-rich fluid in association with fever, leukocytosis, sepsis, or the need for percutaneous drainage.

Table III. Method of vascular reconstruction

Vessel resected and method of reconstruction	No. of patients
SMV or SMPV confluence	56
Saphenous vein patch	8
Primary end-to-end anastomosis with ligation and division of the splenic vein	16
Primary end-to-end anastomosis without ligation of the splenic vein	5
Internal jugular vein interposition graft	26
Preservation of splenic vein confluence	19
Resection of splenic vein confluence	7
Gore-Tex interposition graft	1
Inferior vena cava	3
Saphenous vein patch	2
Gore-Tex interposition graft	1
Hepatic artery	8
Primary anastomosis	4
Reverse saphenous vein interposition graft	4
TOTAL	67*

SMV = superior mesenteric vein; SMPV = superior mesenteric–portal vein.
*Four of the 63 patients had resection and reconstruction of two vascular structures resulting in a total of 67 vessel reconstructions.

prior to referral, the patient had undergone two previous operations and received radiation therapy (including external-beam and intraoperative radiation therapy) and chemotherapy for a localized adenocarcinoma of duodenal origin. Definitive tumor extirpation required en bloc resection of the SMV. A subclinical leak at the pancreaticojejunostomy led to sudden gastroduodenal artery stump blowout approximately 5 weeks after pancreaticoduodenectomy, resulting in exsanguination from intra-abdominal and gastrointestinal hemorrhage. All other patients were successfully discharged from the hospital after a median hospital stay of 14.5 days.

Twenty-two percent of patients had perioperative complications, but only one patient had a complication related to the vascular resection and reconstruction. This patient had required resection of the hepatic artery, and reconstruction was completed with a reverse saphenous vein graft from the aorta. Because of coexistent hereditary dysfibrinogenemia, the patient was given anticoagulant therapy postoperatively. Bleeding at the aortic anastomosis necessitated reoperation. His postoperative course was further complicated by segmental areas of hepatic necrosis and resulting abscess formation secondary to embolic events following arterial reconstruction. This patient's underlying hypercoagulable state probably contributed to the complications. He nevertheless recovered and was able to receive postoperative adjuvant 5-flourouracil–based chemoradiation. There were no other complications clearly related to vascular resection and reconstruction.

No patient was found to have a grossly positive retroperitoneal margin of excision. A microscopically positive retroperitoneal margin was found in only 13% of cases.

In patients who underwent venous resection, left internal jugular vein interposition grafting was the most common method of reconstruction (Table III). The infrequent use of tangential excision with saphenous vein (patch) repair suggests that most patients had apparent tumor involvement of greater than one third of the circumference of the vein wall.

The need for venous resection was predicted by preoperative CT in 84% of patients who underwent resection and reconstruction of the SMV or SMPV confluence (Table IV). In the 49 patients with adenocarcinoma of the pancreas, 42 (88%) of 48 evaluable CT scans predicted the need for venous resection (the

Table IV. Radiologic and pathologic evidence of vascular invasion

Vascular structure resected	No. of patients (%)		
	Total	Invasion seen on CT	Invasion seen on histologic examination
SMV or SMPV confluence	56	46/55 (84)*	24/34 (71)†
Inferior vena cava	3	2/3 (67)	1/2 (50)
Hepatic artery	8	5/8 (63)	NA

SMV = superior mesenteric vein; SMPV = superior mesenteric–portal vein; NA = not available.
*CT scans were not available for re-review in 1 of the 56 patients.
†Includes only patients who underwent segmental venous resection (saphenous vein patch reconstructions excluded) and pathologic analysis of the resected vessel.

hypodense tumor was inseparable from the vessel wall). Following the adoption of a standardized pathologic examination method, the most recent operative specimens from patients who required segmental venous resection were evaluated for histologic evidence of tumor invasion of the wall of the SMV or SMPV confluence (see Table IV). Neoplastic cells were found to invade the vein wall in 24 (71%) of 34 specimens. In cases of adenocarcinoma of pancreatic origin, neoplastic cells penetrated into at least the tunica adventitia in 19 (66%) of 29 specimens.

DISCUSSION

The management of tumor adherence to the lateral wall of the SMV or SMPV confluence represents the most challenging technical aspect of pancreaticoduodenectomy. This finding is often unexpected and not visible until after pancreatic and gastric transection, a point in the operation at which the surgeon has committed to resection. When confronted with this finding intraoperatively, the surgeon has three options: (1) leave tumor attached to the vein, resulting in a grossly positive margin of resection; (2) continue with dissection of the vein in an attempt to separate the vein from the pancreatic tumor (this, however, often results in venous injury, uncontrolled hemorrhage, and the necessity for rapid and oncologically unsatisfactory removal of the primary tumor); or (3) perform a partial or segmental resection of the involved venous segment with reconstruction. When tumor is left on the lateral wall of the SMV or SMPV confluence, local recurrence and short survival are to be expected. Recent data have clearly demonstrated that patients who undergo pancreaticoduodenectomy with a positive margin of resection have a survival duration similar to that of patients who have locally advanced disease treated nonsurgically with 5-fluorouracil–based chemotherapy and irradiation.[25-30] As demonstrated in this article, partial or segmental venous resection and reconstruction are the ideal means of managing tumor adherence to the SMV or SMPV confluence. In our series, treatment-related death occurred in only one patient, and complications were similar to those in other series of patients treated with pancreaticoduodenectomy without vascular resection. However, blood loss was higher than in our published experience with standard pancreaticoduodenectomy,[11] and operative times were long, suggesting that vascular resection adds significantly to the complexity of pancreaticoduodenectomy, even when performed at a specialty center.

Preoperative high-quality contrast-enhanced CT suggested the need for resection of the SMV or SMPV confluence in 84% of patients in this series (see Table IV). Although histologic evidence of tumor invasion of the resected segment of SMV or SMPV confluence was present in only 71% of specimens, in all cases venous resection was deemed necessary by the operating surgeon based on the intraoperative determination that the SMPV confluence (or other vessel) could not be separated from the pancreatic neoplasm. In agreement with our data, Furukawa et al.[14] also found that the relationship of the tumor to the SMPV confluence as seen on high-quality contrast-enhanced CT can accurately predict the need for venous resection. When the low-density tumor is inseparable from the SMPV confluence and forms a concavity against the vessel[15] or involves more than 25% of the circumference of the vein,[14] surgery should be considered only by surgeons experienced in venous resection and reconstruction. The standard preoperative evaluation of patients with malignant tumors of the pancreas should routinely involve contrast-enhanced CT to assess the relationship of the tumor to the SMV, SMPV confluence, SMA, and celiac axis.

As seen in Table III, interposition grafting with the internal jugular vein was the most common method of reconstruction of the SMV or SMPV confluence. This is because we prefer to preserve the splenic vein–portal vein junction whenever possible. Sinistral

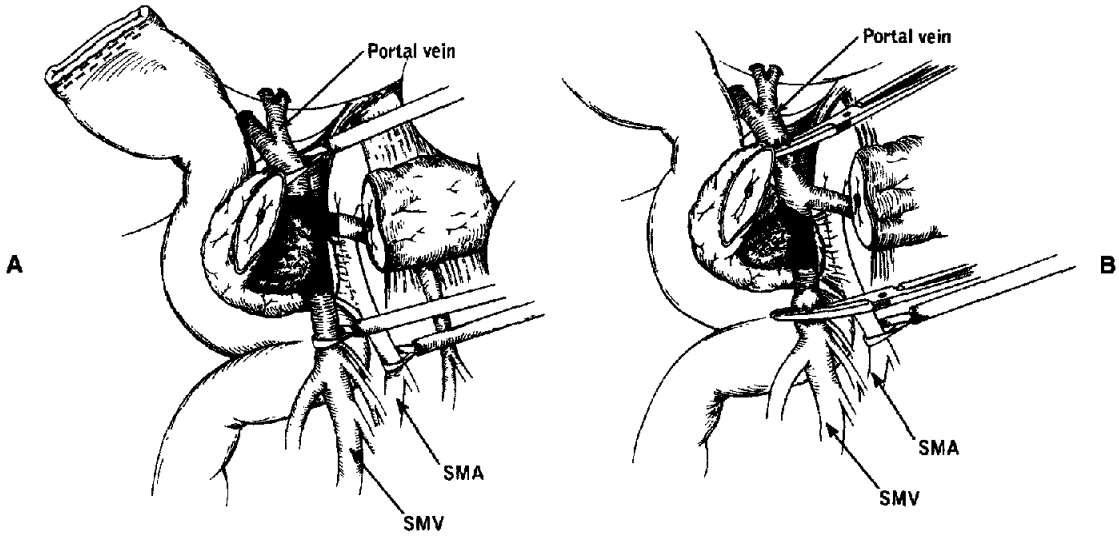

Fig. 3. Technique of venous resection. **A,** Segmental resection of the superior mesenteric–portal vein confluence when tumor involves a long segment of the vein including the splenic vein confluence. In such a situation there is no good option for splenic vein preservation. SMA = superior mesenteric artery; SMV = superior mesenteric vein. **B,** In contrast, isolated involvement of the superior mesenteric vein can be treated with segmental venous resection with preservation of the splenic vein confluence. Unless the segment of resected vein is less than 2 cm, an interposition graft is used; we prefer to use internal jugular vein.

portal hypertension resulting in upper gastrointestinal hemorrhage is rare but can occur with splenic vein ligation.[6,11] The intact splenic vein–portal vein junction significantly limits mobilization of the portal vein and prevents primary anastomosis between the SMV and portal vein unless excision of the SMV is limited to less than 2 cm. Therefore splenic vein preservation results in the need for interposition grafting for most segmental resections of the SMV. However, extensive tumor involvement of the SMPV confluence (Fig. 3) often makes splenic vein preservation impossible. In such cases, which usually require resection of a long segment of SMPV confluence (and interposition grafting), we ligate the splenic vein. It is a mistake to accept less than a perfect SMV–portal vein anastomosis (with or without an interposition graft) in an attempt to preserve the splenic vein–portal vein junction.

Direct extension of a pancreatic head neoplasm to the anterior surface of the inferior vena cava is uncommon. Only three patients who had primary or recurrent tumors that were otherwise resectable required resection of the inferior vena cava. In two patients, saphenous vein was used as an onlay patch (Fig. 4), and in one patient with a gastrointestinal stromal sarcoma, the inferior vena cava was resected and replaced with a reinforced Gore-Tex interposition graft. We prefer to complete all venous anastomoses using autologous tissue (saphenous vein or internal jugular vein) with interrupted 6-0 Prolene sutures; however, a running suture is needed when interposing a Gore-Tex graft.

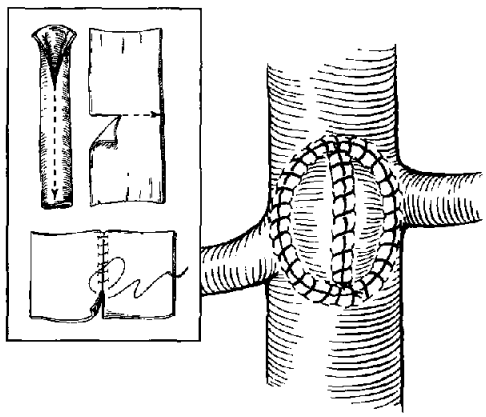

Fig. 4. Resection and reconstruction of the anterior wall of the inferior vena cava using saphenous vein. *Inset* shows how saphenous vein can be used to close a large defect in the anterior wall of the inferior vena cava.

The need for hepatic arterial resection and revascularization is also uncommon. We do not consider operation in patients with direct tumor extension to the celiac axis origin. However, in the absence of direct extension of tumor to involve the SMA or celiac axis, tumor encasement of the hepatic artery can occur in an isolated fashion, most commonly at the origin of the gastroduodenal artery. Isolated encasement

Table V. Reports of venous resection during pancreaticoduodenectomy or total pancreatectomy (excluding the Japanese experience)

Reference	No. of patients	Mortality rate (%)	Median survival (mo)	Positive retroperitoneal margin (%)
Sindelar[32] (1989)	20	20	12	NA
Trede et al.[27] (1990)	12	0	NA	NA
Launois et al.[33] (1993)	9	0	6.1*	NA
Allema et al.[34] (1994)	20†	15	8	20‡
Yeo et al.[29] (1995)	10	NA	NA§	NA
Fortner et al.[35] (1996)	51	9	NA	‖
Harrison et al.[12] (1996)	50	6	13	24¶
Roder et al.[36] (1996)	22	0	8	68¶
Leach et al.[13] (1998)	31	0	22	13

NA = not available.
*Mean.
†Eight of 20 had adenocarcinoma of the distal bile duct or ampulla of Vater.
‡Seventeen (85%) of 20 had positive resection margins overall.
§Three-year survival rate was 13%.
‖Patients with positive margins were excluded from the study.
¶Site of positive margin not defined.

of a short arterial segment at this level is treated with segmental arterial resection. Because of the redundancy of the hepatic artery, a primary anastomosis is often possible; if not, a reverse saphenous vein interposition graft is used. In more complicated reoperative cases, often in patients who have been heavily treated with radiation and chemotherapy, the proximal common hepatic artery may prove to be an unsuitable vessel for anastomosis. In such cases a saphenous vein graft can be taken directly off of the aorta just inferior to the left renal vein. We routinely cover arterial grafts with the falciform ligament or omentum, in the same way we cover the stump of the gastroduodenal artery when arterial resection is not performed, so as to separate these vessels from the afferent limb used for the pancreatic and biliary anastomoses. This is done to prevent arterioenteric fistula, which although rare is a known complication of pancreaticoduodenectomy.[31]

An aberrant hepatic artery arising from the SMA courses posterior to, or through, the head of the pancreas and is prone to tumor encasement at the posterosuperior border of the pancreatic head. An aberrant right hepatic artery may arise from the SMA as an accessory right hepatic artery, in which case a normal right hepatic artery also arises from the celiac axis; ligation of the accessory artery in such a case would not affect hepatic or bile duct arterial flow. In contrast, the right hepatic artery may arise from the SMA as a replaced right hepatic artery, or the entire common hepatic artery may arise from the SMA. Because the proximal bile duct receives virtually all of its arterial supply from the right hepatic artery following interruption of cephalad flow from the gastroduodenal artery, we prefer to perform revascularization in patients who have a replaced right hepatic artery when tumor involvement of this vessel requires resection. Resection of a replaced common hepatic artery would also require revascularization. Reconstruction is performed with a reverse saphenous vein interposition graft. The proximal anastomosis can be done at the origin of the replaced hepatic artery (from the SMA) or directly at the anterior surface of the aorta just inferior to the left renal vein.

Our previous work demonstrated that patients with adenocarcinoma of the pancreatic head who required resection of the SMV or SMPV confluence at the time of pancreaticoduodenectomy had a survival duration no different from that of patients who underwent pancreaticoduodenectomy without vascular resection.[13] This is consistent with the hypothesis that tumor adherence to, or invasion of, the SMPV confluence is a function of tumor location and possibly tumor size but not an adverse prognostic factor associated with early tumor recurrence and short patient survival. Other reports of venous resection during pancreaticoduodenectomy are difficult to interpret because they lack information about the retroperitoneal margin of resection. However, the high frequency of positive margins in reported patients who underwent venous resection (Table V[12,13,27,29,32-36]) suggests that many of these patients had locally advanced tumors with arterial encasement and were poorly selected for pancreaticoduodenectomy. Dis-

tinction between tumor involvement of the SMA and of the SMV is critical. Venous resection and reconstruction should be performed as part of pancreaticoduodenectomy only when pretreatment imaging studies and intraoperative findings reveal isolated involvement of the SMV or portal vein. Under these circumstances, venous resection can allow one to achieve a negative-margin resection, as demonstrated by our data. Venous resection should not be performed when pretreatment imaging demonstrates tumor extension to the SMA or celiac axis; pancreaticoduodenectomy with a grossly positive retroperitoneal margin, performed as part of either standard pancreaticoduodenectomy or extended regional pancreaticoduodenectomy, is associated with a median survival of less than 1 year.[1,21] Reports of regional pancreatectomy from Japan continue to confirm the belief that venous resection has no impact on survival duration if the patient is left with a positive margin.[37]

In summary, our data demonstrate that the need for venous resection at the time of pancreaticoduodenectomy for presumed or biopsy-proved malignancy can be predicted by high-quality CT. Furthermore, histologic evidence of tumor cell infiltration of the vein wall will be found in most resected specimens. Vascular resection and reconstruction at the time of pancreaticoduodenectomy add significant complexity to an already lengthy operation and should be performed at centers with experience and expertise in complex pancreatic surgery.

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Discussion

Dr. D. Fromm (Detroit, Mich.). Did you, in fact, state that there was no significant difference in survival between vein resection and no vein resection? So why go through all this?

Dr. D. Evans. These are situations where we divide the pancreas, divide the duodenum or stomach, and the vein is inseparable from the tumor.

Dr. Fromm. I understand, but if it doesn't make any difference in survival, why not just leave that little bit of tumor behind?

Dr. Evans. There are clear data in the literature that patients with positive resection margins do poorly; they have a median survival of 9 to 10 months, which is no different than the median survival of patients treated with no surgery and 5-fluorouracil and radiation.

Dr. W. Nealon (Galveston, Tex.). I have wondered a bit about your rate of vein resection. Out of 190 patients, 68 required vein resection. Knowing that you often use neoadjuvant radiation therapy, have you been concerned that radiation therapy when used preoperatively may create an inflammatory reaction and play a role in adhesion to the vein. Could radiation lead to a relatively higher rate of histologic noninvolvement?

Dr. Evans. I think it is reasonable to consider whether radiation therapy or previous laparotomy plays a role in these results, since 30% of the patients who underwent resection of the SMV or portal vein had no histologic evidence of venous involvement.

Therapy for Microcirculatory Disorders in Severe Acute Pancreatitis: Effectiveness of Platelet-Activating Factor Receptor Blockade vs. Endothelin Receptor Blockade

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Many of the complications of severe acute pancreatitis are the result of the amplifying effects of microcirculatory disruption. The factors causing microcirculatory disorders in acute pancreatitis involve vasoactive mediators such as platelet-activating factor (PAF) and endothelin-1 (ET) activated during the inflammatory response to pancreatic injury. To further evaluate the potential therapeutic role of specific receptor antagonists (RA) to these mediators, the present study compares the effect of PAF and ET receptor blockade on microcirculation and organ function in a well-established rodent model of severe acute pancreatitis. Six hours after acute pancreatitis induction, rats were randomized to therapy with ET-RA (50 mg/kg LU-135252), PAF-RA (82 μg/kg WEB-2170), or NaCl 0.9% (volume equivalent). After 18 hours of fluid resuscitation, animals were relaparotomized for intravital microscopic determination of capillary blood flow, leukocyte rolling, and capillary permeability in the pancreas and colon. Other measurements included cardiorespiratory parameters, hematocrit, pleural effusions, ascites, urine production, and survival. Compared to saline treatment both ET-RA and PAF-RA significantly improved capillary blood flow in the pancreas and colon, reduced leukocyte rolling, and stabilized capillary permeability. The beneficial effects of receptor antagonist treatment on microcirculation were associated with decreased fluid loss into the third space, improved renal and respiratory function, and survival. Although both receptor antagonists likewise improved capillary blood flow, ET-RA was significantly more effective in counteracting leukocyte rolling and capillary leakage, thereby further reducing fluid sequestration. The present study confirms the beneficial effects of PAF and ET receptor blockade on microcirculation inside and outside the pancreas, organ function, and survival when given at the early stage of severe pancreatitis. Because ET-RA was more effective in stabilizing capillary permeability and avoiding subsequent fluid loss into the third space, we propose that ET-RA should be tested in a clinical trial (either in comparison or in addition to PAF-RA). (J GASTROINTEST SURG 1999;3:244-251.)

KEY WORDS: Acute pancreatitis (experimental), microcirculation, vasoactive mediators, endothelin, platelet-activating factor

Microcirculatory disorders are a hallmark of severe acute pancreatitis. They are not confined to the pancreas, where they may promote acinar cell necrosis, but also have been demonstrated in the colon, lung, and liver, where they are believed to contribute to organ dysfunction.[1-4] Assuming that many of the complications and much of the mortality of acute pancreatitis result from the amplifying effects of organ-specific microcirculatory impairment, improving microcirculation appears to be a logical therapeutic approach. In addition to adequate fluid resuscitation, which is essential for compensating hypovolemia and hemoconcentration, novel strategies are aimed at inhibiting the vasoactive substances that cause vasoconstriction, higher vascular permeability, and pathologic interaction between blood cells and the endothelium.

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Vasoactive substances activated during acute pancreatitis include cytokines such as interleukins (IL-1, IL-6, and IL-8), tumor necrosis factor, and platelet-activating factor (PAF), as well as secondary mediators such as bradykinin, prostaglandin, endothelin, and nitric oxide.[5-8]

This study is part of a series evaluating which vasoactive mediator inhibitors are the most beneficial in severe acute pancreatitis. It also investigates whether the amelioration of disturbed microcirculation is accompanied by improved tissue morphology and organ function and what microcirculatory component (capillary blood flow, capillary permeability, or leukocyte-endothelial interaction) is mainly involved. The study applies a well-established rat model of acute necrotizing pancreatitis that has been proven suitable for evaluating therapy in a number of previous studies,[9-11] and involves extended organ monitoring and intravital microscopy with a novel computer-assisted image analysis system for quantitative assessment of microcirculation.[12] The tested vasoactive mediator inhibitors are a specific endothelin-1 (ET) antagonist previously shown to be most effective in this model of acute pancreatitis[13] and a platelet-activating factor (PAF) antagonist comparable to that currently being tested in severe human pancreatitis.[14]

MATERIAL AND METHODS

Animal Preparation, Induction of Pancreatitis, Randomization, and Measurements

All experiments were conducted in accordance with the national guidelines for the use and care of laboratory animals and approved by the local ethics committee. After overnight fasting, 30 male Sprague-Dawley rats (330 ± 20 kg) housed individually in metabolic cages were anesthetized with intraperitoneal pentobarbital (20 mg/kg) and ketamine (40 mg/kg). Polyethylene catheters (inside diameter 0.5 mm) were inserted into both jugular veins and the left carotid artery, subcutaneously tunneled to the neck, and advanced through a steel tether, which allowed blood sampling and intravenous access in the unrestrained animals.

Severe (necrotizing) acute pancreatitis was induced by a standardized retrograde infusion of 0.5 ml of 10 mmol/L glycodeoxycholic acid (GDOC; Sigma, St. Louis, Mo.) into the biliopancreatic duct for 10 minutes, followed by intravenous infusion of 5 μg/kg/hr cerulein (Farmitalia, Freiburg, Germany) over 6 hours.

After 6 hours of acute pancreatitis induction and monitoring of cardiorespiratory parameters, blood was drawn to determine hematocrit and trypsinogen activation peptides (TAP) in plasma. Thereafter animals were randomized to therapy with the specific ET antagonist LU 135252[15,16] (Knoll AG, Ludwigshafen, Germany; 50 mg/kg; slow intravenous injection; n = 9), the PAF antagonist WEB 2170 (Boehringer, Ingelheim, Germany; 50 μg/kg initial intravenous bolus plus 1.8 μg/kg/hr continuous infusion for 18 hours; n = 9), or normal saline solution (volume equivalent; n = 12).

After 24 hours (18 hours after the start of therapy), during which animals received continuous fluid resuscitation with Ringer's lactate (6 ml/kg/hr), cardiorespiratory and laboratory parameters were reevaluated, and animals were relaparotomized to determine ascites and perform intravital microscopy. Other measurements included determination of pleural effusion at autopsy, urine collection from the metabolic cages, and histologic examination of pancreatic specimens harvested at autopsy.

Healthy sham-operated animals (intraductal and intravenous saline infusion) treated with NaCl, ET-RA and PAF-RA served as additional controls (data not shown).

Therapy

The dosage and types of antagonist administration were chosen according to the references in the literature and the manufacturers' recommendations, and had been previously tested in a series of pilot studies.

The LU 135252 dose used (50 mg/kg) is known to selectively block endothelin A receptors mainly found on vascular smooth muscle cells, which selectively bind ET-1 (over ET-2). It is well tolerated in rats and affects the capillary blood flow in various organ beds without significantly changing systemic hemodynamic parameters.[15,16] The WEB 2170 dose has been shown to effectively block PAF-induced platelet aggregation ex vivo and capillary permeability without changing cardiorespiratory parameters in vivo.[17,18] Since maximal PAF inhibition by WEB 2170 is limited to a few hours (half-life approximately 1 to 2 hours), WEB 2170 was given continuously using a Harvard infusion pump, whereas LU 135252 providing ET-A receptor blockade for at least 12 hours was injected only once after induction of acute pancreatitis.

Assessment of disease severity included the amount of trypsinogen activation and the extent of acinar cell necrosis as indicators of pancreatic injury, as well as systemic measurements representing the animals' fluid status and organ functions. TAPs in plasma reflecting premature intrapancreatic protease activation were measured 6 hours after the start of acute pancreatitis induction (before therapy) and at the end of the observation period using an enzyme-linked immunosorbent assay with affinity-purified rabbit anti-

TAP antibodies.[19] Pancreatic acinar cell necrosis was evaluated by histologic examination and scored according to previously described critieria.[9] Fluid sequestration into the third space was estimated from repeated hematocrit measurements and the amount of free fluid collected from the abdomen before intravital microscopy and the thorax at autopsy. Cardiorespiratory function was assessed by monitoring arterial blood gases, mean arterial pressure, and heart rate at several time points during the experiment (0, 6, 12, 18, and 24 hours before and after intravital microscopic examination) and renal function by collecting urine from the metabolic cages and determining creatinine levels in urine and serum. (NOTE: Creatinine data were not of any informational value because of high standard deviations.)

For intravital microscopy, animals were placed on a heated operating table. The abdomen was opened through a small midline incision. First, the duodenum and the head of the pancreas were mobilized and exteriorized, placed in an immersion chamber with Ringer's lactate maintained at 37° C, and positioned under a fluorescence microscope (Leitz, Wetzlar, Germany) with a heat protection and excitation filter (450 to 490 nm) connected to a video recorder. Following pancreas exposure, 0.5 ml/kg erythrocytes labeled with fluorescein isothiocyanate (FITC; Sigma, Deisenhofen, Germany) was injected intravenously, and after a 5-minute stabilization period, three randomly chosen regions in the head of the pancreas (400 × 325 μm) were recorded for off-line analysis of pancreatic capillary blood flow. Thereafter the duodenal loop was repositioned and the ascending colon exposed for assessment of colonic capillary blood flow in the mucosa. Following the assessment of capillary blood flow, leukocyte-endothelial interaction was recorded in venules of the adjacent mesentery after injection of 1 ml/kg 0.02% rhodamine (Sigma, Deisenhofen, Germany). Leukocyte rolling, defined as the number of cells passing a previously defined vessel segment within 1 minute, was assessed off line in 8 to 10 video segments per animal. Leukocyte sticking (defined as the number of cells attached to the endothelial lining with no movement over a longer observation period) was not assessed in this experiment to limit recording time. Capillary permeability was determined in the ascending colon following an injection of 0.2 ml of 5% FITX-Dextran 150 (Sigma, Deisenhofen, Germany; molecular weight 150,000 daltons) by quantifying the increase in perivascular fluorescein intensity in the same field (5 fields per animal) over 30 minutes by means of CAP-Image (Zeintl, Heidelberg, Germany).[12] This novel computer-assisted video frame analysis system for dynamic capillaroscopy allows the off-line analysis of a variety of microcirculatory parameters and calculates capillary permeability from the changes in perivascular density caused by extravasation of the fluorescent-labeled dextran (molecular weight 150,000 daltons) over a defined observation period. Details of the equipment, techniques, and methods of calculating the microcirculatory parameters have been described elsewhere.[4,20,21]

Because continuous extravasation of FITX-dextran causes halation of the microscopic picture, which compromises further measurements, capillary permeability can only be determined at the end of the experiment in one organ bed per animal. We chose the colon (rather than the pancreas, which we examined in previous studies) because its extensive surface and capillary density make it a major site of fluid extravasation in capillary leakage syndrome. The total time needed for exposure and recording of the microcirculatory beds was 90 to 120 minutes per animal. Heart rate, arterial pressure, and blood gases were measured before and after intravital microscopy. Only data from the animals with stable cardiorespiratory conditions were included in the analysis of the microcirculatory parameters to avoid bias possibly resulting from systemic cardiorespiratory derangement. Exclusion criteria were mean arterial pressure <80 mm Hg, pO_2 <80 mm Hg, pCO_2 >50 mm Hg, and pH <7.3 or >7.5.

Forty-eight-hour survival was determined in an additional set of 36 animals with severe acute pancreatitis receiving ET-RA, PAF-RA, or saline solution (according to the same protocol but without intravital microscopy). These animals received fluid resuscitation with Ringer's lactate in the first 24 hours, and were given free access to water and food thereafter.

All results are expressed as mean ± standard error of the mean (SEM). Variables were tested for group differences with the Student's *t* test, Mann-Whitney rank-sum test, and chi-square test when appropriate. A P value ≤ 0.05 was considered significant.

RESULTS

Deaths and Dropouts

Four of 12 saline-treated animals died before intravital microscopy at 24 hours (experiment 1); another two developed signs of severe cardiorespiratory distress during intravital microscopy and were subsequently excluded from further analysis (see exclusion criteria). With PAF-RA and ET-RA, one animal died and one had to be excluded in each group. Forty-eight-hour mortality (experiment 2) was 50% in animals with severe acute pancreatitis treated with saline, and 25% and 16% in animals treated with PAF-RA and ET-RA, respectively ($P = 0.15$ NaCl vs. ET-RA).

Table I. Target parameters 18 hours after therapy with normal saline, WEB 2170 (PAF-RA), or LU 135252 (ET-RA) (mean ± SEM)

	Saline (n = 8)	PAF-RA (n = 8)	ET-RA (n = 8)
MAP (mm Hg)	115 ± 6	114 ± 7	116 ± 4
pO_2 (mm Hg)	89 ± 3	96 ± 8	97 ± 5
pH	7.44 ± 0.02	7.38 ± 0.03	7.39 ± 0.02
Urine (ml)*	2.9 ± 0.7	3.9 ± 1.1	4.8 ± 0.6
Hematocrit (%)	45 ± 1	40 ± 1†	38 ± 1†
Ascites (ml)	8.0 ± 0.5	6.7 ± 0.7	5.3 ± 0.6†
Pleural effusion (ml)	4.0 ± 0.4	2.7 ± 0.5	1.5 ± 0.4†
TAP (nmol/L)	4.5 ± 0.9	3.3 ± 0.7	3.8 ± 0.6
Necrosis (points)	2.8 ± 0.2	2.5 ± 0.2	2.6 ± 0.2

PAF-RA = platelet-activating factor receptor antagonist; ET-RA = endothelin-1 receptor antagnoist; MAP = mean arterial pressure; TAP = trypsinogen activation peptides.
*Collected between 6 and 24 hours.
†$P < 0.05$ compared to normal saline.

Systemic Parameters

Compared to healthy controls, animals with severe acute pancreatitis developed a significant decrease in urine production and an increase in hematocrit and plasma TAP (data not shown). The comparison of mean arterial pressure, respiratory parameters, urine production, hematocrit, and TAP in plasma before the administration of the test substances showed no significant group differences, indicating that the severity of pancreatitis was comparable in all animals at the time of randomization (data not shown).

At 24 hours (18 hours after the start of therapy), animals treated with either PAF-RA or ET-RA had a higher urine output, lower hematocrit, and less ascites and pleural effusion than animals given normal saline solution (Table I). Whereas only two animals given PAF-RA or ET-RA showed signs of cardiorespiratory distress (2 of 16), four out of eight saline-treated survivors had a pO_2 <80 mm Hg, pCO_2 >50 mm Hg, and/or a pH indicating (respiratory) alkalosis at at least one point during the follow-up period (two of which had to be excluded from intravital microscopy according to the defined criteria).

Local Parameters

TAP values determined in plasma at the end of the experiment were lower than those measured after 6 hours of acute pancreatitis induction but did not differ between the treatment groups. Scores for acinar cell necrosis were not significantly lower in animals given the antagonists (see Table I).

Differences between animals treated with PAF-RA and those given ET-RA were only significant ($P < 0.05$) for fluid loss into the third space (total amount of ascites and pleural effusions).

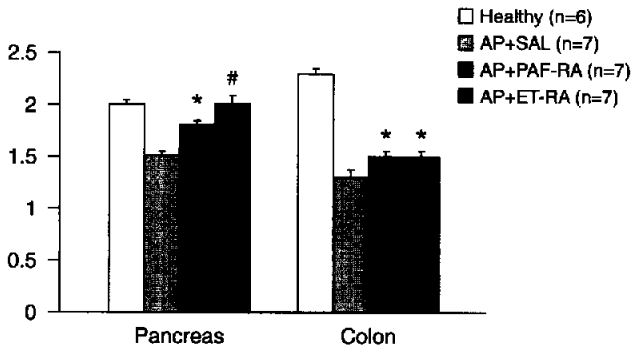

Fig. 1. Pancreatic and colonic capillary blood flow (nl/min/cap) in healthy control animals and animals with severe acute pancreatitis *(AP)* treated with saline *(SAL)*, PAF-RA, and ET-RA (mean ± SEM).* = $P < 0.05$ compared to SAL; # = $P < 0.05$ compared to control and PAF-RA vs. ET-RA.

Capillary Blood Flow

Compared to values in healthy control animals, pancreatic capillary blood flow (Fig. 1) was significantly reduced 24 hours after induction of acute pancreatitis (2.2 ± 0.1 vs. 1.5 ± 0.1 nl/min/cap; $P < 0.05$). PAF-RA improved pancreatic capillary blood flow by 20% (1.8 ± 0.1 vs. 1.5 ± 0.1 nl/min/cap; $P < 0.05$) and ET-RA by 33% (2.0 ± 0.1 nl/min/cap; $P < 0.05$ vs. PAF-RA). The increase in capillary blood flow in the colon was 15% in both treatment groups ($P < 0.05$).

Leukocyte-endothelium interaction (Fig. 2) was markedly enhanced in saline-treated animals with acute pancreatitis (16.3 ± 1.6 vs. <5 ± 1 rolling cells/min in healthy controls). PAF-RA therapy had no effect on the number of rolling leukocytes (12.5 ± 1.6 vs. 16.3 ± 1.6), whereas ET-RA reduced the number by 50% (7 ± 1.6; $P < 0.01$ vs. NaCl; $P < 0.05$ vs. PAF-RA).

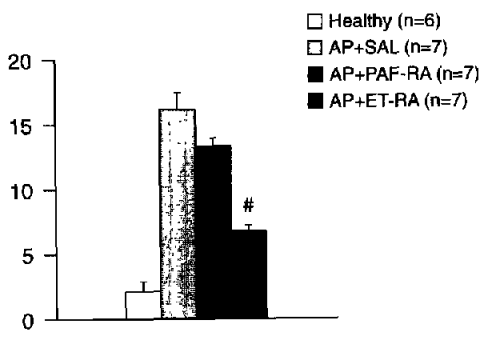

Fig. 2. Leukocyte rolling (cells/min) in mesenteric venules in healthy control animals and animals with severe acute pancreatitis *(AP)* treated with saline *(SAL)*, PAF-RA, and ET-RA (mean ± SEM). # = $P < 0.05$ compared to SAL and PAF-RA.

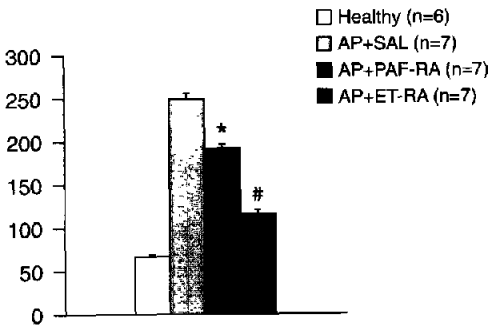

Fig. 3. Capillary permeability (%) in the colonic mucosa in healthy control animals and animals with severe acute pancreatitis *(AP)* treated with saline *(SAL)*, PAF-RA, and ET-RA (mean ± SEM). * = $P < 0.05$ compared to SAL; # = $P < 0.05$ compared to SAL and PAF-RA.

Capillary permeability in the colon (Fig. 3) was significantly increased in saline-treated animals with acute pancreatitis (248 ± 6 vs. <70 ± 5% in healthy control animals). PAF-RA reduced permeability by 24% (188 ± 4 vs. 248 ± 6; $P < 0.001$) and ET-RA by 54% (114 ± 5; $P < 0.001$ vs. PAF-RA).

DISCUSSION

Impairment of pancreatic blood supply, microcirculation, and tissue oxygenation contributes to the severity of acute pancreatitis and is considered a hallmark of the severe (necrotizing) forms of the disease.[1] The microcirculatory disorders are, however, not confined to the pancreas, at least in animals; they can also be demonstrated in the colon, lung, and liver where they have been associated with organ dysfunction.[2-4] If it is assumed that many of the complications in acute pancreatitis result from the amplifying effects of microcirculatory disorders, improving microcirculation appears to be a logical therapeutic approach. This is underlined by the observation that microcirculatory disorders persist for a longer period of time, whereas the parameters reflecting the initial intracellular events that trigger the damage subside within a few hours after disease onset.[4] Since the pathogenesis of microcirculatory disorders in acute pancreatitis involves several components and causes, different therapeutic measures may be beneficial. Adequate fluid resuscitation is essential to compensate hypovolemia and hemoconcentration, thereby improving blood velocity.[21,22] Hemodilution therapy is even more effective since it reduces hematocrit more rapidly than conventional fluid therapy and the low-molecular-weight colloids used as diluents directly counteract hypercoagulability and reduce abnormal leukocyte-endothelium interaction at the microcirculatory level.[23] Better understanding of the cytokine cascade activated during the inflammatory response has now given rise to novel strategies for directly interfering with the vasoactive substances that cause vasoconstriction, increased vascular permeability, and pathologic interaction between blood cells and the endothelium. Candidates that may be influenced include various (proinflammatory) cytokines such as IL-1, IL-6, IL-8, tumor necrosis factor, and plasma-activating factor, which are mainly released from tissue macrophages and monocytes, as well as secondary mediators such as endothelin and nitric oxide directly released from the endothelial cells in response to cytokine activation, endothelial injury, circulating substances (including proteases liberated from the injured pancreas), and changes in tissue perfusion.[5,6,24] Modulating these factors in experimental acute pancreatitis by specific antagonists has yielded the first positive effects on local as well as systemic measures of disease severity (e.g., serum amylase, pancreatic morphology, mortality[25-29]). However, a direct impact on organ-specific microcirculation, which would explain improved organ function and outcome, has not yet been demonstrated. Thus the present study was designed to quantify the effect of specific antagonists to various vasoactive mediators on microcirculation inside and outside the pancreas using a model of severe acute pancreatitis previously shown to be suited for evaluating therapy.[9-11] In addition to the pancreas, we focused on microcirculation in the colon because previous findings suggested that decreased capillary blood flow in the colonic mucosa contributes to gut failure with increased bacterial translocation.[10]

Capillary permeability was assessed with regard to clinical sequelae, suggesting that capillary leakage is a decisive factor in fluid loss into the third space and other subsequent complications early in severe acute pancreatitis. The endothelin-1 antagonist LU 135252 was chosen for the experiment because of our positive experiences with this specific compound in previous

studies,[13,30] the platelet-activating factor antagonist because of the positive results recently reported with lexipafant in both animal studies and clinical trials.[14,25] Because lexipafant was not available, we used WEB 2170 because it has proved to be effective in many disease-related in vivo models in rats.[17,18]

The only information we found suggesting that the effect of WEB 2170 is substantially different from that of lexipafant was the study by Jancar et al.,[31] who compared the effect of WEB 2170 and BN 52021 in cerulein-induced mild acute pancreatitis. They reported that both receptor antagonists significantly reduced extravasation of Evans blue in the pancreas, whereas serum amylase, water content, edema, and inflammatory cell infiltration of the pancreas were only attenuated by BN 52021. BN 52021 has been described as a nonspecific protease inhibitor,[32] which may explain its special effect in acute pancreatitis. However, this should not be relevant for our study focusing on the systemic response to pancreatic injury in a model with fulminant protease activation in which edema and necrosis develop within the first 6 hours,[9] that is, prior to antagonist therapy.

Despite comparable macrohemodynamic parameters (with adequate fluid resuscitation), animals given either PAF-RA or ET-RA had significantly better microcirculatory parameters than saline-treated rats. Stabilizing microcirculation was associated with increased intravascular volume (as assessed by hematocrit), decreased fluid loss into the third space (as assessed by ascites and pleural effusions), and improved renal and respiratory function and survival. In contrast (and despite the improvement of capillary blood flow in the pancreas), treatment with the receptor antagonists had no significant impact on trypsinogen activation and the extent of acinar cell injury. These findings agree with previously discussed observations[13,30] and suggest that the antagonists improve the systemic rather than the local sequelae of severe acute pancreatitis, and that the systemic sequelae of acute pancreatitis determine outcome. The latter is supported by a recent clinical study[33] that could not confirm the relationship between the extent of pancreatic necrosis and outcome in a series of 47 patients with acute pancreatitis. Improved pancreatic microcirculation may only limit the extent of pancreatic necrosis once perfusion is restored in ischemic (low-flow) areas (at risk of undergoing irreversible necrosis) early in the course of the disease.

At a later stage, disturbed pancreatic microcirculation may be a systemic rather than a local phenomenon. This would explain why pancreatic injury did not regress when we started therapy 6 hours following disease onset after confluent necrosis had already developed, whereas other groups investigating the effect of prophylactic mediator blockade (administration of PAF antagonists before or during acute pancreatitis induction) succeeded in ameliorating pancreatic injury.[25,34] Prophylactic ET-RA administration investigated in our model was also associated with a significant reduction in acinar cell necrosis,[30] whereas Rivera et al.[35] found no such beneficial effect with lexipafant in the same model. In their study, 9-hour administration of PAF-RA from the start of acute pancreatitis induction was not accompanied by adequate fluid resuscitation. This differed from our approach (18 hours of continuous infusion of PAF-RA and 6 ml/kg/hr Ringer's lactate) and may explain the difference in mortality rates (50% vs. 25%). In our experience, animals only profit from stabilization of capillary permeability if additional fluid is resuscitated to restore the intravascular volume and to stabilize microcirculation by decreasing blood viscosity and increasing blood cell velocity.

The present experiments underline the role of microcirculatory disorders in the development of systemic sequelae of severe acute pancreatitis and confirm PAF and ET as decisive mediators in the pathogenesis of these disturbances, thus explaining the beneficial effect of the antagonists. Improved capillary blood flow and capillary permeability and reduction of altered leukocyte-endothelium interaction, both in the pancreas and the colon, suggest that many of the beneficial effects seen in the present experiment and previous studies with these antagonists result from the stabilization of systemic microcirculation, especially increased capillary permeability. The effect of PAF and especially ET on vascular permeability has been emphasized elsewhere.[5,7,25,36-38]

Although both PAF-RA and ET-RA likewise improved capillary blood flow, ET-RA was significantly more effective in counteracting leukocyte rolling and capillary permeability. The latter was associated with the further reduction of fluid loss into the third space (as assessed by ascites and pleural effusions) and lower hematocrit levels halfway through the experiment.

The hypothesis that capillary leakage caused by ET and PAF activated during the inflammatory response to pancreatic injury is a major factor in the development of microcirculatory disorders and organ dysfunction in severe acute pancreatitis would not only account for the beneficial effects of the receptor antagonists but would also explain why ET-RA was more effective than PAF-RA.

CONCLUSION

Although we cannot conclusively explain the effect of ET-RA in severe acute pancreatitis and its advantage over PAF-RA at this point, the present experiments together with our experience with other treatment modalities proven effective in this model and in

severe human pancreatitis[39] suggest that ET-RA may become a powerful new tool in the treatment of severe acute pancreatitis.

This assumption is supported by the fact that ET-RA was effective in delayed therapy, that is, when the events triggering pancreatic injury had already subsided (reflecting the clinical situation). It also improved microcirculation inside and (perhaps more important) outside the pancreas, especially stabilizing increased capillary permeability, which contributes to many of the early sequelae of severe acute pancreatitis. Therefore we feel strongly that ET-RA should be tested in a clinical trial (either in comparison or in addition to PAF-RA).

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Discussion

Dr. E. Klar (Heidelberg, Germany). It is well known that leukocyte-endothelium interaction is a main determinant of microvessel perfusion. On the other hand, it has been shown that PAF exerts its main effect via the activation of leukocytes, which will instantly lead to an increase in permeability. From this one would expect a PAF antagonist to show a profound effect on permeability changes. Can you explain why the endothelin antagonist was much more effective in preventing an increase in permeability than the PAF antagonist? This is astonishing since endothelin is mainly a vasoconstrictor.

Dr. T. Foitzik. Endothelin is much more than a vasoconstrictor. At the capillary level it has tremendous effects on permeability, which are independent from its vasomotor action and which appear to be also independent from leukocyte-endothelium interaction. This has been shown by many other groups in the past. We are currently performing experiments in which we compare the effect of endothelin on capillary permeability with other substances such as PAF and bradykinin, and our preliminary results suggest that endothelin is indeed the dominant mediator.

Dr. J. Werner (Heidelberg, Germany). You showed that endothelin antagonist prevents capillary leakage. Could you show an effect on leukocyte adhesion or infiltration into the tissue as measured by, for example, myeloperoxidase activity?

Dr. Foitzik. We showed that leukocyte rolling was significantly reduced by the endothelin receptor antagonist. Myeloperoxidase was not measured.

Dr. J. Peters (Los Angeles, Calif.). Have you or anyone else conducted the same experiment with resuscitation more like the clinical situation?

Dr. Foitzik. Animals were adequately resuscitated with 6 ml/kg/hr Ringer's lactate.

Dr. Klar. Dr. Werner from our group has shown that there is an expression of ICAM-I on microvascular endothelium in necrotizing pancreatitis not only within the pancreas but also in distant organs. This occurs early and might represent an initial mechanism for the ubiquitous increase in permeability resulting from the induction of endothelial leukocyte adherence. The action of PAF might be further downstream. Can you correlate this with your findings?

Dr. Foitzik. I agree that adhesion molecule activation may be an initial event, whereas endothelin activation appears to affect microcirculation somewhat later during the systemic inflammatory response. This may explain why therapy with endothelin receptor antagonists started 6 hours after the onset of acute pancreatitis is effective.

Dr. Werner. You mentioned that chemokines play an important role in the pathogenesis of acute pancreatitis. PAF is just one of them; at least as important are IL-1, IL-8, and tumor necrosis factor-alpha. Have you measured those chemokines or have you investigated what effect they have in your model and what relationship they have to endothelin? In previous studies you have shown that endothelin antagonists cannot prevent the local injury in the pancreas when given after a 6-hour therapy-free interval. Do you see any role for the chemokines mentioned earlier to prevent this?

Dr. Foitzik. We did not measure other chemokines in this study. We concentrated on PAF and endothelin because recent studies suggest that they are the dominant mediators and that PAF and endothelin receptor antagonists are most effective in preventing local and systemic complications in acute pancreatitis. We have previously shown that prophylactic administration of endothelin receptor antagonists reduces local injury, but the aim of the present study is to prove efficacy with delayed therapy. I do not think that there is a therapeutic role related to chemokines because chemokine activation is an initial event that may not be influenced by the time the patient arrives at the hospital.

Early Increase in Intestinal Permeability in Patients With Severe Acute Pancreatitis: Correlation With Endotoxemia, Organ Failure, and Mortality

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Sepsis accounts for 80% of deaths from acute pancreatitis. This study aimed to investigate early changes in intestinal permeability in patients with acute pancreatitis, and to correlate these changes with subsequent disease severity and endotoxemia. The renal excretion of enterally administered polyethylene glycol (PEG) 3350 and PEG 400 was measured within 72 hours of onset of acute pancreatitis to determine intestinal permeability. Severity was assessed on the basis of APACHE II scores and C-reactive protein measurements. Serum endotoxin and antiendotoxin antibodies were measured on admission. Eight-five patients with acute pancreatitis (mild in 56, severe in 29) and 25 healthy control subjects were studied. Urinary excretion of PEG 3350 (median) was significantly greater in patients who had severe attacks (0.61%) compared to those with mild disease (0.09%) and health control subjects (0.12%) ($P < 0.0001$), as was the permeability index (PEG 3350/400 excretion) ($P < 0.00001$). The permeability index was significantly greater in patients who subsequently developed multiple organ system failure and/or died compared with other severe cases (0.16 vs. 0.04) ($P = 0.0005$). The excretion of PEG 3350 correlated strongly with endotoxemia ($r = 0.8$; $P = 0.002$). Early increased intestinal permeability may play an important role in the pathophysiology of severe acute pancreatitis. Therapies that aim to restore intestinal barrier function may improve outcome. (J GASTROINTEST SURG 1999;3:252-262.)

KEY WORDS: Pancreatitis, endotoxemia, intestinal permeability, PEG, intestinal barrier

Acute pancreatitis is an enigmatic disease, the pathophysiology of which remains poorly understood. It continues to claim an overall mortality of approximately 10%, despite improvements in intensive therapy and radiologic and surgical interventions.[1-3] Sepsis, which is usually secondary to infection of pancreatic or peripancreatic necrosis, accounts for 40% to 80% of deaths.[4,5] Secondary infection is a complication in 30% to 70% of patients with pancreatic necrosis[6,8] and is associated with a significantly increased mortality. In a series of 114 patients with acute necrotizing pancreatitis, mortality rates varied from 9% in patients with sterile necrosis to 38% in those with infected necrosis.[6] Gram-negative enteric organisms are responsible for most of these infections,[6,9-12] which suggests a gastrointestinal origin. Although the actual route of migration of microorganisms from the intestinal lumen to the pancreatic sequestrum is unclear,[13-19] bacterial translocation appears most likely,[17] suggesting failure of intestinal barrier function.

Intestinal barrier failure has long been implicated in the development of sepsis and multiple organ system failure (MOSF) in a variety of severely ill patients.[20-24] Endotoxin, an important product of gram-negative microorganisms, which often originates from the gut,[25] is associated with known and quantifiable pathophysiologic effects.[26,27] In particular, endotoxemia appears to have a considerable impact on the immune system, as it is associated with a reduced proportion of T helper lymphocytes in peripheral blood[28] and increased generation of suppressor T cells,[29] thus depressing cellular immunity. It may also induce liver injury and impair reticuloendothelial function.[30] Endotoxemia can be detected in the peripheral blood of

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most patients with severe acute pancreatitis, and in excess of 90% of those dying of the disease.[31-33] Circulating antiendotoxin antibodies bind endotoxin to form complexes, which are subsequently removed from the circulation.[34] These antibodies have been observed to fall significantly during severe attacks of acute pancreatitis as compared with mild attacks, suggesting a higher endotoxin exposure.[35]

More compelling evidence to implicate the gut as a source of bacterial and endotoxin translocation has been gained from studies of experimental pancreatitis in animal models. Bacteria were observed to translocate from the intestinal lumen to mesenteric lymph nodes, and subsequently to other extraintestinal sites, following experimental induction of acute pancreatitis.[36-38] Furthermore, reduction of colonic bacterial load by formation of a cecostomy and colonic irrigation after induction of acute necrotizing pancreatitis resulted in significantly lower serum endotoxin levels and a reduction in mortality compared to control values.[39]

Our hypothesis was that severe pancreatic inflammation may inflict an ischemic or inflammatory insult on neighboring parts of the gastrointestinal tract,[40] particularly the closely related transverse colon. Subsequent disruption of the functional integrity of the mucosa might then lead to increased permeability. Bacteria and endotoxin may thus translocate *locally* to infect the adjacent inflamed and necrotic pancreas and *systemically* to overwhelm and suppress immune defenses.[41-43] This may perpetuate the inflammatory process resulting in systemic microvascular injury.[44] Both effects could lead to the development of sepsis, the systemic inflammatory response syndrome, and MOSF.

The aims of the present study were (1) to examine intestinal barrier function in patients with acute pancreatitis by measuring intestinal permeability to macromolecules, and (2) to examine the relationship of permeability changes to severity of disease and evidence of endotoxemia.

Polyethylene glycol (PEG) 3350 was employed for this study as a permeability probe. Unlike sugar probes such as lactulose, which is a molecule of 340 daltons (Da), PEG 3350 is a macromolecule of 3350 Da that more closely approximates the size of gram-negative endotoxin.[45-47] Moreover, it is not metabolized by intestinal bacteria, as sugar probes are, and it is not normally absorbed. If it is absorbed, it is excreted rapidly and is quantitatively unchanged in the urine,[48] where it can be detected and measured accurately by high-performance liquid chromatography.[49] Furthermore, it has an established safety record and is nontoxic,[50] being often used in high doses for preoperative bowel preparations and as a component of some drugs and food products. PEG 400 was added as an internal control for changes in intestinal motility and absorption. The differential urinary excretion of the two PEG molecules (PEG 3350/PEG 400 ratio) was calculated to provide an index of intestinal permeability.[51,52]

MATERIAL AND METHODS

Studies in humans were approved by the ethics committees of the following participating institutions: The General Infirmary at Leeds, Bradford Royal Infirmary, Huddersfield Royal Infirmary, York District Hospital, Pontefract General Infirmary, and the Friarage Hospital, Northallerton.

Adults admitted consecutively with a clinical diagnosis of acute pancreatitis and hyperamylasemia (serum amylase value more than three times the upper limit of normal) were considered for inclusion if their symptoms had been present for no longer than 48 hours. Written informed consent was obtained from all patients. Patients with inflammatory bowel disease, previous bowel resection (except appendectomy), renal disease, and those with established organ failure at the time of admission to the hospital were excluded.

Attacks were classified as mild or severe according to the Atlanta criteria of 1992, which are based on clinical outcome.[1] Mild disease was defined as that associated with minimal organ dysfunction and uneventful recovery, whereas severe disease was associated with organ failure and/or the development of localized complications such as pancreatic necrosis, abscess, or pseudocyst. Healthy volunteers matched for age and sex served as control subjects.

METHODS

All samples for measurement of intestinal permeability were obtained within 72 hours of onset of severe abdominal pain. Serum endotoxin and antiendotoxin core antibodies were measured at the time of recruitment, that is, within 24 hours of admission to the hospital. Acute Physiology and Chronic Health Enquiry (APACHE) II scores were determined within the first 24 hours of admission.[53] C-reactive protein was measured daily (see below), and the highest level recorded during the initial 5 days was identified for the purpose of analysis.

Measurement of Intestinal Permeability

A urine sample was obtained immediately before the administration of the PEG solution. This sample served as a baseline for each patient. A mixture of 40 g PEG 3350 (Sigma-Aldrich Co., Ltd., Dorset, U.K.)

and 5 g PEG 400 (Sigma-Aldrich Company) was dissolved in 100 ml of sterile pyrogen-free water, to which 0.1 ml of lemon flavoring was added. The prepared solution was administered either orally or through a nasogastric tube if one was already in place. Urine was collected over the subsequent 24 hours in chemical-free plastic containers (Laboratory Sales U.K. Ltd., Rochdale, Lancashire, U.K.), its volume measured and recorded, and a 100 ml sample retained for analysis in a chemical-free plastic container. The pH of the urine sample was then adjusted to 7.4, and the sample was stored as four 20 ml aliquots at −20° C for later analysis.[49]

The urine samples (20 ml each) were centrifuged through prewashed Amicon Centricon-50 concentrators (Millipore U.K. Ltd., Watford, U.K.) at 1000 g for two spins of 30 minutes each, to eliminate potentially confounding molecules of more than 50 kDa. Two aliquots (2 ml each) from each filtrate were vortexed with 2 g of a uniformly hydrated, mixed-ion exchange resin (RG 501-X8, Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, U.K.) for 1 hour and then allowed to stand for an additional hour to remove charged molecules that could interfere with the analysis. The urine-resin mixture was filtered, to remove the resin, by centrifugation through a prewashed Vectaspin-3 molecular weight sieve separation system (Whatman International Ltd., Maidstone, Kent, U.K.), at 1000 g for 5 minutes, to yield approximately 2 ml of filtrate per aliquot. To enhance detection of PEG 3350, 1.6 ml of the Vectaspin-3 filtrate was concentrated fourfold by centrifugation through a washed Amicon Centricon-3 separation system (Millipore U.K. Ltd.) designed to retain substances greater than 3000 Da at 3500 g for 60 minutes. The residue (≤0.4 ml) was weighed and adjusted to a volume of 0.4 ml by adding and mixing with distilled water. PEG 400 concentration was determined from the remaining paired sample of Vectaspin-3 filtrate. All samples were then prepared by injection through 0.45 μm syringe filters (Acrodisc 13, Gelman Sciences Ltd., Northampton, U.K.) into sealed vials and mounted on an autosampler for analysis by high-performance liquid chromatography.

The complete system comprising autosampler, pump, and analogue-digital converter was run by a computer workstation equipped with STAR chromatography software (Varian Associates Ltd., Walton-on-Thames, Surrey, U.K.). Samples were injected on a single gel filtration column (Polysep-GFC-P2000, 300 × 7.8 mm, Phenomenex Ltd., Macclesfield, Cheshire, U.K.) using glass-collected, double-distilled, and filtered deionized water, at a flow rate of 0.6 ml/min, to allow for the chromatographic separation of PEG 3350, PEG 400, and other molecules. The elute was monitored continuously by a refractive index detector (Altex model, Anachem, Luten, Beds, U.K.). Test and standard samples were run in tandem, and the chromatograms obtained were analyzed by reference to previously determined calibration standards. PEG urinary excretion was reported as a percentage (median) of the administered dose.

Endotoxin and Antiendotoxin Core Antibody Assays

Peripheral venous blood samples were collected into endotoxin-free vacuum blood collection tubes (Quadratech, Epsom, Surrey, U.K.). Blood was centrifuged and the supernatant stored at −20° C for subsequent batch analysis. Levels of endotoxin were determined using a *Limulus* amebocyte lysate method that employed a kinetic-chromogenic assay (COATEST Endotoxin, Chromogenix, Molndal, Sweden).

Endogenous immunoglobulin (Ig)G and IgM antiendotoxin core antibody (EndoCAb) levels to core glycolipid antigens were measured by a direct enzyme-linked immunosorbent assay, as previously described by Barclay et al.[34,54] The normal ranges of antibodies were determined from 1024 healthy adult blood donors and the results expressed as a percentage of the median (MU = median unit) of the normal adult range: the median IgG EndoCAb was 100.2 MU (10th to 90th percentile 32.9 to 240.8 MU; lower and upper quartiles 56.7 and 151.8 MU, respectively) and the median IgM EndoCAb was 100.5 MU (10th to 90th percentile 38.8 to 258.8 MU; lower and upper quartiles 63.8 and 156.6 MU, respectively).

C-Reactive Protein Assay

C-reactive protein was measured using an enzyme-linked immunosorbent assay (Dako, High Wycombe, U.K.) as previously described.[42] The normal C-reactive protein concentration in serum is less than 10 mg/L.

Statistical Analysis

Results are expressed as medians with interquartile ranges. Comparison between the groups was performed using the Mann-Whitney U test and the chi-square test with Yates correction as appropriate. The Pearson correlation coefficient was calculated where indicated. Significance was accepted at the 5% level.

RESULTS

Between June 1996 and June 1997, 85 patients with acute pancreatitis (mild in 56, severe in 29) and 25

Table I. Comparison of patients and control subjects

	Mild pancreatitis (n = 56)	Severe pancreatitis (n = 29)	Controls (n = 25)
Median age in years (range)	60 (22-93)	57.5 (17-83)	55.7 (23-82)
Sex ratio (M/F)	24/32	20/9	13/12
Etiology			
Gallstones	34	14	
Alcohol	15	9	
ERCP	4	3	
Hyperlipidemia	1	0	
Idiopathic	2	3	
APACHE II score on admission			
Median (range)*	5 (1-16)	10 (4-27)	
Prophylactic antibiotics	27 (48%)	19 (66%)	

ERCP = endoscopic retrograde cholangiopancreatography.
*P = 0.001, Mann-Whitney U test.

Table II. Diagnosis and clinical outcome in 29 patients with severe acute pancreatitis

Diagnosis	No. (%)	Died (%)
Pseudocyst	6 (20.7)	0 (0)
Pancreatic necrosis	11 (37.9)	5 (17.2)
Pancreatic abscess	2 (6.9)	0 (0)
Single organ failure	9 (31.0)	0 (0)
MOSF	8 (27.6)	6 (20.7)

MOSF = multiple organ system failure.

control subjects were studied. The median interval between the onset of severe abdominal pain and admission to the hospital was 21 hours (range 2 to 43 hours). A detailed comparison of patients and control subjects is presented in Table I. Groups were similar in age and sex distribution. The cause of the attacks was identified in 80 of 85 patients (gallstones in 48, alcohol abuse in 24, endoscopic retrograde cholangiopancreatography in 7, and hyperlipidemia in 1). Prophylactic antibiotics were administered in the hospital in 19 patients with severe attacks (66%) and 27 patients with mild attacks (48%) (P = 0.129).

APACHE II scores within 24 hours of admission were significantly higher in patients with severe attacks (median 10; range 4 to 27) compared to those with mild attacks (median 5; range 1 to 16) (P = 0.001; Mann-Whitney U test).

The diagnosis and clinical outcome in 29 patients with severe pancreatitis are shown in Table II. Contrast-enhanced CT was performed within 72 hours of onset of severe abdominal pain in 37 of 85 patients and thereafter in an additional 14 patients. Necrosis involved more than two thirds of the gland in 6 of the 11 patients with demonstrable pancreatic necrosis at dynamic contrast-enhanced CT, and two of the patients who subsequently died became secondarily infected with gram-negative enteric organisms later in the course of the disease. The diagnosis of pancreatic necrosis (n = 11) was established within 1 to 5 days of the onset of severe abdominal pain, 3 to 5 weeks of pancreatic abscess (n = 2), and 5 to 7 weeks of pancreatic pseudocyst (n = 6). MOSF developed in eight patients, five of whom had necrosis affecting more than two thirds of the gland; these five patients later died.

Intestinal Permeability

Intestinal permeability to macromolecules, as measured by the percentage of urinary excretion of PEG 3350, was significantly increased in patients with severe acute pancreatitis compared to those with mild disease and the healthy control subjects (median [interquartile ranges]: 0.61% [0.26% to 2.21%], 0.09% [0.06% to 0.14%], and 0.12% [0.08% to 0.16%], respectively; P <0.0001) (Fig. 1). There was no significant difference in the excretion of the micromolecule PEG 400 among patients with severe or mild attacks and healthy control subjects (median [interquartile ranges]: 19.2% [6.2% to 25.9%], 15.2% [7.7% to 18.3%] and 15.8% [11.3% to 21.4%], respectively, P = NS).

The ratio of the urinary excretion of the two PEG molecules (PEG 3350/PEG 400) corrects for possible changes in intestinal motility and absorption between groups. It was significantly increased in patients with severe disease compared to those with mild disease and control subjects (median [interquartile ranges]: 0.06 [0.01 to 0.19], 0.008 [0.005 to 0.013], and 0.009

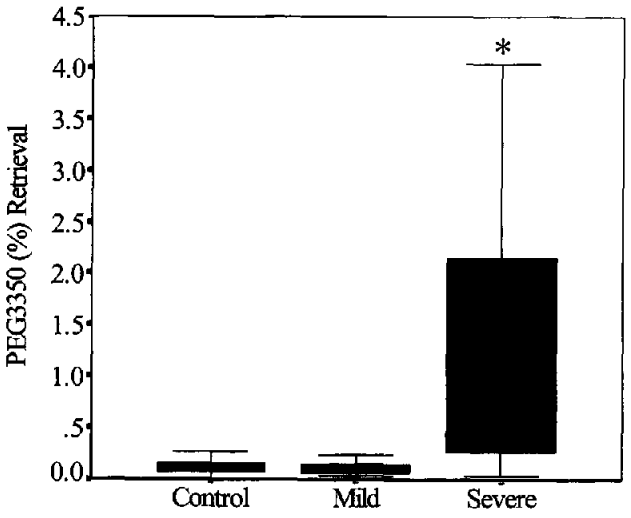

Fig. 1. The percentage urinary excretion of PEG 3350 was significantly increased in patients with severe attacks compared to those with mild disease and healthy control subjects (*P <0.0001). Boxes represent medians and interquartile ranges.

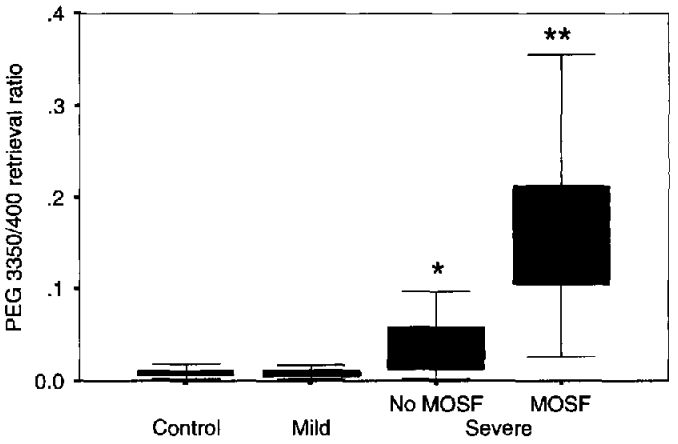

Fig. 2. PEG 3350/400 urinary excretion ratio (permeability index). Bars represent medians and interquartile ranges. **P = 0.0005 (comparing the two groups with severe disease); *P <0.001 (comparing the group with less severe attacks with the mild and control groups).

[0.005 to 0.012], respectively; P <0.00001). No significant differences in the excretion of either PEG molecule were observed between patients with mild disease and control subjects, suggesting minimal changes in intestinal permeability, motility, and absorption during mild acute pancreatitis.

The ratio of excretion of PEG molecules was also significantly higher in patients with severe acute pancreatitis who later developed MOSF (n = 8, of whom 5 died) compared to those with severe disease who developed single organ system failure and/or localized pancreatic complications and survived (n = 21) (median [interquartile ranges]: 0.16 [0.1 to 0.24] vs. 0.04 [0.01 to 0.59]; P = 0.0005), as was the ratio in the latter group compared with the mild pancreatitis and control groups (P <0.001) (Fig. 2).

Endotoxemia

Peripheral venous blood was obtained for endotoxin and antiendotoxin core antibody measurements on admission from 22 of 29 patients with severe disease and 42 of 56 with mild disease. Endotoxemia was present in 50% (11 of 22) of patients with severe acute pancreatitis and in 21% (9 of 42) with mild disease (χ^2 = 5.5; P <0.02). Endotoxemia was more common in nonsurvivors than in survivors and in those who developed MOSF than in those who did not (5 of 6 [83%] vs. 15 of 58 [26%], P <0.005; and 6 of 8 [75%] vs. 14 of 56 [25%], P <0.005).

Serum endotoxin levels (median [interquartile ranges]) were higher in patients with severe attacks compared to those with mild attacks (0.035 [0.005 to 0.165] endotoxin units [EU]/ml vs. 0.01 [0.003 to 0.029] EU/ml), although this difference was not statistically significant. However, significantly higher endotoxin levels were detected in nonsurvivors (0.113 [0.037 to 8.5] EU/ml vs. 0.015 [0.003 to 0.034] EU/ml; P = 0.02, Mann-Whitney U test) and in patients who developed MOSF (0.113 [0.038 to 3.155] EU/ml vs. 0.01 [0.003 to 0.029]; P = 0.001).

Correlation Between Endotoxemia and Macromolecular Permeability

A strong positive correlation was observed between the level of endotoxemia in patients with measurable endotoxin levels on admission (n = 20) and the percentage urinary excretion of the macromolecule PEG 3350 (r = 0.8, P = 0.002; Pearson correlation test) (Fig. 3). The latter did not correlate with endogenous IgG antiendotoxin antibodies (r = −0.2, P = 0.5).

Antiendotoxin Core Antibodies

On admission, the endogenous IgG antiendotoxin core antibody concentrations were significantly lower in patients with severe acute pancreatitis compared to patients with mild attacks and healthy control subjects (median: 57.8, 111.2, and 100.2 GMU, respectively; P <0.01 and P <0.03, respectively; Mann-Whitney U test). Levels of endogenous IgM antiendotoxin core

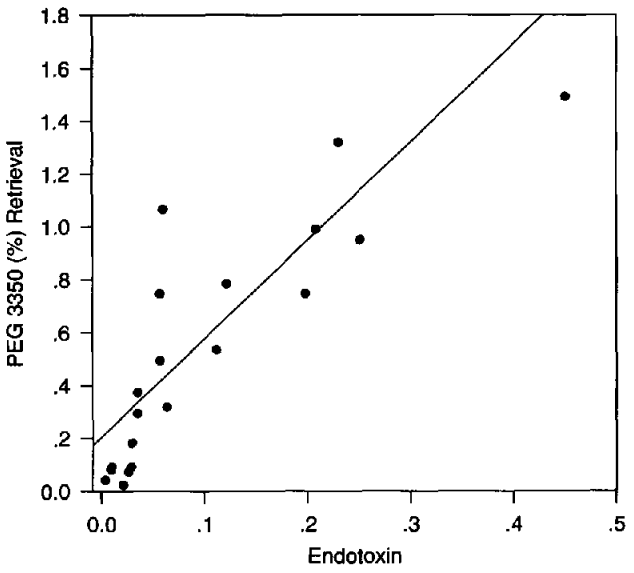

Fig. 3. Strong positive correlation between the percentage urinary excretion of the macromolecule PEG 3350 and endotoxemia ($r = 0.08$, $P = 0.002$).

antibodies were similarly and significantly reduced in both the severe and mild groups compared to healthy control subjects (median: 44.4, 54.5, and 100.5 MMU, respectively; $P < 0.001$ and $P < 0.001$, respectively; Mann-Whitney U test).

C-Reactive Protein

Peak serum C-reactive protein was significantly raised in patients with severe acute pancreatitis compared to those with mild disease (median [interquartile ranges]: 260 [155.5 to 327.5] mg/L vs. 93 [23.5 to 163.3] mg/L; $P < 0.0001$, Mann-Whitney U test).

DISCUSSION

This study is the first to investigate intestinal permeability to macromolecules in patients with acute pancreatitis. Permeability remained unchanged early in the course of mild acute pancreatitis, but was markedly and significantly abnormal within 72 hours of onset of severe attacks. This increased permeability correlated with disease severity, being significantly greater in severe attacks that culminated in MOSF or death. Similarly, significantly higher levels of endotoxemia were detected in patients who developed MOSF or died. There was also a strong correlation between intestinal permeability to PEG 3350 and endotoxemia.

In patients with severe acute pancreatitis, MOSF and sepsis are the major causes of death.[55] Derangement of intestinal barrier function leading to an increase in intestinal permeability to bacteria and bacterial fragments as well as other large and potentially toxic molecules are central mechanisms to the hypothesis that implicates the intestine in the development of sepsis and MOSF.[24] Previous studies of experimental pancreatitis have also demonstrated an increase in intestinal permeability to macromolecules[56,57] and have identified the gut as an important source of infection during acute pancreatitis in animal models.[36,37] Early in the course of severe attacks, we have demonstrated a sevenfold rise in the intestinal macromolecular permeability index, as measured by the ratio of excretion of PEG 3350 to PEG 400 in urine. This change in permeability is consistent with failure of the essential protective barrier function of the gastrointestinal tract rather than a phenomenon that develops as part of an already established and more generalized multisystem failure. Abnormal permeability was demonstrated in this study at an early stage of the disease and prior to the onset of MOSF, as well as in patients with severe disease uncomplicated by organ failure.

The core role of the gut in the development of sepsis and MOSF is further supported in the current study by the strong correlation between increased intestinal permeability to macromolecules and levels of endotoxemia ($r = 0.8$, $P = 0.002$), a correlation that was particularly marked in patients who developed MOSF or who later died. Furthermore, there was a significant drop in serum IgG antiendotoxin antibodies only in patients with severe disease, suggesting a greater prior endotoxin exposure. The changes in antiendotoxin antibodies are not dissimilar to those observed by Windsor et al.[35] in patients with acute pancreatitis.

An understanding of the mechanisms responsible for this derangement in gut barrier function is important if effective therapies are to be evaluated to reduce its consequences. Several causes have been identified from experimental and clinical studies, and include mucosal ischemia,[21,58-62] reperfusion,[63,64] impaired immune defenses,[65-69] and a disturbed indigenous intestinal flora that leads to abnormal bacterial overgrowth.[70-73] The development of systemic endotoxemia may, in turn, act through positive feedback mechanisms, directly or through cytokine release,[74] to further increase intestinal permeability,[75,76] impair host immunity,[77] and promote bacterial translocation.[78,79]

Such pathophysiologic disturbances are known to occur during acute pancreatitis. Intestinal blood flow, particularly that of the colonic mucosa, is decreased at an early stage of acute pancreatitis.[40,57] This is reflected clinically and, in extreme cases, by the development of colonic necrosis, fistulization, or ischemic

stricture formation.[80,81] Immunosuppression in patients with severe acute pancreatitis is evident in the reduction in circulating levels of CD4-positive (T helper) lymphocytes,[42] the impairment of reticuloendothelial system clearance of alpha-2 macroglobulin-protease complexes,[41] and the decrease in delayed-type skin hypersensitivity,[43] the latter correlated with septic morbidity and mortality. Disruption of intestinal microflora was found in the small intestine and cecum of animals following the induction of acute pancreatitis, with a significant increase in gram-negative bacterial counts.[37]

Although starvation and malnutrition may also result in increased intestinal permeability,[82] it is unlikely that they have contributed to the permeability changes observed in patients with severe acute pancreatitis. Patients with mild acute pancreatitis who were similarly starved for up to 72 hours had no changes in intestinal permeability; their values remained similar to those in healthy control subjects. We did not starve the healthy volunteers in the control group, however, as a similar period of starvation is neither ethical nor practical.

This study describes a state of "leaky bowel," which develops early in the course of severe acute pancreatitis in humans and correlates with endotoxemia, organ failure, and disease mortality. The increase in intestinal permeability to macromolecules in patients with severe acute pancreatitis, however, does not necessarily equate with bacterial translocation—a theory that remains unproved in humans. These findings, nonetheless, are of potential clinical significance since the gastrointestinal tract has been implicated as a source of infection of pancreatic necrosis in patients with severe acute pancreatitis.[6,9-13] If the early changes in intestinal permeability demonstrable in patients with severe acute pancreatitis do result in bacterial translocation, then specific therapeutic measures to counteract these changes will be required and should be instituted soon after admission. Therapeutic manipulations that aim to restore intestinal mucosal functional integrity or target the intestinal bacterial population may therefore improve disease outcome. Cecostomy and colonic irrigation,[39] digestive decontamination,[83,84] and prophylactic parenteral antibiotics[85] have been associated with a reduction in bacterial translocation and death in experimental models of acute pancreatitis. Prospective randomized clinical studies in patients with severe acute pancreatitis have also demonstrated the beneficial effects of prophylactic parenteral antibiotics[86-88] and selective digestive decontamination[89] with reductions in gram-negative pancreatic infections and mortality.

Antibiotic treatment or selective decontamination regimens target bacteria within the intestinal lumen or during the presumed phase of translocation, but would probably do little to rectify the changes in intestinal permeability observed in this study. Platelet-activating factor antagonists preserved and restored intestinal permeability and barrier function in experimental pancreatitis[90] and reduced pancreatic infections and mortality in a prospective randomized trial of lexipafant in patients with severe acute pancreatitis.[91] Enteral nutrition, on the other hand, offers several advantages. It preserves gastrointestinal mucosal mass and microbial ecology, reduces bacterial translocation,[92-95] maintains immunocompetence, and attenuates harmful exaggerated stress responses to injury.[92] A meta-analysis of eight prospective randomized trials comparing the efficacy of total enteral versus parenteral nutrition in high-risk surgical patients demonstrated a reduction in postoperative septic complications.[96] Three prospective randomized clinical studies have demonstrated the safety and feasibility of enteral nutrition during attacks of acute pancreatitis.[97-99]

CONCLUSION

Early changes in intestinal permeability described in the present study, and their correlation with endotoxemia and the subsequent development of MOSF and death, further implicate failure of intestinal barrier function in the pathophysiology of severe acute pancreatitis. Studies are required to establish the site of the degraded intestinal barrier, to investigate the underlying mechanism, and to examine potential therapeutic measures.

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Discussion

Dr. F. Moody (Houston, Tex.). Our laboratory has had trouble relating the permeability of the mucosal barrier to macromolecules to bacterial translocation. Bacterial translocation occurs frequently in all of these conditions, but only gets to the nodes. Do you have any bacteriology correlates to these permeability studies? Macromolecules may not move the same way as the bacteria. They use a different route.

Dr. B. Ammori. As you are aware, there are few patients with severe pancreatitis who have positive blood cultures for gram-negative bacteria. Among all of our patients, we had two who developed pancreatic sepsis with gram-negative organisms out of eight who developed multiorgan failure. What we need is a more sensitive method of detecting bacterial translocation. Crude culture methods are probably not sensitive enough, and it might be that the use of methods such as polymerase chain reaction techniques to look for organisms captured within the leukocytes might be able to better demonstrate bacterial translocation. So far there has been no evidence to correlate permeability changes with evidence of bacteria in the blood. I think the clinical data, which show the distribution of organisms in pancreatic sepsis to be very similar to that within the colon, strongly suggest that when permeability is increased, bacteria translocate locally rather than systemically and infect the pancreas. It might be that the systemic effect or increase in permeability relates to substances such as endotoxins rather than bacteria.

Dr. R. Prinz (Chicago, Ill.). You are implying, as many believe, that the colon is the source of translocation in severe necrotizing pancreatitis. Do you have any information on where the small-molecular-weight and large-molecular-weight polyethylene glycol markers are being absorbed along the gastrointestinal tract? In some of our experimental studies in dogs, the small intestine appears to be the site of bacterial translocation.

Dr. Ammori. This method measures the whole intestinal tract and cannot specifically detect where the marker is leaking, that is, whether in the small bowel or in the large bowel, or both. The markers do not leak from the stomach; this technique was used in burn patients and those who had delayed gastric emptying had less retrieval of these markers in their urine. There is some evidence from studies in animals that leakage of macromolecules is occurring in the terminal ileum and in the ascending or transverse colon, but thus far in humans we cannot determine the site. When we operate on patients with pancreatic sepsis and perform debridement, we routinely bring out a terminal ileostomy or cecostomy and wash out the colon. This gives us the potential opportunity in these advanced cases of already established organ failure to measure the small bowel permeability separate from the colon, and that might give us a better idea of where the leakage is occurring.

Dr. J. Fischer (Cincinnati, Ohio). There is some evidence of an immunologic defect in mild and moderate pancreatitis and it comes from an entirely different source. We

performed two studies of total parenteral nutrition in patients with pancreatitis at two different institutions and found that there was a significant increase in the catheter infection rate, which seemed to be unrelated to technique, suggesting that there may be an immunologic defect and the problem may not be limited to permeability, as Dr. Moody suggested. I wonder whether you have done any other studies suggesting that the defect you are looking at may in fact be systemic and not related to permeability or bacteria from the colon, which does occur, but may occur only in a very small subset of patients.

Dr. Ammori. It is true that patients with pancreatitis, particularly severe attacks, have evidence of impaired immune function. We see this clinically when we operate on these patients, as most of them are slow to improve and require a long stay in the intensive care unit. We have studied immune function in patients with pancreatitis and demonstrated that alpha-2 macroglobulin, which is an important scavenger for proteases and other products of inflammation, is reduced in patients with severe pancreatitis compared to those with mild disease, so there is a systemic immune defect in these patients, which makes them particularly vulnerable to sepsis. We also know from studies in animals that immunosuppression is associated with an increase in permeability, so it is not only a systemic factor but also has a localized effect that might encourage translocation of bacteria and endotoxin from the bowel.

Prognostic Factors in Resectable Pancreatic Cancer: p53 and Bcl-2

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The *p53* tumor suppressor gene and the *Bcl-2* proto-oncogene regulate cell cycle progression and apoptosis. We evaluated the expression of these molecular markers with standard pathologic prognostic variables in patients who received multimodality therapy for resectable adenocarcinoma of the pancreas to study the effect of p53 and Bcl-2 on survival duration. Immunohistochemical staining of archival material was performed to determine levels of expression of p53 and Bcl-2 proteins in 70 patients with adenocarcinoma of pancreatic origin. All patients underwent a potentially curative pancreaticoduodenectomy and standardized pathologic analysis of resected specimens. Potential pathologic and molecular prognostic variables were assessed for their effect on survival duration. Nuclear staining for p53 was observed in 33 (47%) of 70 specimens. Immunostaining for Bcl-2 was observed in 23 specimens (33%). A trend toward improved survival duration was seen in patients whose tumors stained positive for either p53 or Bcl-2. Negative staining for both markers predicted short survival ($P = 0.01$). By univariate and multivariate analyses, no single pathologic factor was associated with survival duration. Immunohistochemical staging using both p53 and Bcl-2 significantly predicted survival duration by univariate and multivariate analysis; patients whose tumors stained positively for p53 and/or overexpressed Bcl-2 had a significantly longer survival than those whose tumors stained negative for both proteins. (J GASTROINTEST SURG 1999;3:263-277.)

KEY WORDS: Pancreatic cancer, p53, Bcl-2, pancreaticoduodenectomy

Adenocarcinoma of the pancreas is characterized by local tumor growth that infiltrates vascular structures, nerves, and lymphatics, and causes early hematogenous spread to the liver.[1] Studies of pathologic characteristics of the primary or metastatic tumor have failed to define high- and low-risk subgroups of clinical significance, mainly because most patients experience rapid disease progression. The lack of effective cytotoxic therapy has caused investigators to focus on innovative therapies based on the rapidly evolving knowledge of molecular alterations in pancreatic tumorigenesis. The *p53* tumor suppressor gene and the *Bcl-2* proto-oncogene are critically important in control of cell cycle progression and regulation of apoptosis.[2,3] However, their potential as targets of molecular-based therapy is largely unknown.

The tumor suppressor gene *p53* is critical to normal cellular function, and its amino acid sequence is highly conserved among many species. Following DNA damage, p53 protein levels increase because of post-translational changes in protein stability. The p53 response to DNA damage leads to both cell cycle arrest and apoptosis.[2] Increased p53 protein levels lead to transcriptional activation of $p21^{waf1/cip1}$, which inhibits cyclin-dependent kinase activity and prevents cell cycle progression from G1 to S-phase.[2,4] During this period of G1 arrest, GADD45, a p53-regulated

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protein, stimulates DNA repair. In addition to its effect on the cell cycle, p53 also modulates apoptosis in response to DNA damage through the transcriptional activation of additional genes. This likely leads to production of reactive oxygen species, resulting in damage to mitochondria and stimulation of caspases.[5] The downstream effect of p53 on the mitochondrial membrane may then inactivate Bcl-2 and its associated anti-apoptotic proteins while activating pro-apoptotic proteins (Bax, Bak, and Bad).[2,6] The absence of a normal p53 response to DNA damage results in continued growth of abnormal cells, often leading to neoplastic transformation.[2]

Bcl-2 belongs to a growing family of apoptosis-regulating gene products that include anti-apoptotic proteins (Bcl-2, Bcl-X_L, Bcl-w, BFl-1, Brag-1, Mcl-1, and A1) and pro-apoptotic proteins (Bax, Bak, Bcl-X_S, Bad, and Bid).[7] The relative abundance of pro-apoptotic and anti-apoptotic proteins determines the susceptibility of a cell to respond to an apoptotic signal. Bcl-2–related proteins undergo selective dimerization; this competitive dimerization results in a competitive equilibrium between homodimers and heterodimers. Resistance to regulated or programmed cell death is associated with neoplastic transformation and tumor progression.[2,3,5]

In an effort to study the potential impact of mutations in *p53* and dysregulated Bcl-2 expression on the duration of patient survival, we analyzed a carefully selected population of patients with localized, resectable adenocarcinoma of the pancreatic head or uncinate process. In contrast to previous reports, preoperative staging, operative technique, and pathologic analysis of resected specimens were standardized.

MATERIAL AND METHODS

Patients and Surgical Specimens

The prospective pancreatic tumor database was used to identify all patients who underwent pancreaticoduodenectomy for adenocarcinoma of pancreatic origin from June 1990 to April 1997. We excluded all patients who had undergone pancreatic resection for primary adenocarcinomas that did not arise within the head or uncinate process of the pancreas. We also excluded from analysis all patients who underwent operations other than pancreaticoduodenectomy (i.e., total pancreatectomy or distal pancreatectomy).

To be considered for operation, patients were required to fulfill the following objective CT criteria for resectability: (1) absence of extrapancreatic disease; (2) no evidence of tumor extension to the superior mesenteric artery or celiac axis as defined by the presence of a normal fat plane between the tumor and these arterial structures; and (3) a patent superior mesenteric-portal vein confluence. All surgical resections were performed using a standardized technique as previously described.[8] Intraoperative radiation therapy (10 Gy) was delivered to the bed of the resected pancreas (following tumor removal) in a dedicated intraoperative radiation therapy–surgical suite, obviating patient relocation.[9] A standardized pathologic evaluation of the pancreaticoduodenectomy specimen was performed by the surgeon and the pathologist in a pathology suite in the operating room complex.[10] Histologic evaluation began with frozen-section analysis of the bile duct and pancreatic transection margins. A positive bile duct or pancreatic transection margin was treated with re-resection. The retroperitoneal margin was defined as the soft tissue margin directly adjacent to the proximal 3 to 4 cm of the superior mesenteric artery. A 2 to 3 mm full-face (en face) section of the margin was evaluated by permanent-section microscopic examination, and the margin was interpreted as positive if tumor cells were seen in this section. Samples of multiple areas of each tumor, including the interface between the tumor and adjacent uninvolved tissue, were submitted for paraffin embedding and histologic examination (5 to 10 blocks per case). Final pathologic evaluation of permanent sections included an assessment of lymph node status, a description of tumor histology and differentiation, and gross and microscopic evaluation of the tissue of origin (pancreas, bile duct, ampulla of Vater, or duodenum). This study included only patients who had adenocarcinoma of pancreatic origin arising from the head or uncinate process of the pancreas. Tumor size was calculated following surgical resection by measuring the greatest transverse diameter of the tumor.

Most of the patients received adjuvant chemotherapy and radiation therapy (chemoradiation).[11] Radiation therapy was delivered with 18 MeV photons either preoperatively to a total dose of 50.4 Gy (standard-fractionation) or 30.0 Gy (rapid-fractionation) or postoperatively to a total dose of 50.4 Gy. Concurrent with either standard- or rapid-fractionation radiation therapy, 5-fluorouracil was given by continuous infusion at a dosage of 300 mg/m^2/day, five days a week, through a central venous catheter. All patients who received protocol-based preoperative chemoradiation were required to have biopsy proof of adenocarcinoma and a low-density mass in the pancreatic head. This was done to exclude patients with nonpancreatic, periampullary cancer, who have a superior survival duration compared to patients with adenocarcinoma of the pancreas. Therefore, by definition, the patients who received preoperative therapy had larger, more locally advanced primary tumors.

Postoperative follow-up consisted of physical examination, chest radiography, and CT at 3- to 4-month intervals. Survival duration was calculated from the time of cytologic or histologic diagnosis of malignancy.

Immunohistochemical Analysis

Immunohistochemical analysis was performed on formalin-fixed, paraffin-embedded tissue sections. Slides were deparaffinized and rehydrated through graded alcohols and rinsed with phosphate-buffered saline solution. Antigen retrieval was performed using the microwave technique in either 10 mmol/L citrate buffer, pH 6 (for Bcl-2), or phosphate-buffered saline (for p53). The primary anti-Bcl-2 monoclonal antibody (Dako Corp., Carpinteria, Calif.) is a murine antihuman monoclonal antibody, subclass IgG1, that recognizes a cytoplasmic epitope of Bcl-2. The anti-p53 monoclonal antibody D01 (Ab-6; Oncogene Research Products, Cambridge, Mass.) is a murine antihuman monoclonal antibody, subclass IgG2A, that recognizes an amino-terminal epitope (wild-type and mutant) between amino acids 21 and 25 of the human p53 molecule. Visualization was performed using a biotinylated horse-antimouse, streptavidin–HRP system (Vector Labs, Inc., Burlingame, Calif.) followed by diaminobenzidine (Sigma Chemical, St. Louis, Mo.). Sections were counterstained with Gill's hematoxylin and fixed. Positive controls (lymphocytes from human lymph nodes for Bcl-2, and a colon cancer known to harbor a *p53* mutation for p53 staining) and negative controls (omission of the primary antibody) were done for all slides. Histologically normal pancreas was uniformly negative for both p53 and Bcl-2, although immunostaining for Bcl-2 was observed in acinar cells of patients with adjacent areas of chronic pancreatitis.

All immunostained slides were examined by one histopathologist (K.R.C.) who was blinded to clinical outcome. A minimum of 10 high-power fields or the total number of cancer cells in the biopsy specimen were evaluated. Bcl-2 and p53 staining were evaluated and scored separately. Specimens were scored as positive for Bcl-2 or p53 when 25% or more of the cancer cells stained positive.[12-14]

Statistical Methods

Survival was measured from the time of tissue diagnosis. Survival curves were estimated using the Kaplan-Meier method. Univariate and multivariate Cox proportional-hazards regression analysis was used to estimate hazard ratios, confidence intervals for hazard ratios, and *P* values. Assumptions were verified using appropriate residual analysis. Analyses were performed using S-Plus (version 3.3, Statistical Sciences Inc., Seattle, Wash.). Statistical significance was assumed for a *P* value less than 0.05.

RESULTS

Formalin-fixed, paraffin-embedded tumor blocks of the original tumor were available for 70 (64%) of 110 patients who underwent pancreaticoduodenectomy for pancreatic adenocarcinoma; adequate tissue for immunostaining was not available for 40 patients. Median survival of the 70 patients studied was 21 months, which did not differ statistically from that of the 40 patients whose tumor tissue was not available for immunohistochemical analysis (Fig. 1). Patient demographics are shown in Table I. Nuclear staining for p53 was observed in 33 (47%) of 70 patients (Fig. 2). A trend toward improved survival was seen in patients whose tumors overexpressed p53 proteins, but this did not reach statistical significance (Fig. 3;

Table I. Patient demographics

Variable	No. of patients (%)
Sex	
Male	36 (51)
Female	34 (49)
Median age	64 years
Intraoperative radiation therapy	
Yes	47 (67)
No	23 (33)
Adjuvant therapy	
Preoperative chemoradiation	43 (61)
Postoperative chemoradiation	19 (27)
No adjuvant therapy	8 (12)
Median tumor size	3.0 cm
Retroperitoneal margin	
Positive	13 (19)
Negative	57 (81)
Lymph node status	
Positive	32 (46)
Negative	38 (54)
Degree of differentiation (tumor grade)*	
Well differentiated	15 (21)
Moderately differentiated	37 (53)
Poorly differentiated	15 (21)
Disease status	
Alive, no evidence of disease	16 (23)
Alive, with disease	10 (14)
Dead, no evidence of disease	4 (6)
Dead, with disease	40 (57)
Median survival	21 months

*Three specimens could not be graded because of extensive treatment effect from preoperative chemoradiation.

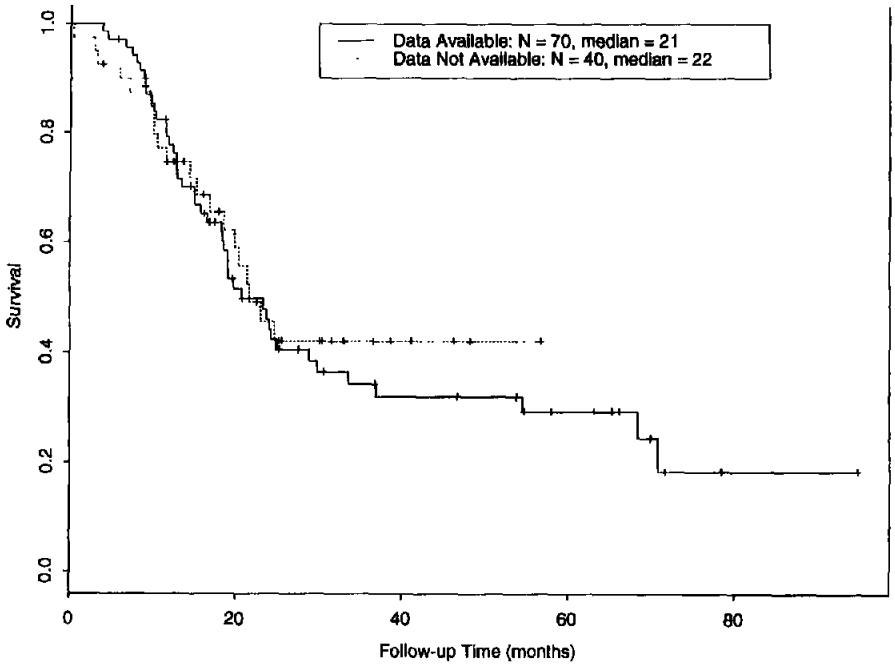

Fig. 1. Kaplan-Meier survival analysis of 70 patients whose tumors were available for immunohistochemical staining and 40 patients treated during the same time period whose tumors were not available for molecular analysis. Survival duration between subgroups did not differ ($P = 0.67$).

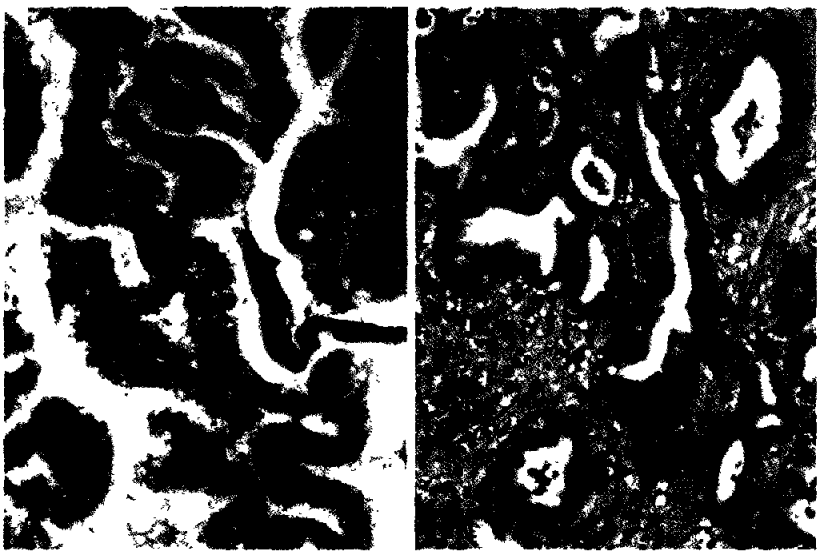

Fig. 2. Negative **(A)** and positive **(B)** immunohistochemical staining for p53 in pancreatic adenocarcinoma. Positive staining suggests a mutant p53 gene with accumulation of mutant p53 proteins.

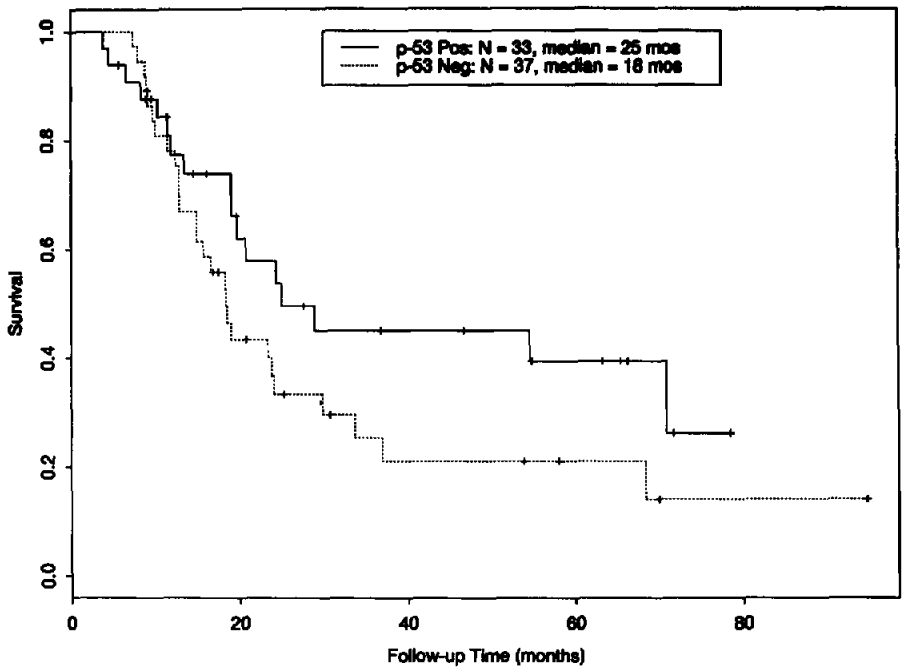

Fig. 3. Kaplan-Meier survival analysis according to immunohistochemical staining for p53 ($P = 0.11$).

Table II. p53 and Bcl-2 immunostaining

	Bcl-2		
p53	Positive	Negative	Bcl-2 Positive/total in row
Positive	14	19	14/33 (42%)
Negative	9	28	9/37 (24%)
p53 Positive/total in column	14/23 (61%)	19/47 (40%)	

$P = 0.11$). The median survival of patients whose tumors stained positive for p53 was 25 months vs. 18 months for patients whose tumors were negative for p53. The p53-positive and p53-negative groups showed no difference in the number of patients who had lymph node metastases or in the number of poorly differentiated tumors. In addition, no difference was seen in the number of patients found to have mutations in codon 12 of the K-*ras* oncogene in p53-positive vs. p53-negative groups (data not shown).

Immunostaining for the Bcl-2 protein was observed in 23 (33%) of 70 tumor specimens; cellular distribution of the positive staining was restricted to the cytoplasm, which is typical for Bcl-2 (Fig. 4). A trend toward improved survival was seen in patients whose tumors overexpressed the Bcl-2 protein, but this did not reach statistical significance (Fig. 5; $P = 0.15$). Patients with Bcl-2–positive tumors had a median survival of 37 months, whereas those with negative tumors survived a median of 19 months.

No statistically significant correlation was observed between Bcl-2 and p53 immunostaining (Table II; $P = 0.13$). Immunostaining for both markers was negative in 28 tumors (40%), and one or both markers were positive in 42 tumors (60%). In the p53-negative subgroup, most of the tumors (76%) were also negative for Bcl-2; this expected association did not reach statistical significance. A significant effect on survival was seen in tumors expressing p53, Bcl-2, or both compared with tumors that were negative for both markers. In this regard, median survival of the 42 patients whose tumors had at least one positive marker was 29 months, compared to 18 months for

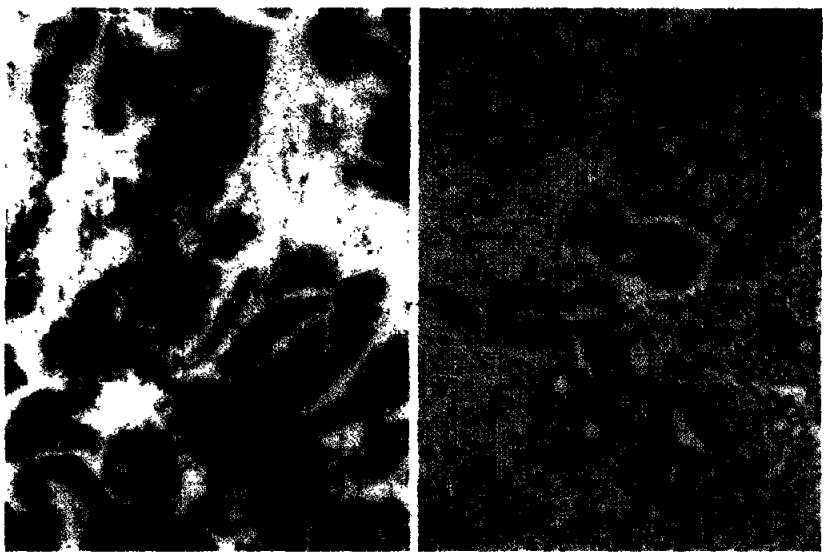

Fig. 4. Negative **(A)** and positive **(B)** immunohistochemical staining for Bcl-2 expression in pancreatic adenocarcinoma. Positive staining indicates overexpression of the Bcl-2 protein.

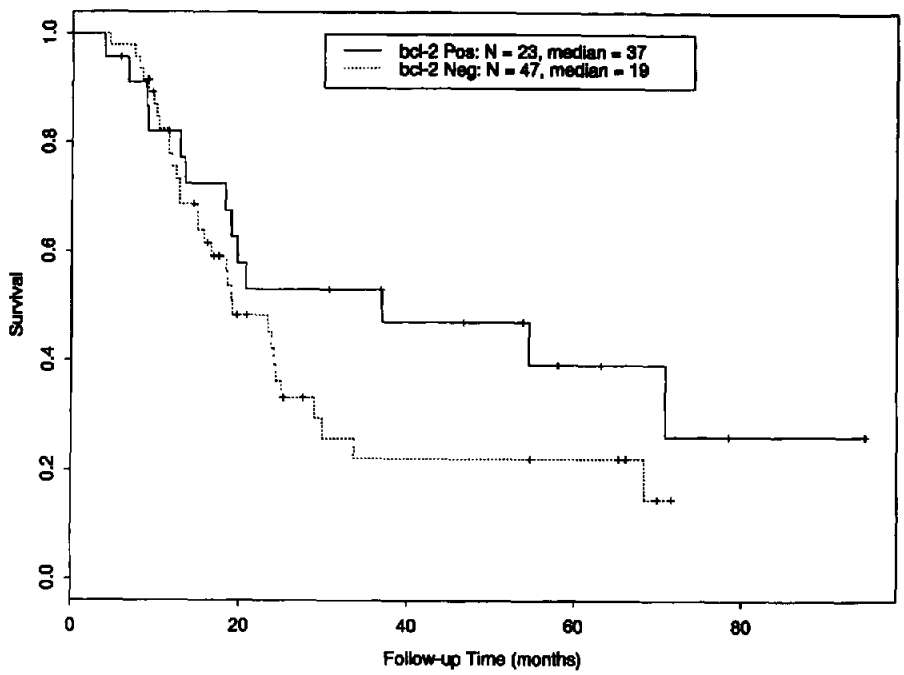

Fig. 5. Kaplan-Meier survival analysis according to immunohistochemical staining for Bcl-2 ($P = 0.15$).

the 28 patients in whom both markers were negative (Fig. 6; $P = 0.019$).

Subset analysis based on the presence or absence of preoperative chemoradiation was performed because of the potential effect of preoperative therapy on immunohistochemical analysis. Forty-three patients had received preoperative 5-fluorouracil–based chemoradiation, and therefore their tumors were studied by histopathologic analysis and immunostaining after treatment. In this subset of 43 patients, no statistically significant difference in survival was calculated based on p53 (Fig. 7, *A*; $P = 0.9$) or Bcl-2 (Fig. 7, *B*; $P = 0.18$) immunostaining. In the remaining 27 patients who had not received preoperative

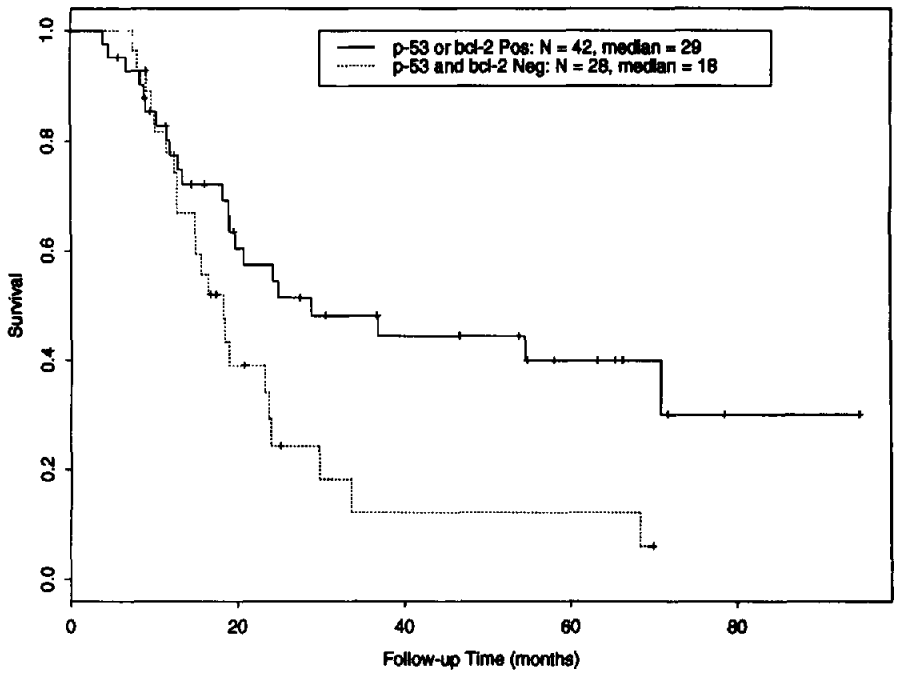

Fig. 6. Kaplan-Meier survival analysis of patients whose tumors stained positive for either p53 or Bcl-2 *(p53 or bcl-2 Pos)* compared with patients whose tumors lacked immunohistochemical staining for both p53 and Bcl-2 (*p53 and bcl-2 Neg*; $P = 0.019$).

Table III. Cox proportional-hazards regression analysis

Variable	Univariate hazard ratio (*P* value)	Multivariate hazard ratio (*P* value)
Adjuvant therapy (before or after vs. none)	1.3 (0.40)	1.5 (0.24)
Retroperitoneal margin (positive vs. negative)	1.2 (0.55)	1.4 (0.41)
Lymph node status (positive vs. negative)	1.4 (0.22)	1.6 (0.14)
Differentiation (good/moderate vs. poor)	0.6 (0.13)	0.6 (0.096)
p53 (positive vs. negative immunostaining)	0.6 (0.11)	0.6 (0.086)
Bcl-2 (positive vs. negative immunostaining)	0.6 (0.15)	0.6 (0.11)
p53/Bcl-2 (either positive vs. both negative)	0.5 (0.02)	0.4 (0.01)
70 Study patients vs. 40 nonstudy patients	0.9 (0.67)	

chemoradiation, p53-positive immunostaining was associated with improved survival duration (Fig. 8, *A*; $P = 0.016$). In this subset of 27 patients, there was no difference in pathologic prognostic factors (such as nodal status, degree of differentiation, etc.) between p53-positive and p53-negative subgroups. In contrast, no statistically significant difference in survival duration was seen in these 27 patients on the basis of Bcl-2 immunostaining (Fig. 8, *B*; $P = 0.54$). Overall there was no difference in median survival duration between patients who received preoperative chemoradiation (21 months) and those who received postoperative chemoradiation or no adjuvant therapy (23 months; $P = 0.4$). Selection bias related to protocol entry criteria makes it impossible to further analyze the potential effects (on survival) of adjuvant therapy.

Neither the absence of lymph node metastases (Fig. 9) nor the absence of poorly differentiated histology (Fig. 10) predicted improved survival duration, although a trend in that direction was noted.

Using a Cox proportional-hazards model, univariate and multivariate analyses were performed to determine whether any of the above-mentioned potential prognostic tumor variables were predictors of survival. The only factor that significantly predicted survival by univariate or multivariate analysis was molecular staging using both p53 and Bcl-2 (Table III). Specifically, patients with tumors that overexpressed

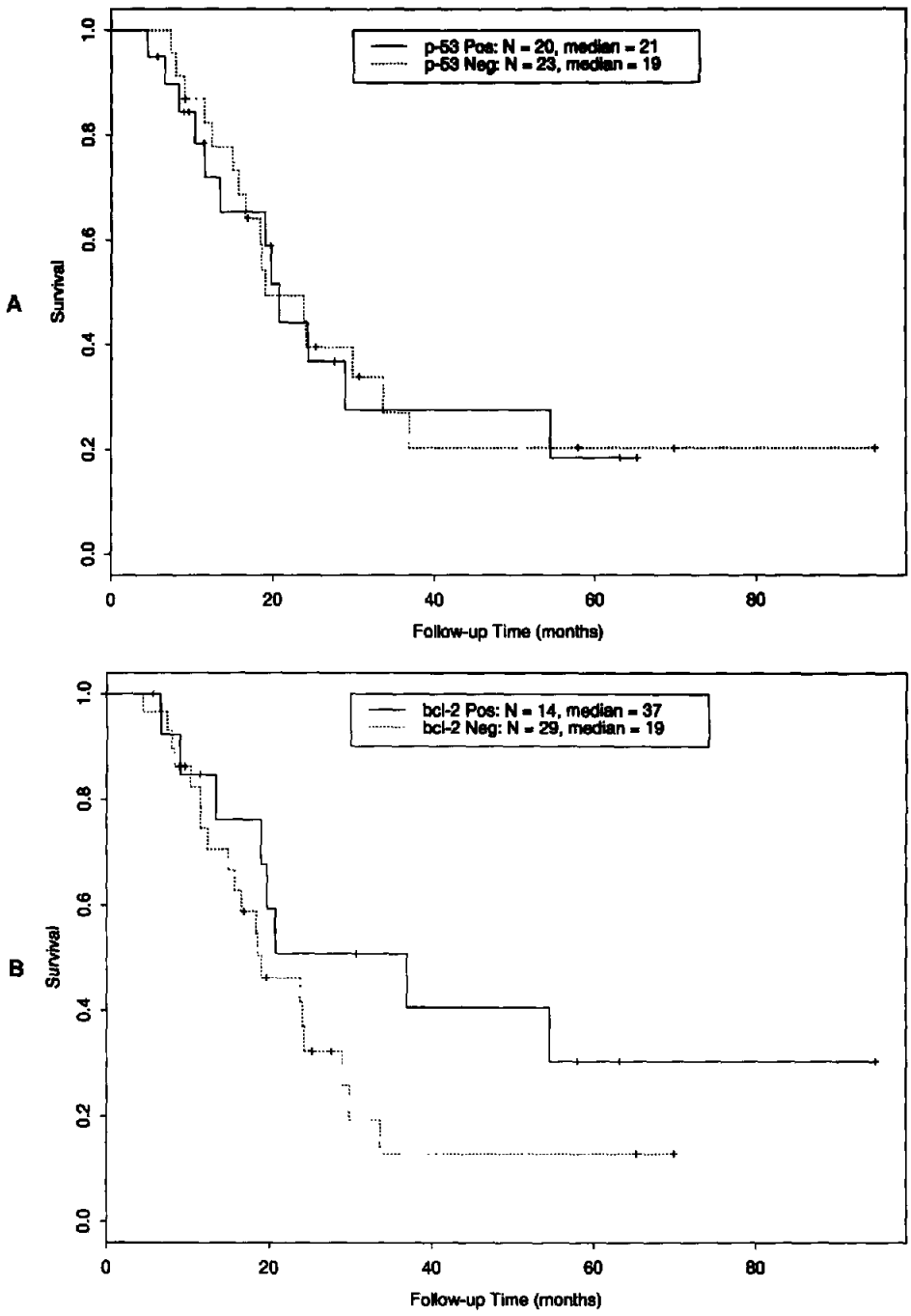

Fig. 7. Kaplan-Meier survival analysis according to immunohistochemical staining for p53 (**A**, $P = 0.9$) and Bcl-2 (**B**, $P = 0.18$) in patients who received preoperative chemoradiation.

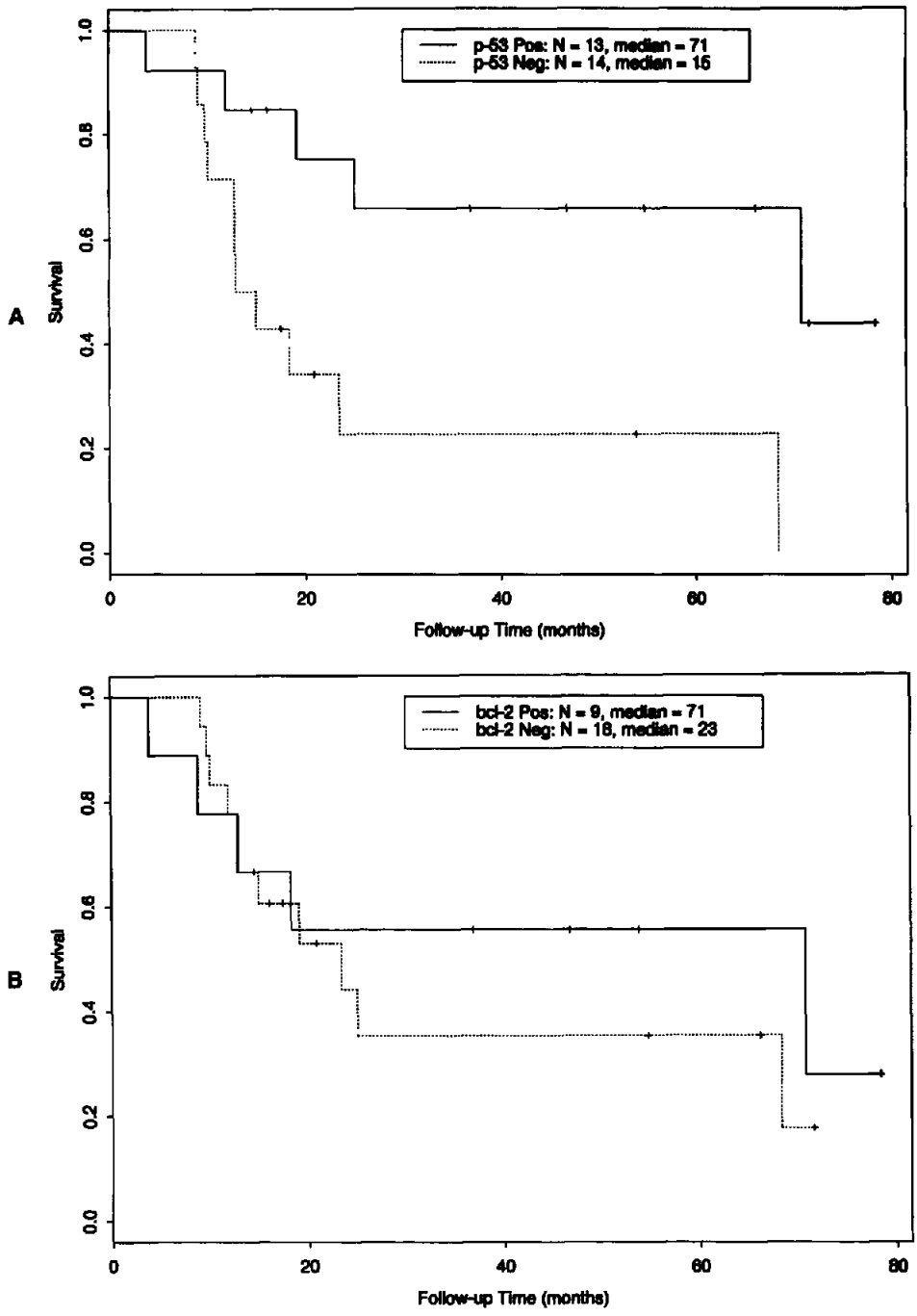

Fig. 8. Kaplan-Meier survival analysis according to immunohistochemical staining for p53 (**A**, $P = 0.016$) and Bcl-2 (**B**, $P = 0.54$) in patients who received preoperative chemoradiation.

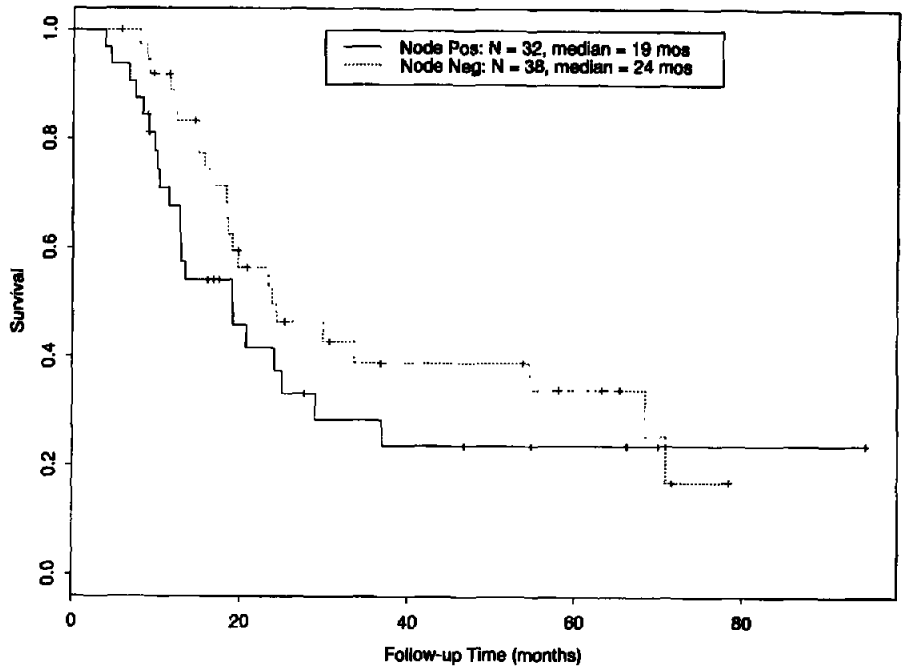

Fig. 9. Kaplan-Meier survival analysis of 70 study patients based on presence or absence of lymph node metastases ($P = 0.22$).

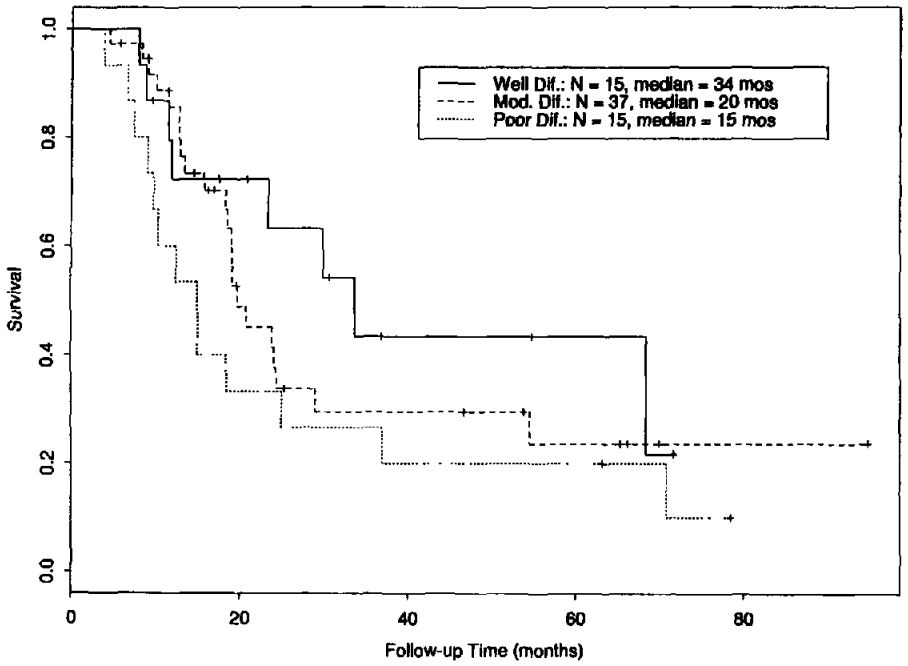

Fig. 10. Kaplan-Meier survival analysis of 70 study patients based on histologic degree of differentiation ($P = 0.24$). Data not available for three patients.

p53 or Bcl-2 or both had significantly better survival rates than did patients whose tumors were negative for both markers.

DISCUSSION

In the vast majority of patients with pancreatic adenocarcinoma, tumor progression results in liver, peritoneal, and occasionally lung metastasis.[1] Improvements in local-regional therapies have not been paralleled by innovations in systemic therapy; most patients with pancreatic cancer die of extrapancreatic metastatic disease. Molecular-based therapy represents an exciting new potential treatment for patients with tumors that are resistant to cytotoxic chemotherapy. However, gene therapy strategies require a knowledge of which oncogenes or tumor suppressor genes are most critical to tumor growth and metastasis. In the current study we examined the immunohistochemical expression of p53 and Bcl-2 as prognostic factors in patients who underwent a potentially curative pancreaticoduodenectomy.

The normal *p53* gene has a half-life of approximately 20 minutes. However, the production of mutant *p53* as a result of amino acid substitutions caused by mis-sense mutations leads to stabilization of the p53 protein so that its half-life is several hours. The abnormal p53 accumulates in the nucleus of the cell and can be detected by immunohistochemical assay.[15] However, frame-shift and non-sense mutations are generally not detected by immunohistochemical methods. DNA sequencing of exons 5 through 8, which contain the majority of inactivating mutations, will also underestimate the frequency of *p53* mutations. The desmoplastic reaction associated with pancreatic adenocarcinomas causes both mutant alleles and normal DNA (or RNA) to be isolated from human pancreatic tumors (fresh, frozen, formalin-fixed, or paraffin-embedded) selected for amplification. In this situation the high proportion of background normal tissue relative to mutant allele may result in a false negative result by direct sequencing.[16,17] Therefore false negative results may occur with both immunohistochemistry and direct sequencing. This is supported by the higher frequency of *p53* mutations in human pancreatic cancer cell lines than in human tumor specimens.[16] In addition, abrogation of downstream effector genes could give the same functional result (with regard to cell cycle control and apoptosis) as a mutation in the *p53* gene.

Our results demonstrated a trend toward improved survival in patients whose tumors stained positive for p53. Previous investigators have reported similar results with squamous cell carcinoma of the tongue,[18] non–small-cell carcinoma of the lung,[13,19,20] and adenocarcinoma of the colorectum.[21] In addition, recent studies of tumor specimens from patients with pancreatic adenocarcinoma showed either no effect of p53 immunoreactivity on survival or a trend toward improved survival in patients whose tumors were p53 immunopositive (when compared to a subset lacking alterations in both p53 and K-*ras*).[21,22] Assuming that positive immunostaining indicates a mutant *p53* gene, one would expect the opposite. As shown in this study, detectable p53 protein seems to be associated with less aggressive tumor biology, but it may not indicate mutation of the *p53* gene. Immunohistochemical staining may produce false positive results because of stabilization of the p53 protein through mechanisms other than mutation of the *p53* gene.[17] This could occur as a normal response to DNA damage induced by irradiation through stabilization of the wild-type protein within cells.[4] In addition, the acute pancreatitis that often accompanies pancreatic cancer has been associated with ductal cells that stain positive for p53.[17]

Clearly the genetic cascade necessary for formation and growth of human pancreatic cancer involves more than changes in a single oncogene or tumor suppressor gene. However, it was the subset of p53-positive specimens from patients who did not receive preoperative therapy that had the longest median survival duration. There is no obvious explanation for this finding. Subset analysis is hindered by small patient numbers; future analysis of our expanding database will confirm or refute these findings.

The *Bcl-2* gene is one member of a family of apoptosis-regulatory genes that control the process of programmed cell death. Tumors with a relative balance of apoptotic gene members favoring the pro-apoptotic group have been postulated to be more sensitive to standard antineoplastic therapy and associated with improved survival.[23] The former hypothesis was, therefore, that low Bcl-2 expression would be compatible with favorable tumor biology and longer patient survival. Previous studies used histochemical analysis to compare the survival duration of patients with solid tumors based on the presence or absence of Bcl-2 overexpression in the primary tumor. Paradoxically, overexpression of Bcl-2 protein was associated with longer survival duration in patients with breast,[24-26] lung,[27,28] and colon cancer.[29] In a recent large study of patients with advanced-stage pancreatic cancer, Bcl-2 overexpression in the primary tumor was associated with a statistically significant improvement in survival duration.[30] Our previous report also suggested that high expression (immunopositive cells >25%) of Bcl-2 was associated with improved survival in a small group of patients with pancreatic and periampullary carcinoma.[31] However, this reached statistical significance only when combined with de-

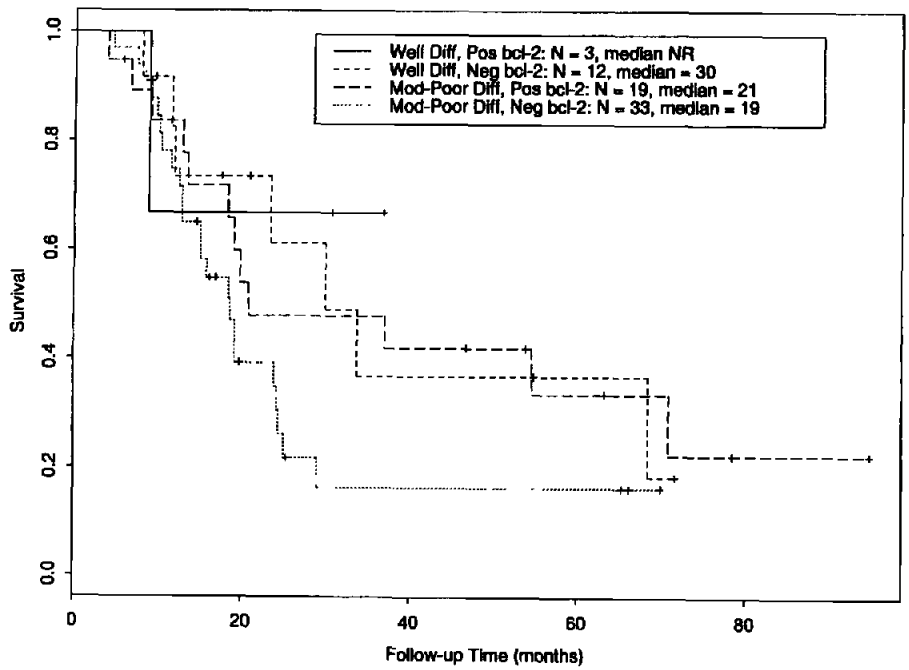

Fig. 11. Kaplan-Meier survival analysis for 70 study patients based on immunohistochemical staining for Bcl-2 and histologic degree of differentiation ($P = 0.27$). Data not available for three patients.

gree of differentiation. In the larger group included in this study, the association persisted but no longer achieved statistical significance (Fig. 11). The *Bcl-2* gene is likely to be only one part of a complicated system designed to regulate cell death. Clearly a complex interaction exists between the members of the *Bcl-2* gene family, so that relative expression levels of one gene may not be sufficient to interpret apoptotic sensitivity to chemotherapy or irradiation. Instead, expression of several members of the gene family should be examined to construct an apoptotic profile, which may be more accurate in predicting tumor behavior and responsiveness to standard cytotoxic therapies.[32] Furthermore, the anti-apoptotic proteins can also suppress cell growth by inhibiting the entry of quiescent cells into the cell cycle. The effects of Bcl-2 overexpression may therefore result in either cell proliferation or growth inhibition based on many factors including tumor type, the ratio of anti-apoptotic to pro-apoptotic proteins, and the influence of such upstream regulators as p53.[7]

Our results demonstrated a significant difference in survival when results of Bcl-2 and p53 immunostaining were combined—that is, patients whose tumors overexpressed one or both proteins had a significantly longer survival than those whose tumors stained negative for both Bcl-2 and p53. The relationship of p53 to Bcl-2 has been the subject of extensive investigation. The promoter region of *Bcl-2* has several putative p53 binding sites, and transcription has been shown to be downregulated by wild-type p53.[33] The downregulation of Bcl-2 by wild-type p53 may partially explain the ability of p53 to induce apoptosis.[34] Wild-type p53 may keep Bcl-2 expression at a relatively low level; when the gene is mutated, this inhibitory control may be released. Positive p53 immunostaining (suggesting a mutation in the *p53* gene) has been associated with overexpression of Bcl-2 in small-cell carcinoma of the lung,[35] yet the opposite has been reported in breast cancer,[24-26,34-36] and no relationship between p53 and Bcl-2 immunostaining has been reported in colon cancer[14] and in non–small-cell carcinoma of the lung.[37] Similarly we found no relationship between p53 and Bcl-2 immunostaining in pancreatic adenocarcinoma. Our finding of short survival in patients whose tumors failed to stain for p53 and Bcl-2 remains unexplained; it is certainly the opposite of what was expected assuming that a functional p53 gene and repressed Bcl-2 expression should inhibit tumor growth and be associated with improved survival duration. The interaction of p53 and Bcl-2 is clearly more complex and the impact of downstream regulators of cell growth more important than is currently understood. For example, Bcl-2 has been shown to inhibit nuclear import of p53 following DNA damage, thereby abrogating the transcriptional regulatory function that requires nuclear localization.[34] Bcl-2 also is capable of suppressing expres-

sion of $p21^{WAF1/CIP1}$, a known cell cycle regulator that may be an effector of p53 function.[38] Bcl-2 also downregulates NF-κB, another transcriptional regulator that effects many downstream target genes including *p53*.[39] It is also possible that a more potent regulator of pancreatic cancer progression, such as K-*ras*, is the dominant factor in predicting tumor biology. In fact, activation of K-*ras* has been shown to upregulate both p53 and Bcl-2.[40]

Our data on the molecular genetics and histopathologic characteristics of early-stage pancreatic cancer demonstrated both the complexity of pancreatic tumorigenesis and the aggressive tumor biology seen in the majority of patients with pancreatic cancer. In contrast to all other studies examining the immunohistochemical profile of resected pancreatic cancers, our study included objective CT criteria for resection and a standardized pathologic evaluation of all pancreaticoduodenectomy specimens. The absence of positive staining for p53 and Bcl-2 was associated with a statistically significant decrease in survival duration. In contrast, standard histopathologic factors had no significant effect on survival duration. Studies such as this one will provide information on the central molecular events involved in the neoplastic process. Once the critical steps are identified, they become potential targets for molecular therapy; a variety of techniques for gene delivery are currently undergoing active investigation and phase I testing. Whether the mechanism of molecular manipulation is gene therapy, antisense techniques, use of intracellular antibodies, or other evolving modalities, a detailed understanding of the molecular events associated with tumorigenesis and metastasis is critical for success.

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Discussion

Dr. C. Yeo (Baltimore, Md.). This study will help us to further understand the molecular genetics of pancreatic cancer. It is very important that these correlations be made, so that we can learn how molecular genetic abnormalities will affect therapy in the future. Your patients largely receive preoperative chemoradiation; how do you think that affects the molecular markers? For example, you found K-ras mutations in only two thirds of the tumors. We and others have reported an incidence of up to 90% K-ras mutations. Is it possible that the cellularity of your tumors affects your DNA retrieval? Second, the immunocytochemical analysis of p53 is fraught with problems. The accumulation of the p53 gene product to immunocytochemically detectable levels is neither sensitive nor specific for the p53 gene mutation. You used the word "counterintuitive," in trying to interpret why the outcome was better with the p53 positivity. It may simply be that those data are not accurate and that you are going to have to look at p53 mutational status in the DNA. When that is done, and it has been done now by two groups that I am aware of, there is a clear correlation between p53 mutations, particularly microdeletions, and poor outcome. Finally, how do you put all this together in reality in the next few years? Do you think that these mutational events will be reasonable therapeutic targets? How can we make use of this mutational information to help us stage patients, treat patients, or be innovative in our therapies?

Dr. R. Bold. Neoadjuvant therapy probably does affect the detection of K-ras mutations because of the cellularity in our specimens. We used mutant-enriched polymerase chain reaction amplification from paraffin blocks. As a result, those patients who had significant treatment effect, that is, the tumor decreased to nearly undetectable amounts, probably would not be scored as K-ras positive. If the molecular markers had been measured in the initial fine-needle aspiration or initial biopsy prior to neoadjuvant treatment, and not at the time of resection, the K-ras mutational rate would probably have been more along the lines of what other groups have noted. With respect to the immunocytochemical results of p53, there is a lot of hesitation in interpreting a positive immunohistochemical result

as a mutation of the gene. We are going back to do the same type of assay that we use for K-ras mutations, that is, elution of DNA, direct polymerase chain reaction, and sequencing. Finally, I believe that these molecular events are realistic targets for therapy.

Dr. R. Swanson. (Worcester, Mass.). How do these molecular markers interact with standard clinical prognostic factors such as nodal status? For example, do K-ras–negative, node-positive patients live longer?

Dr. Bold. In our multivariate analysis, we included nodal status, differentiation, tumor size, and microscopic margin of resection. All of those fell out and K-ras remained the most significant predictor, independent of those other histopathologic criteria.

Force-Feedback Grasper Helps Restore Sense of Touch in Minimally Invasive Surgery

Mark MacFarlane, M.D., Jacob Rosen, Ph.D., Blake Hannaford, Ph.D., Carlos Pellegrini, M.D., Mika Sinanan, M.D., Ph.D.

The age of minimally invasive surgery has brought forth astounding changes in the health care field. Less pain and quicker patient recovery have been demonstrated with several types of operations that were once performed by an open technique. With these changes have come reports of complications. The decreased sense of touch is just one of several limitations inherent to current techniques of minimally invasive surgery that limit detection of subtle or unapparent lesions on palpation, such as common duct stones and liver lesions. The purpose of this study is to demonstrate the ability of a force-feedback–equipped grasper to restore some of the sense of touch that is lost in minimally invasive surgery. To demonstrate this ability, we created six silicone phantoms of identical dimensions but graded compliance, and asked 10 subjects to place them in increasing/decreasing order of compliance. They used three tools (their dominant gloved hand, a standard laparoscopic Babcock grasper, and our force-feedback device fitted with the identical Babcock grasper) to rate the compliance of the samples in a blinded fashion. These conditions thus approximated the conditions of open surgery, minimally invasive surgery, and minimally invasive surgery fitted with a force-sensing device, in terms of palpating tissues. Five surgeons skilled in minimally invasive surgery and five nonsurgeons participated in the study. The results indicate that the force-feedback device is significantly ($P < 0.05$) better than a standard Babcock grasper at rating tissue compliance, but was not as successful as a gloved hand (mean of squared errors = 1.06, 3.15, and 0.25, respectively). There was no significant difference between surgeons and nonsurgeons in rating compliance. We conclude that this force-feedback instrument is able to partially restore the sense of touch in minimally invasive surgery. This restored ability may thus potentially result in more efficient operations with improved diagnostic capabilities and fewer complications during minimally invasive surgery. (J GASTROINTEST SURG 1999;3:278-285.)

KEY WORDS: Haptic, surgical simulation, force feedback, touch

The current age of minimally invasive surgery has brought forth astounding changes in the health care system. Patients have benefited by faster recovery,[1-4] less patient discomfort,[5,6] and improved cosmesis because of the smaller incisions. Insurance companies and employers alike have also benefited by way of shorter hospital stays[7-9] resulting in lower hospital charges[10,11] and a quicker return to work.[12] Unfortunately this new technology has also been accompanied by reports of endoscopic complications such as gastrointestinal and colon perforation, as well as injuries to other organs.[13] In addition, some diagnostic information may be lost when endoscopic surgery is performed[14] because of the inability of the surgeon to feel the tissues with the hand. This may result in underestimated or unrecognized tissue inflammation or inability to detect solid and hollow organ masses. The preceding disadvantages of minimally invasive surgery are a result (at least in part) of the need to use long instruments that leave the surgeon at a mechanical disadvantage in terms of the haptic interface or sense of touch. Other investigators have proposed ways to improve the haptic feedback in minimally invasive surgery by incorporating a sleeve,[14] which allows passage of the hand into the abdomen, but this requires a larger incision, thus partly defeating the purpose of minimally invasive surgery. Visual cues can supply information on tissue deformation and com-

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pliance,[15,16] but the information is highly subjective and incomplete.

To address the deficit of haptic feedback during minimally invasive surgery, we have designed and tested an endoscopic grasper with force-feedback capabilities,[17] to improve the sense of touch in minimally invasive surgery. This prototype instrument is our initial attempt to enhance the sense of touch during minimally invasive surgery.

MATERIAL AND METHODS

Computerized Grasper Design

The grasper design utilizes "master" and "slave" components, which are linked by a computer interface. The master component is manipulated by the surgeon through a standard set of endoscopic finger loops. By this movement the surgeon determines the corresponding position of the instrument tool tip on the slave component. The driving force to move the tool tip is an electromagnetic coil actuator on the slave. The position of the tool tip and finger loops is measured by identical optical encoder position sensors on the master and slave. Thus, as the surgeon manipulates the finger loops on the master, the position is measured by the master position sensor. This position is then transferred via the computer interface to the slave position sensor, and the slave actuator then moves the tool tip to the corresponding position of the finger loops. The force-feedback capability (haptic interface or sense of touch) of this device is produced by a second and identical actuator on the master which is linked to the finger loops. Therefore as the slave actuator creates a force to move the tool tip and compress whatever is in the tool tip, the identical force is generated in the master actuator, which is linked to the surgeon's hand by means of the finger loops. This constant and simultaneous interplay of position and force between the master and slave is outlined in Fig. 1.

The computer interface allows transmission of signals of position and force between the master and slave. In addition, it also allows real-time display and recording of force and position data, and thus allows one to measure how much force is required to displace a tissue a given distance. The computer interface also has the capability to perform a task with the slave in an automated preprogrammed manner (no involvement of master) or in a bimanual mode (response of the slave is controlled by the action of the master). The force-feedback mode is the bimanual mode. More technical aspects of the grasper system have been described in detail previously.[17]

The tool tip used throughout the experiments consisted of a nondisposable endoscopic Babcock grasper tip and shaft with a tool tip surface area of 9 × 9 mm. The prototype is displayed in Figs. 2 and 3.

Creation and Objective Testing of Silicone Phantoms

Six silicone phantoms of uniform shape and color (15 mm diameter × 150 mm length cylinders) but of varying compliance (Fig. 4) were custom manufactured by a local private company (Simulab Inc., Seattle, Wash.). The compliance of the materials was altered by varying the percentage by weight of the catalyst during manufacture of the phantoms. To ob-

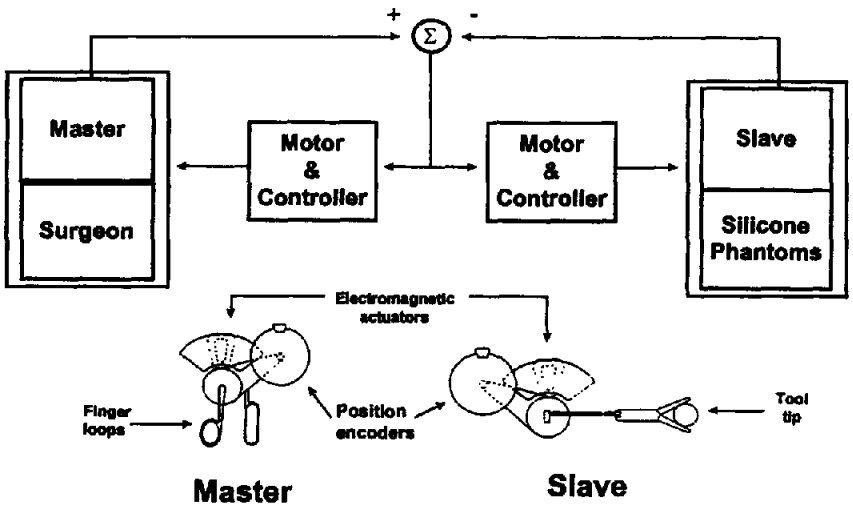

Fig. 1. Flow diagram for interaction of master and slave components of the grasper system. Optical encoder position sensors and electromagnetic actuators are identical on the master and slave components.

Fig. 2. Master and slave components of the computerized grasper. Both parts can be detached from the metal base as needed.

Fig. 3. Detailed views of the master (A and B) and slave (C and D) components. Each component contains an actuator and position sensor.

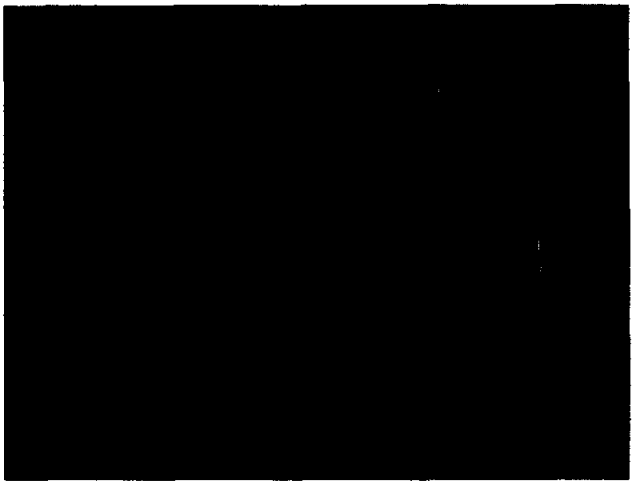

Fig. 4. Photograph of the six silicone phantoms of graded compliance. Dimensions are identical for each of the six phantoms.

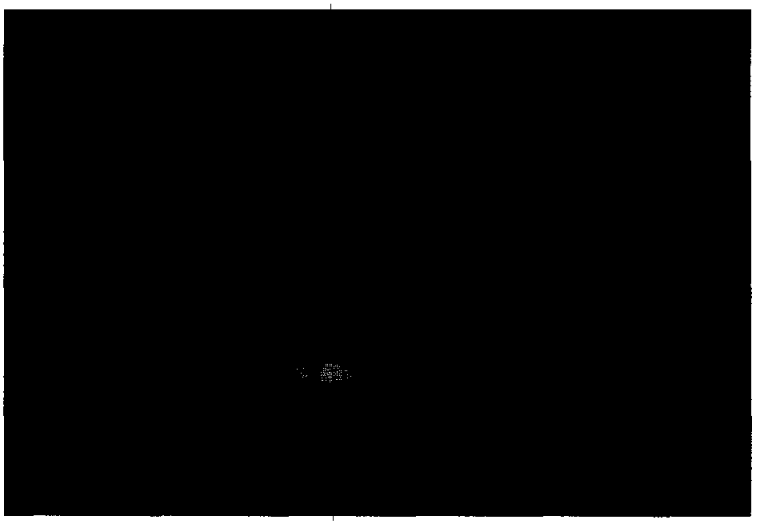

Fig. 5. Device used to carry out objective stress/compression studies of the six silicone phantoms.

tain an objective measure of the compliance ratings, the phantoms were subjected to stress/compression testing. This was performed by sequentially compressing each phantom in increments of 1.1 mm over a total distance of 9.9 mm using a device that held and compressed the materials. We measured the force required to compress the materials by means of a force meter attached to the compression device (Fig. 5). Testing was performed three times on each silicone phantom. The data were used to construct stress/compression curves for each phantom (Fig. 6).

Subjective Compliance Rating of Silicone Phantoms

To test for possible improvement in haptic feedback by the force-feedback grasper compared to a standard laparoscopic instrument, we performed experiments as outlined below. Ten subjects (five experienced laparoscopic surgeons and five electrical engineers with experience in haptic technology) performed palpation experiments on the six silicone phantoms. Subjects were asked to place the phantoms in the correct order of increasing or decreasing compliance. This was performed a total of four times for each palpation tool used. The three tools for palpation included a dominant surgical gloved hand (simulating open surgery palpation), a standard 10 mm nondisposable laparoscopic Babcock grasper (simulating endoscopic surgical palpation), and an identical Babcock grasper tip fitted to our force-feedback device in the bimanual mode (simulating possibly improved endoscopic surgical palpation). Subjects were allowed a 3-minute unblinded practice period for each

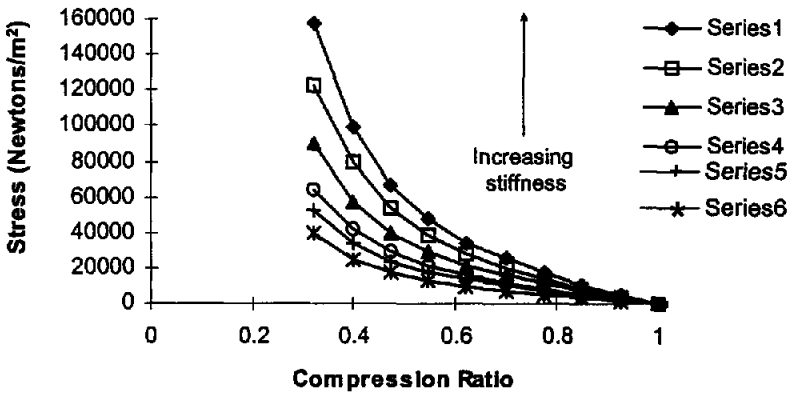

Fig. 6. Stress/compression ratios for each of the six silicone phantoms. Standard deviations (not shown) indicate minimal to no overlap, even at the low end of the compression ratio, although this is not appreciated on the graph secondary to the large y-scale.

of the three tools so they could become aquainted with the palpation of the phantoms. The phantoms were presented to the subjects in random order, and the order was changed on each presentation. The phantoms were assigned a number of 1 through 6 (1 = hardest, 6 = softest) based on the preceding objective compliance testing. Subjects attempted to choose the correct order. The difference of the chosen order (subjective) from the known order (objective) was then squared to give a positive integer for each choice. Each squared value was summed, and the total divided by six (6 phantoms) to yield the mean squared error. Testing resulted in a total of 12 rounds of compliance ratings for each of the six phantoms for each subject (a possibility of 72 errors in compliance rating for each subject and 720 possible errors for the 10 subjects). Although the tool type obviously could not be blinded, the subjects were not allowed to visualize any interaction of the tool type/phantom interface. Likewise the subjects were not allowed to visualize any part of the three tools or phantoms during the experiments; thus visual cues were eliminated in their subjective evaluation of phantom compliance.

Data Analysis

Objective stress/compression ratio curves were generated from 36 data points for each silicone phantom after graduated compression. A best curve fit using Matlab (The MathWorks, Inc., Natick, Mass.) was used to construct the stress/compression curves.

Subjective compliance ratings of the six silicone phantoms by 10 subjects were scored as the mean of the squared difference (error) of the subjective order from the known correct order as described previously. Results were analyzed with a two-tailed t test with significance reported as a P value of ≤ 0.05.

RESULTS

Data points for the six different silicone phantoms generated from compression of the samples were fitted to the stress/compression curves as illustrated in Fig. 6. Increasing stress is applied to the samples (y-axis) in to produce a given compression ratio. Series 1 is thus the hardest material (least compliance) and series 6 is the softest silicone phantom (greatest compliance). This serves as the basis for comparing the subjective rating of the silicone phantoms by the 10 subjects to the correct order of varying compliance.

Fig. 7 depicts the mean of the squared errors of the subjective order of compliance (compared to the known order) for surgeons and nonsurgeons for each of the three tool types. Although the nonsurgeons (electrical engineers with extensive experience in haptic technology) appeared to have fewer errors in determining the correct order (lower error score) than the surgeons, this difference was not significant ($P > 0.05$).

Fig. 7 shows pooled data from both groups (n = 10 subjects, surgeons and nonsurgeons) on the subjective rating of sample compliance. The force-feedback Babcock grasper yielded improved force feedback when compared with the standard nondisposable Babcock grasper ($P \leq 0.05$) with a tool tip of identical mechanics and surface area. The human hand was significantly better ($P \leq 0.05$) than the other two in determining the correct order of sample compliance.

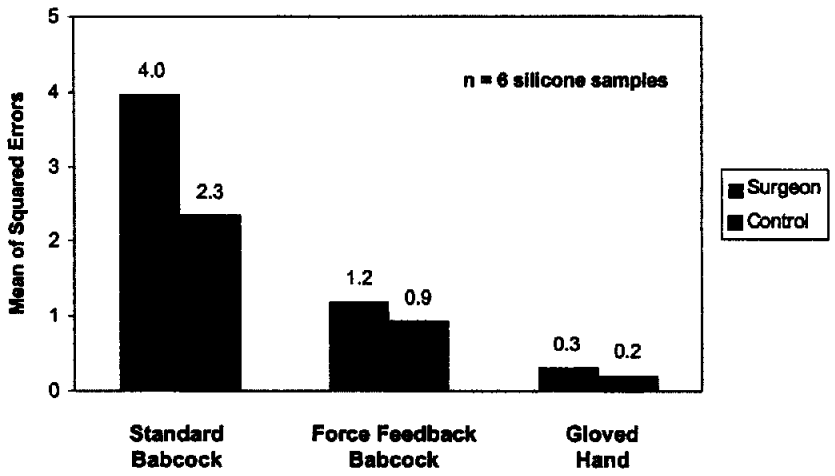

Fig. 7. Palpation experiments of six silicone phantoms by surgeons and nonsurgeons *(control)*. Three different tools (standard laparoscopic Babcock grasper, force-feedback Babcock grasper, and gloved hand) were used. No significant difference is observed between the two (surgeon and nonsurgeon) groups.

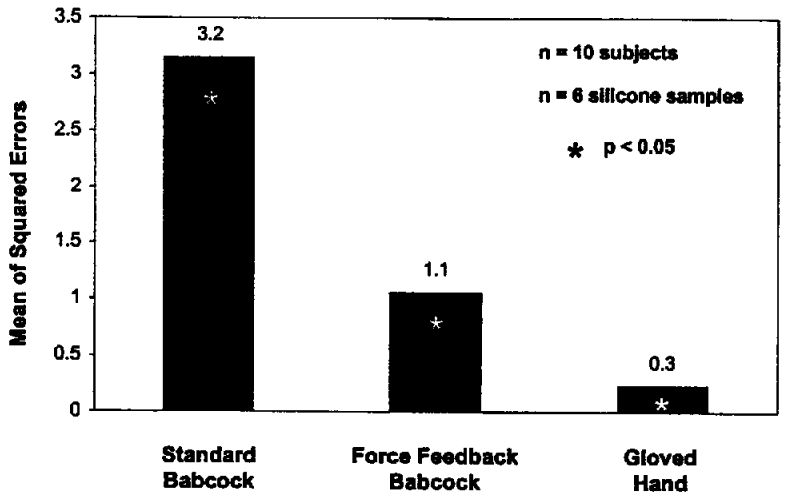

Fig. 8. Silicone phantom palpation error scores. Pooled data (surgeon and nonsurgeon) from palpation experiments on the six silicone phantoms. Significant differences are observed based on the palpation tool used.

DISCUSSION

Endoscopic-based operations continue to expand into new areas of surgery. Operations that were once thought possible to complete only by means of an open approach are now being performed commonly via an endoscopic approach.[18] In order for an endoscopic approach to surgery to be beneficial, it must also be safe. Although most endoscopic surgery proceeds without incident, there are reports of injury to organs resulting in significant complications.[13] Surgeons need to rely on visual cues to compensate for lack of depth perception and poor haptic feedback secondary to reliance on long instruments to perform the operation.

To potentially improve the force feedback during endoscopic surgery, we have designed and tested a prototype surgical endoscopic instrument that has the advantage of easy tool tip interchangeability with existing marketed tool tips. Although the prototype design is bulky, the intracavitary portion of the tool is identical to that of conventional endoscopic instruments.

As depicted in Fig. 8, the human hand is superior to both the force-feedback Babcock instrument and the standard Babcock endoscopic grasper at determining the correct order of silicone phantom compliance. This is not surprising given the fact that the human hand is a highly complex diagnostic tool and has

multiple haptic properties (sense of position, proprioception, temperature, texture sensation). What is surprising is the degree of haptic sensation improvement of the force-feedback Babcock vs. the standard Babcock grasper. Although the time to complete each rating of the six samples was not measured, there was a noticeable time difference to complete each subjective rating by all subjects (human hand, force feedback grasper, standard grasper with increasing time requirements, respectively). This further underscores the differences in difficulty in determining the correct order of phantom compliance for each tool type.

The premise that improved force feedback will result in less tissue injury during endoscopic operations is difficult to assess since improved force feedback is not yet available to the endoscopic surgeon. A similar premise is that three-dimensional imagery may produce increased efficiency in endoscopic operations.[15,16] It seems apparent that visual cues are very powerful in filling in the gaps of visual and haptic deficits in endoscopic surgery, but these "compensations" may not be necessarily accurate or safe.[14-16] Until improved force-feedback capabilities can be made available for testing, we will not know if improved haptic feedback will result in fewer injuries to soft tissues. In addition, it is not known how much force and torque applied to a given tissue by laparoscopic operations will result in tissue injury, either reversible or irreversible injury. This aspect is currently undergoing study in our laboratory.

The inability to palpate tissues accurately during endoscopic surgery because of inadequate force feedback undoubtedly results in loss of diagnostic information. As surgeons of the open surgery era, we have been spoiled by the luxury of the human hand to supply this diagnostic information. Common examples would include hand palpation of common bile duct stones, lung and liver nodules, and intestinal masses. As endoscopic surgery further displaces open surgery as the standard of care, we will be additionally handicapped in our diagnostic intraoperative capabilities. Although endoscopic ultrasound imaging has excellent potential for bridging some of this gap of intraoperative diagnostic limitations,[19,20] it does not work well on hollow organs because of artifacts of shadowing from air/tissue interfaces. Future generations of force-feedback devices will surely improve in their compactness and fidelity of information. Furthermore, we have previously reported with a computerized grasper that normal biologic organs have characteristic force profiles[17] based on their intrinsic tissue properties. If the force profile is not "normal" for a given biologic tissue, then this could represent tissue inflammation, fibrosis, foreign body, or cancer, thus improving diagnostic yield.

Another potential application of this technology is in the area of tissue protection. If the degree of forces and torques that result in tissue injury can be determined (work in progress), then "smart endoscopic force-feedback instruments" can be developed that will apply force/torque limitations resulting in tissue protection from iatrogenic operative tissue injury.

CONCLUSION

Although minimally invasive surgery techniques have brought forth astounding changes in surgical care, with benefit to all participants who provide and receive health care, we need to continue to strive to make operations safer, more efficient, and with fewer complications. We have demonstrated that haptic feedback can be potentially improved during minimally invasive surgery. Whether this will translate into fewer episodes of tissue injury and improved diagnostic capabilities remains unclear. However, the first step is to be able to make this technology available for testing. The ultimate goal is to perform operations which are performed more efficiently, less invasively, and with fewer complications to patients.

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Discussion

Dr. L. W. Traverso (Seattle, Wash.). Of the three common laparoscopic procedures we do—cholecystectomy, Nissen fundoplication, or appendectomy—which do you think will be helped the most by this technology?

Dr. M. MacFarlane. We certainly perform many laparoscopic Nissen fundoplications and I can say that we see a fair number of serosal tears, although perforations are very rare in our hands. So I think the more advanced cases are going to be the best application for this technology.

Dr. B. Schirmer (Charlottesville, Va.). This is a very interesting concept, but I am a little concerned about whether it is going to be generally applicable. Do you see this as being something that every grasper will be equipped with eventually and at what cost? Will equipping these graspers be cost-effective? Second, if you were to design just one type of instrument, in which specific situations would you use it? Ultrasound imaging does very well for solid organs—would you, for example, want to use it in the colon to palpate lesions? What else do you envision for this application?

Dr. MacFarlane. Laparoscopic ultrasound is a wonderful modality. It is very sensitive, especially on solid organs, so I think the grasper would be most applicable, in terms of diagnostic information, on hollow organs. Second, if the cost of new technology were the sole determining factor, we would not have any new technology. Until this technology is developed further, I do not think we will know the answers to your questions. In its current prototype form, our instrument is very bulky. It needs to have stronger actuator forces to carry out the steps in the operation that are performed.

Dr. N. Soper (St. Louis, Mo.). You are at the low end of the development of this instrument and I think there is a long way to go. In terms of the bulkiness of the instrument as currently designed, it looks like there will need to be something at the actuator end, which will bulk up the end that is going through these little incisions we are going to make. Is there any possibility of placing that actuator back out, near the handpiece for instance, rather than down at the tip?

Dr. MacFarlane. The actuator itself does not limit our capabilities inside the abdomen because the length of the intra-abdominal portion is identical to what is currently used in laparoscopic surgery. As with all prototypes, future models will become more compact and more efficient. I cannot currently estimate what the final product will look like.

Laparoscopic Cholecystectomy in Patients With Hepatic Cirrhosis: A Five-Year Experience

Charles M. Friel, M.D., Jenny Stack, R.D., R. Armour Forse, M.D., Ph.D., F.A.C.S., Timothy J. Babineau, M.D., F.A.C.S.

Our institution is a tertiary referral center that specializes in hepatobiliary surgery. To evaluate the safety, efficacy, and conversion rate of laparoscopic cholecystectomy in patients with hepatic cirrhosis, we conducted a retrospective analysis of all cirrhotic patients undergoing attempted laparoscopic cholecystectomy during the period from 1991 to 1996. The diagnosis of cirrhosis was made on the basis of either a preoperative history, a liver biopsy, or the surgeon's operative description of the liver. All patients had early, well-compensated cirrhosis (Child's class A or B). A total of 30 patients underwent attempted laparoscopic cholecystectomy and five patients were converted to an open procedure (17%). The conversion rate for elective cases was 5% compared with 36% for urgent procedures. Two patients were converted because of varices and three because of unclear anatomy. No patients were converted because of bleeding. There were no operative deaths. The complication rate for elective procedures was 16%, with an average length of stay of 2.1 days, compared with 36% and 4.8 days, respectively, for urgent cases. Laparoscopic cholecystectomy in patients with early, well-compensated cirrhosis is safe and should be the treatment of choice for these patients. (J GASTROINTEST SURG 1999;3:286-291.)

KEY WORDS: Laparoscopic cholecystectomy, hepatic cirrhosis

Biliary stone disease in patients with hepatic cirrhosis has long been a therapeutic and diagnostic challenge. The incidence of cholelithiasis in patients with cirrhosis has been reported to be as high as 46%,[1] compared with approximately 5% to 15% in the general population.[2] Fortunately, most patients remain asymptomatic and do not require surgical intervention.[3] However, if surgery is required, the mortality rate may be quite high. In fact, the mortality rate for open cholecystectomy in patients with cirrhosis is reported to be 10% to 30%.[4-7] Most of these deaths occur in patients with advanced, complicated cirrhosis (Child's class C) who suffer either excessive blood loss during the operation or develop postoperative hepatic complications.[4,5] Patients with Child's class A and B cirrhosis have much less morbidity and mortality,[4,8] but the rates are still higher than those for the general population. For these reasons it is generally accepted that patients with cirrhosis and cholelithiasis should be closely monitored and operated on only for significant symptomatic disease.

However, in the laparoscopic era the role of cholecystectomy in patients with hepatic cirrhosis is still being defined. The Beth-Israel Deaconess Medical Center is a tertiary care center that specializes in hepatobiliary surgery. To better define the safety and efficacy of laparoscopic cholecystectomy in patients with cirrhosis, we retrospectively reviewed our experience with this procedure over the past 5 years.

PATIENTS AND METHODS

A retrospective chart review was performed from September 1991 to August 1996. Approximately 100 charts were examined for patients whose discharge diagnoses included cholecystectomy and liver disease. Of those, 30 patients had a diagnosis of cirrhosis and either an attempted laparoscopic cholecystectomy with conversion to an open procedure or a successful laparoscopic cholecystectomy. These patients formed the study group. A diagnosis of cirrhosis was made on the basis of either a preoperative history, as docu-

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mented in the medical chart, an intraoperative biopsy, or the gross appearance of the liver as determined by the attending surgeon at the time of operation. The operations were performed by one of six attending surgeons with the assistance of a resident. The following data were collected from the charts when available: preoperative diagnosis, prothrombin time, serum albumin, total bilirubin, history of encephalopathy, history or presence of ascites, operative time, estimated blood loss, and length of stay. Data on nutritional status were generally not available from the chart. Therefore all patients were assumed to have a good nutritional status unless otherwise indicated. Using Child's criteria, the severity of the liver disease was determined. Not all Child's data were available for every patient. If a particular data point was not available for a patient, it was assumed to be normal for the purpose of determining hepatic disease severity. Patients were classified as having either Child's A, B, or C cirrhosis if they had two or more of the clinical criteria for that category.

All statistical analysis was carried out using a commercially available software package (Microsoft Excel). Student's *t*-test or chi-square test was performed where appropriate. Data are reported as mean ± standard deviation.

RESULTS

Over the 5-year period there were 30 patients with the diagnosis of cirrhosis who underwent either attempted or successful laparoscopic cholecystectomy. The patient characteristics are presented in Table I. Thirteen patients (43%) had a known or suspected history of cirrhosis documented in the medical record prior to surgery (11 known and 2 suspected, secondary to ascites). Preoperative data were not available for all of these patients. However, at the time of operation the attending surgeon noted a grossly cirrhotic liver in 10 of the 13 patients. No mention was made concerning the appearance of the liver in the remaining three patients. However, one patient had a known history of encephalopathy and ascites, one had a previous variceal bleed, and one had varices noted on esophagogastroduodenoscopy 2 years after his cholecystectomy. An intraoperative diagnosis was made in the remaining 17 patients (57%) based on the appearance of the liver. In 15 patients, the diagnosis was confirmed by liver biopsies, the results of which were reported to be consistent with cirrhosis. Five patients had a history or evidence of portal hypertension, four had a history of encephalopathy, and six had a history of ascites (three patients had all three sequelae of cirrhosis, one had a history of encephalopathy and ascites, two had ascites alone, and two had portal hypertension alone). One patient who was taking coumadin had a prothrombin time greater than 2 seconds above the control value. Twenty-three patients (77%) had Child's A, well-compensated cirrhosis and seven patients (23%) had Child's B cirrhosis. There were no patients with Child's C disease.

Five cases (17%) were converted to open procedures—two for varices and three for unclear anatomy secondary to adhesions and inflammation. None of the cases were converted for blood loss. Mean blood loss was 92 ± 88 ml, mean operative time (time of incision to time of arrival in the postanesthesia care unit) was 139 ± 34 minutes, and mean length of stay was 3.1 ± 2.4 days. Overall, there were seven patients (23%) who had complications (Table II).

Mean blood loss, operative time, and length of stay for the converted procedures vs. the laparoscopic procedures are summarized in Table III.

Nineteen patients (63%) were admitted postoperatively after an elective procedure for symptomatic cholelithiasis, whereas 11 hospitalized patients (37%) underwent urgent cholecystectomy (nine patients for acute or chronic cholecystitis, one for gallstone pancreatitis, and one for iatrogenic gallbladder perforation following liver biopsy). The results are summarized in Table IV. Of note, there was a significantly higher conversion rate and greater blood loss in patients undergoing an urgent procedure, which translated into an increased length of stay in the hospital.

The mean blood loss, operative time, and length of stay for patients with Child's A cirrhosis vs. Child's B cirrhosis are shown in Table V. There were no significant differences between these two groups.

Table I. Patient characteristics (N = 30)

Age (yr)	56.2 ± 14.0
Female	14 (47%)
Male	16 (53%)
Preoperative history of cirrhosis	13 (43%)
Intraoperative diagnosis, with liver biopsy	15 (50%)
Intraoperative diagnosis, appearance only	2 (7%)

Table II. Operative and postoperative data for all cases (N = 30)

Converted	5 (17%)
Mean blood loss (ml)	92 ± 88
Mean operative time (min)	139 ± 34
Mean length of stay (days)	3.1 ± 2.4
Complications	7 (23%)

Table III. Converted vs. successful laparoscopic cholecystectomy

	Converted (N = 5)	Laparoscopic (N = 25)	*P* value
Mean blood loss (ml)	270 ± 91	61 ± 25	<0.05
Mean operative time (min)	155 ± 26	135 ± 35	NS
Mean length of stay (days)	6.6 ± 1.8	2.4 ± 1.8	<0.01
Complications	2 (40%)	5 (20%)	NS

NS = not significant.

Table IV. Elective vs. urgent cases

	Elective (N = 19)	Urgent (N = 11)	*P* value
Converted	1 (5%)	4 (36%)	<0.05
Mean blood loss (ml)	62 ± 36	155 ± 122	<0.05
Mean operative time (min)	137 ± 37	141 ± 32	NS
Mean length of stay (days)	2.1 ± 2.0	4.8 ± 2.1	<0.01
Complications	3 (16%)	4 (36%)	NS

Table V. Child's A vs. Child's B cirrhosis

	Child's A cirrhosis (N = 23)	Child's B cirrhosis (N = 7)	*P* value
Converted	3 (13%)	2 (29%)	NS
Average blood loss (ml)	90 ± 84	114 ± 111	NS
Average operative time (min)	140 ± 37	135 ± 26	NS
Average length of stay (days)	2.7 ± 2.1	4.3 ± 3.1	NS
Complications	4 (17%)	3 (43%)	NS

Table VI. Complications

Patient	Major	Minor
1	Wound infection Aspiration pneumonia Encephalopathy	
2	Hematoma	
3		Fungal urinary tract infection
4	Hypotension	
5	Oliguria Ileus	
6	Ascites	
7	Hematoma Ascites	Urinary retention Wound cellulitis

There were no deaths. Seven patients (23%) had a total of 13 complications; 10 were classified as major and three as minor (Table VI). Two patients had hematomas. Neither had hemodynamic compromise, but one patient received two units of packed red blood cells and the other received a single unit.

DISCUSSION

Abdominal operations for patients with cirrhosis have always been a surgical challenge. Aranha et al.[5] and Schwartz[6] have previously documented the high mortality rate associated with cholecystectomy in patients with cirrhosis. Many of these deaths were in patients with advanced disease. In fact, Aranha et al.[5] reported a mortality rate of 9.3% in patients with a prothrombin time less than 2.0 seconds above control, as opposed to 83% in patients with a prothrombin time greater than 2.0 seconds above control. Doberneck et al.[9] reported a mortality rate of 35% in patients with cirrhosis following abdominal surgery. Similar to Aranha et al.,[5] Doberneck et al.[9] noted that patients with advanced disease were at much higher risk than patients with well-compensated cirrhosis. In particular, Doberneck et al.[9] confirmed the observations of Aranha et al.[5] that in patients with cirrhosis prothrombin times greater than 2.0 seconds above control carried a very high risk compared with normal prothrombin times (36.1% vs. 9.8%). All three authors identified excessive blood loss as a predictor of high

mortality. Recently Mansour et al.[7] published similar results in a review of 92 cirrhotic patients undergoing abdominal surgery. They reported an overall mortality rate of 26%, 10% in Child's A patients, 30% in Child's B patients, and 82% in Child's C patients.

Because of the associated high mortality, it is generally accepted that patients with advanced cirrhosis should undergo cholecystectomy only for complications of biliary disease. However, the role of elective laparoscopic cholecystectomy for symptomatic cholelithiasis in patients with early, well-compensated cirrhosis has not been well defined. Kogut et al.[8] reported on 27 patients with early, well-compensated cirrhosis who underwent elective open cholecystectomies and concluded that elective open cholecystectomy could be performed safely in patients with mild cirrhosis. Since the advent of laparoscopic surgery, however, this question has not been extensively examined.

In the late 1980s, surgeons began performing laparoscopic cholecystectomies and, because of its marked advantages, this procedure has quickly become the standard of care for symptomatic cholelithiasis.[10] Initially there were a number of relative contraindications to the laparoscopic approach including obesity, pregnancy, a history of abdominal surgery, and acute cholecystitis.[11] However, as general surgeons have become more experienced with laparoscopic techniques, the indications for a laparoscopic approach have expanded[12] and currently there are few absolute contraindications.

However, patients with cirrhosis who have symptomatic biliary disease continue to present a therapeutic dilemma for many clinicians. In fact, the 1992 National Institutes of Health (NIH) Consensus Conference statement[10] listed advanced cirrhosis with portal hypertension as a relative or absolute contraindication to laparoscopic cholecystectomy. Many clinicians, however, have difficulty classifying hepatic disease because of a lack of specific data and confusion regarding classification schemes. In addition, the presence of hepatic cirrhosis is not always evident preoperatively. Therefore it is not unusual to discover cirrhosis at the time of the laparoscopic procedure, as noted in this series. For these reasons many clinicians have applied the NIH consensus statement to include any patient with even a suspicion of cirrhosis.

Of the 30 patients examined in our series, all were considered to have well-compensated cirrhosis, as demonstrated by a normal prothrombin time. Seven of the patients had Child's B disease, whereas 23 patients had Child's A cirrhosis. There were no operative deaths, which is in agreement with the findings of Kogut et al.,[8] who noted a mortality rate of zero for their series of open cholecystectomies in patients with mild cirrhosis.

Overall the conversion rate was 17% in our series. For elective cases the conversion rate was only 5%, as opposed to 36% for urgent cases. In their reviews of the general population, Scott et al.[13] and Orlando et al.[14] reported conversion rates of approximately 4% to 5% for elective cases, which increases to approximately 15% for urgent cases. For elective cases our conversion rate was the same as that for the general population. However, when an urgent operation was required, our conversion rate was more than twice that of the general population. Moreover, our overall morbidity was 23%, 36% in urgent cases, and 16% in elective cases. Both values are considerably higher than the 4% reported by Scott et al.[13] in the general population. In our series, patients with Child's B cirrhosis had a morbidity of 43%, compared with only 17% in patients with Child's A disease. Interestingly, 50% (3 out of 6) of the patients with ascites experienced a significant complication. Although these numbers did not achieve statistical significance, they do suggest that patients with more advanced disease are apt to have more complications. This is certainly in agreement with previous reports on open cholecystectomy,[5,6,9] which noted that patients with more advanced disease have increased morbidity and mortality.

There have been a number of small clinical series that also concluded that laparoscopic cholecystectomy is safe for patients with well-compensated cirrhosis.[15-17] Recently Sleeman et al.[18] published their clinical experience with 25 cirrhotic patients. They reported no conversions to open procedures. However, most of their patients were being treated for biliary colic, so a low conversion rate is to be expected, as was demonstrated in our series as well. In contrast, our data suggest that a significant higher conversion rate is to be expected when an urgent procedure (e.g., for acute cholecystitis) is necessary. As in our series, Sleeman et al.[18] had no deaths. However, they also had a very high morbidity rate (36%) as compared with the general population, which is comparable to our results.

We have used a number of operative techniques to help minimize the morbidity associated with this procedure. An open technique using the Hassan trocar is essential to prevent inadvertent puncturing of an umbilical varix. If umbilical varices are present, placing the trocar in the right paramedian position may be possible, although conversion to an open procedure may also be necessary. In cases of severe inflammation, problematic bleeding of the liver bed can be encountered. To avoid this liver injury a subtotal cholecystectomy can be performed, leaving the back wall of the gallbladder in the liver bed. This technique is well described with open cholecystectomies,[19] and we found it useful in one laparoscopic case and in one open case, in which there was severe inflammation.

We also have access to an argon beam coagulator, which can be inserted through an operative port and can be very helpful in establishing complete hemostasis in the gallbladder bed.

CONCLUSION

Laparoscopic cholecystectomy is relatively safe and effective in patients with early, well-compensated (i.e., Child's class A or B) cirrhosis. An elective procedure can be performed with a conversion rate similar to that for the general population and an expected length of stay of 1 to 2 days. When an urgent procedure is required, a conversion rate of nearly 40% and a morbidity rate of 40% may be seen, with a length of stay approaching 5 days. Therefore, in patients with early, well-compensated cirrhosis and no evidence of coagulopathy, it may be prudent to perform early laparoscopic cholecystectomy when biliary symptoms become apparent before complications of the disease can develop. The role of laparoscopic cholecystectomy in patients with Child's class C cirrhosis has still not been clearly defined and, as outlined in the NIH consensus statement, should still be considered a relative contraindication.

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Discussion

Dr. H. Pitt (Milwaukee, Wis.). In some of the earlier papers that cited cirrhosis as a risk factor, bleeding was the major complication. I am impressed that you did not have a lot of bleeding problems. Only one sixth of your patients had portal hypertension. I would say that this is the subgroup that is at highest risk. One of the things that I have done over the years when operating on patients with portal hypertension is to place them on a vasopressin drip during the procedure and shortly afterward. Did you do anything in particular to try to reduce bleeding problems in your patients, and in particular in patients with portal hypertension?

Dr. C. Friel. Of the six patients with portal hypertension, two of them actually had preoperative procedures. One had a transjugular intrahepatic portosystemic shunt (TIPS) procedure and one had a distal splenorenal shunt. So at the time of cholecystectomy, they were effectively decompressed. In most of the other patients, varices were actually discovered intraoperatively. In fact, two of our laparoscopic cases were converted to open procedures because varices were discovered at the time of the operation. We did not use intraoperative vasopressin to help control bleeding.

Dr. J. Bowen (New Orleans, La.). At the Ochsner Clinic, in the past 7 years, we have performed more than 3000 laparoscopic cholecystectomies. I do not know exactly how many of these patients had cirrhosis, but my initial impression was the same as Dr. Pitt's—that is, that portal hy-

pertension increased the risk of conversion to an open procedure. However, when we examined our data in an organized and systematic fashion, we came to the same conclusion you did—that conversion was necessary primarily because of a combination of acute cholecystitis with cirrhosis and portal hypertension. The real difficulty is your "urgent" group, which in our experience involves primarily patients with acute cholecystitis. The combination of portal hypertension and acute cholecystitis is very problematic. On the other hand, with regard to elective laparoscopic cholecystectomy, we have found hardly any increased risk for patients with cirrhosis. Incidentally, we routinely perform elective laparoscopic cholecystectomy as an outpatient procedure in patients with or without cirrhosis.

Dr. F. Zaccara (Padova, Italy). Do you always perform intraoperative cholangiography in these patients?

Dr. Friel. No. For patients who have elevated bilirubin levels, we usually perform preoperative endoscopic retrograde cholangiopancreatography (ERCP). We did use intraoperative cholangiography in one patient, but ERCP is not routinely used as part of the procedure.

Dr. J. Roslyn (Philadelphia, Pa.). Have you modified your technique at all in patients with cirrhosis and portal hypertension? The visualization with laparoscopy is much better than with open cholecystectomy and those small vessels that we might not cauterize at the time of open cholecystectomy appear quite large when viewed laparoscopically and we can easily see them. Do you find that you can obtain better hemostasis laparoscopically?

Dr. Friel. We have an argon beam coagulator that we find helpful to achieve hemostasis of the gallbladder bed. Overall, we have not modified our technique, although in several cases we performed a subtotal cholecystectomy when it appeared that we were going to have problems with bleeding.

Multivariate Analysis of Factors Predicting Outcome After Laparoscopic Nissen Fundoplication

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Laparoscopic Nissen fundoplication has been applied with increasing frequency in the treatment of gastroesophageal reflux disease. The aim of this study was to determine the variables that predict outcome of laparoscopic Nissen fundoplication. A multivariate analysis was performed on data from 199 consecutive patients undergoing laparoscopic Nissen fundoplication. Variables included age, sex, body mass index, primary symptoms, clinical response to acid suppression therapy, erosive esophagitis, 24-hour esophageal pH score, and the percentage of time the esophageal pH was less than 4 on 24-hour pH monitoring, lower esophageal sphincter competence, status of the esophageal body motility, hiatal hernia, carditis, intestinal metaplasia of cardiac epithelium limited to the gastroesophageal junction, and Barrett's esophagus of any length. Clinical outcome was obtained from all patients at a median follow-up of 15 months (range 6 to 74 months) after surgery. One hundred seventy-three patients had an excellent or good outcome (87%) and 26 had a fair or poor outcome. Three factors were significantly predictive of a successful outcome: an abnormal 24-hour pH score (odds ratio = 5.4; 95% confidence interval [CI] = 1.9-15.3), a typical primary symptom (odds ratio = 5.1; 95% CI = 1.9-13.6), and a clinical response to acid suppression therapy (odds ratio = 3.3; 95% CI = 1.3-8.7). We conclude that 24-hour pH monitoring provides the strongest outcome predictor of laparoscopic Nissen fundoplication and that outcome is based more on the correct identification of the disease than on its severity. (J GASTROINTEST SURG 1999;3:292-300.)

KEY WORDS: Laparoscopy, antireflux surgery, gastroesophageal reflux, outcome, Nissen

The introduction of laparoscopic access, coupled with the growing recognition that surgery is a safe and durable treatment for gastroesophageal reflux disease (GERD), has resulted in an explosion of patients being referred for laparoscopic fundoplication.[1,2] The threshold for surgical referral is such that increasing numbers of patients without endoscopic esophagitis or other objective evidence of the presence of reflux are now considered candidates for laparoscopic antireflux surgery.[3-5] These facts combine to underscore the importance of selecting patients for surgery who are likely to have a successful outcome. Although a Nissen fundoplication will reliably restore the gastroesophageal barrier and halt reflux of gastroduodenal juice into the esophagus,[6-8] little benefit is likely if the patient's symptoms are not caused by a transient or permanent loss of this barrier. Thus, in large part, the predictability of success following laparoscopic fundoplication is directly proportional to the degree of certainty that gastroesophageal reflux is the underlying cause of the patient's complaints.

With this in mind, we sought to identify the factors that predict outcome after laparoscopic Nissen fundoplication. Virtually all demographic, anatomic, physiologic, and clinical features associated with patients undergoing laparoscopic Nissen fundoplication for GERD were collected in a comprehensive preoperative evaluation. Multivariate logistic regression was then used to determine the factors most associated with symptomatic relief.

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PATIENTS AND METHODS

Study Population

The study population consisted of 233 consecutive patients who underwent a laparoscopic Nissen fundoplication between December 1991 and April 1997 at the University of Southern California. Thirty patients with paraesophageal hernias and four patients who were converted to open procedures were excluded from the analysis. Data were collected on the remaining 199 patients, which included 139 men and 60 women who had a median age of 49 years (range 15 to 77 years). A consistent operative technique was employed by the two senior authors (J.H.P. and T.R.D.) and consisted of the following: crural closure, complete fundic mobilization by routine division of the anterior and posterior short gastric vessels, and a properly oriented 2 cm 360 degree fundoplication constructed over a 60 Fr bougie. The operative technique has been described in detail elsewhere.[6]

Preoperative evaluation included a detailed symptomatic questionnaire, upper endoscopy with routine biopsies of the gastroesophageal junction and lower esophagus, esophageal motility, and 24-hour esophageal pH monitoring. Demographic, anatomic, and physiologic characteristics of the study population are shown in Tables I, II, and III.

Data Collection and Outcome Assessment

The following measures were recorded: age, sex, body mass index, typical or atypical primary symptoms, clinical response to acid suppression therapy, erosive esophagitis, 24-hour esophageal pH score and percentage of time the esophageal pH was less than 4 on 24-hour pH monitoring, lower esophageal sphincter (LES) status, hiatal hernia, carditis, intestinal metaplasia of cardiac-type epithelium limited to the gastroesophageal junction, and Barrett's esophagus of any length. Clinical outcome was obtained from all patients at a median follow-up of 15 months after surgery (range 6 to 74 months). A physician other than the responsible surgeon assessed the outcome by personal interview via telephone or at the follow-up clinic by means of completion of a standardized questionnaire. Outcome was considered excellent if the

Table I. Demographic characteristics of the study population (N = 199)

Median age (yr)	49 (range 15-77)
Sex	
Male	139 (70%)
Female	60 (30%)
Symptoms	
Typical	159 (80%)
Atypical	40 (20%)
Response to acid suppression therapy	
Complete	14 (7%)
Partial	141 (71%)
Minor	34 (17%)
None	10 (5%)
Body mass index	
19-25 (normal)	47 (24%)
25-35 (overweight or moderately obese)	144 (72%)
>35 (severely or morbidly obese)	8 (4%)

Table II. Anatomic characteristics of the study population (N = 199)

Characteristics	No. (%)
Erosive endoscopic esophagitis	
Yes	82 (41.2)
No	117 (58.8)
Hiatal hernia	
Yes	139 (70)
No	60 (30)
Carditis	
Yes	145 (73)
No	54 (27)
CIM	
Yes	41 (20.5)
No	158 (79.5)
Barrett's esophagus	
Yes	47 (23.5)
No	152 (76.5)

CIM = intestinal metaplasia in cardiac-type epithelium restricted to the gastroesophageal junction.

Table III. Physiologic characteristics of the study population (N = 199)

Characteristics	No. (%)
Structurally defective LES	
Yes	149 (75)
No	50 (25)
Distal esophageal amplitude	
Normal	192 (96.5)
Abnormal	7 (3.5)
Abnormal esophageal pH score on 24-hour pH monitoring	
Yes	170 (85.5)
No	29 (14.5)
Abnormal esophageal exposure to pH <4	
Yes	156 (78.5)
No	43 (21.5)

patient was asymptomatic, good if the reflux symptoms were relieved but minor gastrointestinal complaints persisted or developed but required no gastrointestinal medication, fair if reflux symptoms were improved but additional medications were necessary for complete relief, and poor if the symptoms were not improved.

Definition of Study Variables

The most prominent symptom present at the time of surgical referral was recorded as the primary symptom driving the need for operative therapy. Heartburn, regurgitation, and dysphagia were considered *typical* symptoms, whereas hoarseness, cough, wheezing, and chest pain were considered *atypical* symptoms. The response to acid suppression therapy was recorded as complete if the patient reported 100% relief of symptoms, partial if symptoms were lessened but persisted, minor if symptoms were minimally improved, and none if no symptomatic improvement was experienced.

Body mass index (BMI) was calculated using the following formula:

$$\frac{\text{Weight in pounds} \times 703}{(\text{Height in inches})^2}$$

Patients were classified as normal (BMI 19 to 25), overweight or moderately obese (BMI 25 to 35), and severely or morbidly obese (BMI >35).

A hiatal hernia was diagnosed on endoscopy when there was more than 2 cm distance between the gastroesophageal junction, identified as the point where the gastric rugal folds end and the tubular esophagus begins, and the crural impression. Endoscopic esophagitis was defined as the presence of linear or confluent erosions in the distal esophagus. All biopsy specimens underwent routine fixation and staining with hematoxylin and eosin, and all were analyzed for the type and the condition of the epithelium. Cardiac-type mucosa was characterized by a mucosa with glands composed entirely of mucous cells without parietal or chief cells. Inflammation of cardiac-type mucosa or "carditis" was diagnosed in the presence of eosinophil or plasma cell infiltration of the lamina propria and hyperplasia of the mucous cells in the foveolar region. Intestinal metaplasia was identified by the presence of well-defined goblet cells within columnar epithelium. The presence of goblet cells was confirmed by positive staining with Alcian blue at pH 2.5. Intestinal metaplasia limited to the gastroesophageal junction was defined by the absence of visible endoscopic esophageal columnar lining but the presence of intestinal metaplasia in cardiac-type epithelium (CIM) on biopsies from the gastroesophageal junction. The presence of visible endoscopic esophageal columnar lining regardless of its extent and the histologic confirmation of the presence of intestinal metaplasia within the columnar epithelium defined Barrett's esophagus.

LES pressure was measured by stationary pull-through technique at the respiratory inversion point. The resting pressure, overall length, and abdominal length were calculated from the mean of five recordings. A structurally defective sphincter was defined either by resting pressure less than 6 mm Hg, overall length less than 2 cm, abdominal length less than 1 cm, or any combination of these. Esophageal body motility analysis was assessed by positioning the five side holes of the catheter within the esophageal body and with the most proximal side hole placed 1 cm below the lower border of the upper esophageal sphincter. The patients were instructed to take a total of 10 wet swallows (5 ml distilled water) one a time with at least a 20-second interval between each swallow. Poor esophageal body motility was defined by the global presence of distal esophageal contraction amplitudes below the fifth percentile of normal.

Ambulatory 24-hour esophageal pH monitoring was performed using a glass electrode (Ingold Inc., Urdorf, Switzerland) placed 5 cm above the upper border of the manometrically defined LES. Medications were discontinued 72 hours before testing, except for proton pump inhibitors, which were discontinued for at least 2 weeks. An abnormal 24-hour esophageal pH score was defined as greater than 14.7. Abnormal esophageal exposure to pH <4 was defined when it exceeded 4.4% of the recording time.

Statistics

A correlation analysis was performed to evaluate univariate effects of each predictor variable on outcome. The Yates chi-square test or Fisher's exact test, if any of the subgroups had five or less components, was used. Statistical significance was considered at the alpha ≤ 0.05 level.

To determine the statistically best-fitting model, a stepwise logistic regression was performed on outcome. Offered to the regression procedure were age (<50 or ≥50 years), sex, body mass index, typical or atypical primary symptoms, clinical response to acid suppression therapy, erosive esophagitis, 24-hour esophageal pH score, and percentage of time the esophageal pH was less than 4 on 24-hour pH monitoring, LES competence, hiatal hernia (≥2 cm), carditis, CIM, and Barrett's esophagus of any length. To ease clinical interpretation of statistical models, outcome level was coded as a two-level variable (1 = excellent or good; 2 = fair or poor). To stay in the model, covariates were required to be significant at the alpha ≤ 0.05 level.

RESULTS

Symptomatic outcome was rated excellent or good in 173 (87%) and fair or poor in 26 (13%) of the patient group as a whole. Four factors were significantly associated with an excellent or good outcome at the univariate level (Table IV). These included a complete or partial response to acid suppression therapy, a typical primary symptom at presentation, and an abnormal pH score and esophageal exposure to pH <4 greater than 4.4% on 24-hour pH monitoring. Age, sex, BMI, status of the components of the LES, hiatal hernia, and mucosal injury were not predictive. An excellent or good outcome was often achieved independent of these factors.

Table IV. Univariate association with outcome

Variable	Outcome		*P* value
	Excellent/Good No. (%)	Fair/Poor No. (%)	
Response to acid suppression therapy			
Complete/partial	143 (92)	12 (8)	
Minor/none	30 (68)	14 (32)	0.00008
Symptom			
Typical	146 (92)	13 (8)	
Atypical	27 (67.5)	13 (32.5)	0.0001
24-hour esophageal pH score			
Abnormal	154 (91)	16 (9)	
Normal	19 (65.5)	10 (34.5)	0.001
% Time esophageal pH <4			
Abnormal	141 (90)	15 (10)	
Normal	32 (74)	11 (26)	0.01
Structurally defective LES			
Yes	133 (89)	16 (11)	
No	40 (80)	10 (20)	0.14
Age (yr)			
<50	85 (83)	17 (17)	
≥50	88 (91)	9 (9)	0.19
Erosive esophagitis			
Yes	74 (90)	8 (10)	
No	99 (85)	18 (15)	0.33
Carditis			
Yes	128 (88)	17 (12)	
No	45 (83)	9 (17)	0.48
Distal esophageal motility			
Normal	166 (86.5)	26 (13.5)	
Abnormal	7 (100)	0 (0)	0.64
Body mass index			
19-25	41 (87)	6 (13)	
25-38	124 (86)	20 (14)	0.69
>38	8 (100)	0 (0)	
Hiatal hernia			
Yes	122 (88)	17 (12)	
No	51 (85)	9 (15)	0.75
Sex			
Male	120 (86)	19 (14)	
Female	53 (88)	7 (12)	0.89
CIM			
Yes	35 (85)	6 (15)	
No	138 (87)	20 (13)	0.93
Barrett's esophagus			
Yes	41 (87)	6 (13)	
No	132 (87)	20 (13)	1.00

CIM = cardia intestinal metaplasia.

Table V. Stepwise logistic regression results*

Predictor	Adjusted odds ratio (95% confidence interval)	Wald's *P* value
24-hour esophageal pH score		
Abnormal	5.4 (1.9-15.3)	<0.001
Normal	—	—
Primary symptom		
Typical	5.1 (1.9-13.7)	<0.001
Atypical	—	—
Response to acid suppression therapy		
Complete/partial	3.3 (1.3-8.7)	0.02
Minor/none	—	—

*Odds ratios and corresponding *P* values are adjusted for all other factors in the model.

Table VI. Combined effect of predictors on outcome

	24-hour pH score	Primary symptom	Response to acid suppression therapy	Odds ratio
BASELINE	**Normal**	**Atypical**	**Poor/none**	**1.0**
	Normal	Typical	Complete/partial	16.7
	Abnormal	Atypical	Complete/partial	17.7
	Abnormal	Typical	Poor/none	27.2
	Abnormal	Typical	Complete/partial	89.8

Table VII. Clinical evidence of GERD in patients with a normal 24-hour esophageal pH score on 24-hour pH monitoring (N = 29)

Characteristics	No. (%)
Typical symptoms	22 (76)
Structurally defective LES	21 (72)
Carditis	20 (69)
Complete or partial response to acid suppression therapy	19 (66)
Isolated abnormal factor(s) on 24-hour pH monitoring*	15 (52)
High normal 24-hour pH score (>12.9)	8 (28)
Endoscopic erosive esophagitis	8 (28)
CIM	6 (21)
Barrett's esophagus	3 (10)

CIM = cardia intestinal metaplasia.
*Abnormal percentage of time pH <4 (4.4%) and/or abnormal percentage time supine (3.4%), upright (8.4%), or postprandial (8.8%) periods, abnormal number of reflux episodes (>47), abnormal number of episodes >5 minutes (>3), longest reflux episode >19.8 minutes.

At the multivariate level, the factors that significantly predicted a successful outcome were also identified and rank ordered. These included an abnormal score on 24-hour esophageal pH monitoring (odds ratio = 5.4; 95% confidence interval [CI] = 1.9-15.3), a typical primary symptom (odds ratio = 5.1; 95% CI = 1.9-13.6), and a complete or partial response to acid suppression therapy (odds ratio = 3.3; 95% CI = 1.3-8.7) (Table V). Abnormal esophageal exposure to pH <4 fell out as a significant variable. Table VI shows that the odds ratio for a successful outcome increases exponentially if there is a combination of the predictors. One hundred sixteen patients presented with all three outcome predictors defined by the multivariate analyses. An excellent or good outcome was achieved in 97.4% (113 of 117) of these patients.

We also examined the predictors of success limiting the study population to those with an abnormal esophageal pH score on 24-hour pH monitoring (i.e., pH-proved GERD). Two factors were jointly predictive of outcome: the presence of a typical primary symptom (odds ratio = 8.5; 95% CI = 2.5-28.5) and a complete or partial symptomatic response to acid suppression therapy (odds ratio = 5.3; 95% CI =1.6-17.6). Interestingly, neither the magnitude of the 24-hour pH score nor the percentage of time the pH was less than 4 was predictive of a successful outcome.

Twenty-nine patients underwent laparoscopic Nissen fundoplication despite a normal 24-hour esophageal pH score on 24-hour pH monitoring. Table VII shows that most of these patients had a combination of features suggesting GERD, leading to a clinical judgment that the 24-hour pH study was falsely negative. Furthermore, individual components of the 24-

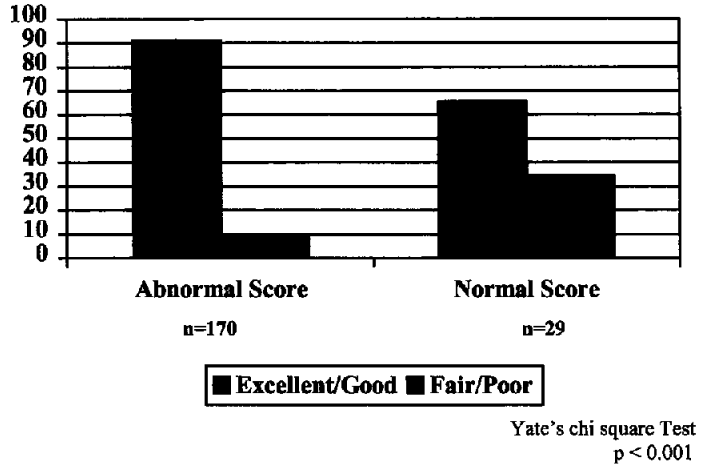

Fig. 1. Outcome after laparoscopic Nissen fundoplication in patients with and without abnormal 24-hour esophageal pH scores ($P < 0.001$; Yates chi-square test).

hour pH monitoring record were abnormal in 15 of these patients (52%). Despite the clinical impression, the symptomatic outcome in these 29 patients was significantly worse than for those with an abnormal 24-hour esophageal pH score (Fig. 1). Both univariate and multivariate analyses were used in an attempt to identify any factor that might predict successful outcome in this group. No factors either singly or in association were found to be significant predictors of a successful outcome.

Twenty-six patients had a fair (n = 12) or poor (n = 14) outcome. Seventeen of the 26 underwent postoperative upper endoscopy, radiography, 24-hour pH monitoring, and motility studies. There were two patterns of failure: (1) mechanical, that is, recurrent herniation or disruption of the fundoplication, which occurred in eight patients (4%), and (2) return or persistence of symptoms without objective evidence of a technical failure, which occurred in 18 patients. Of the latter group, ten patients had a normal 24-hour esophageal pH score on preoperative 24-hour pH monitoring, and the remaining eight all had atypical symptoms that were not relieved or necessitated additional therapy after surgery.

DISCUSSION

Successful antireflux surgery is largely defined by two factors: achieving long-term relief of reflux symptoms and the absence of complications or complaints induced by the operation. In practice, achieving these two deceptively simple goals is difficult. Both are critically dependent on establishing that the symptoms for which the operation is performed are due to excess esophageal exposure to gastric juice, as well as the proper performance of the appropriate antireflux procedure. Success can be expected in the vast majority of patients if these two criteria are met.

All of the patients in this study were selected for and underwent the same antireflux procedure. The operation was a laparoscopic Nissen fundoplication, performed in a standardized manner by two surgeons from the same unit, who jointly worked out the steps of the procedure. As such, the degree to which the selection and technical performance of the procedure affect the outcome was not part of the study.[9] Rather the clinical variables predicting the relief of reflux-induced symptoms were determined. Three predictive factors emerged: an abnormal score on 24-hour esophageal pH monitoring, the presence of typical symptoms of gastroesophageal reflux, and a significant improvement in symptoms with acid suppression therapy prior to surgery. It is immediately evident that each of these factors helps to establish that GERD is indeed the cause of the patient's symptoms. This is in contrast to indicators of the severity of disease, which were not predictive of outcome—that is, the outcome was more dependent on the presence of disease than on its severity.

A positive 24-hour esophageal pH study, as demonstrated by an abnormal score, was the strongest predictor of outcome. Prolonged esophageal pH monitoring was first reported by Miller[10] in 1964, although it was not until 1973 that its clinical applicability and advantages were demonstrated by Johnson and DeMeester.[11] It is considered by many to be the "gold standard" for the diagnosis of GERD, as it has the highest sensitivity and specificity of all tests currently available. The finding that an abnormal study is highly predictive of the relief of symptoms following

laparoscopic Nissen fundoplication argues for its routine use. Some have suggested that 24-hour pH monitoring be used selectively, limiting its use to patients with atypical symptoms and/or no endoscopic evidence of gastroesophageal reflux.[4,12,13] Given present-day referral patterns, more than half of the patients referred for antireflux surgery will have no endoscopic evidence of mucosal injury.[4,7,14,15] For these patients, 24-hour pH monitoring provides the only objective measure of the presence of pathologic esophageal acid exposure. Although it is true that most patients with typical symptoms and erosive esophagitis will have an abnormal 24-hour pH score,[16] the study provides other useful information. It is the only way to quantitatively express the overall degree and pattern of esophageal acid exposure, both of which may direct the decision in favor of surgery.[17] Patients with nocturnal or bipositional reflux have a higher prevalence of complications and failure of long-term medical control.[18] For these reasons we continue to advocate the routine use of 24-hour esophageal pH monitoring in clinical practice.

Patients with GERD can be divided into those with *typical* symptoms of heartburn, regurgitation, and dysphagia, and those with *atypical* symptoms of cough, hoarseness, wheezing, and chest pain. Because there are fewer mechanisms for their generation, typical symptoms are more likely to be secondary to increased esophageal acid exposure than are atypical symptoms. The presence of a typical primary symptom was the second most powerful preditor of a successful outcome. Patients with typical symptoms were five times more likely to be relieved of their symptoms than those with respiratory or other atypical manifestations of GERD. Although it is well documented that the relief of atypical symptoms is less predictable following antireflux surgery,[19] the importance of the presenting symptoms has been underemphasized. Heartburn and regurgitation are reliably relieved. Dysphagia generally improves but may be induced or worsened. Respiratory symptoms may or may not be improved depending on their cause and the patient's underlying esophageal motility.[20] In addition, reflux-induced respiratory symptoms are often due to chronic injury, which resolves slowly and often only partially after antireflux surgery.

Prior to the era of laparoscopic fundoplication, referrals for antireflux surgery were often limited to patients who had continued symptoms or esophagitis despite H_2 blockers and prokinetic agents. Medical therapy for GERD has improved considerably over the past two decades. Proton pump inhibitors, when given in an adequate dose, provide symptomatic relief in the vast majority of patients and heal esophagitis in 75% to 80%.[21] Thus failure of medical therapy is less common and should not be used as a requirement for antireflux surgery; rather, as our study indicated, a good response to acid suppression therapy predicts a successful outcome after antireflux surgery. Patients who have become medication dependent or require escalating doses of medication and those who are young are the ideal candidates for an antireflux procedure.

Equally important are those factors that were not associated with outcome in the multivariate analyses. The fact that LES competence, esophagitis, or Barrett's esophagus did not reach significance indicates that a successful outcome could be achieved independent of these factors. That is, an equal number of patients with and without these anatomic and physiologic abnormalities are relieved of their symptoms. These findings are not requirements for surgery but rather indicators of severe disease. They are risk factors for disease progression and can be used to guide the decision of medical vs. surgical therapy. Factors that predict a poor response to medical therapy, frequent relapses, and the development of complications include supine or bipositional reflux, erosive esophagitis or a columnar-lined esophagus at initial presentation, bile in the refluxate, and a structurally defective LES.[18,22-24] Patients who have these risk factors should be given the option of surgery early in the course of their disease prior to the development of other long-term consequences of GERD.[25,26]

Analysis of the 26 patients who had recurrent or persistent symptoms indicates two patterns of failure. The first was mechanical failure, that is, recurrent herniation or disruption of the fundoplication. Mechanical failure was relatively uncommon, occurring in 4% of the patients, all within the first year after surgery. The second, and by far the most common, was inadequate patient selection. In these patients, symptoms persisted or returned without objective evidence of a technical failure. All of these patients had either atypical symptoms or a normal 24-hour pH study. It is interesting that despite their persistent symptoms, most (79%) were satisfied with the results of the operation and half would make the same decision again. This suggests that even in these patients, some therapeutic effect was obtained.

Last, the study raises the question of the importance of the LES in GERD since it is commonly thought to be the barrier that prevents the reflux of gastric juice into the esophagus.[23] If this is so, then why did it not emerge as an important variable predicting outcome, particularly as the effect of an antireflux procedure is to reestablish the barrier. The explanation lies in the fact that the LES can be either structurally defective or structurally normal but functionally defective. We have no measure of the latter

because it requires identification of the effect of a gastric challenge on LES competence, a test not yet developed for clinical use.[27] It is important to note that a structurally normal LES that is functionally defective results in a specific pattern of reflux—that is, multiple short reflux episodes during the upright and postprandial periods. This is in contrast to the prolonged reflux episodes in the supine position that occur with a structurally defective LES. The former reflux pattern will commonly result in a normal esophageal exposure to pH <4 but an abnormal 24-hour pH score. This is because the score includes in its calculation the number of reflux episodes as well as the time of esophageal exposure to pH <4 in the upright and supine positions. Consequently a 24-hour esophageal pH score identifies reflux through both a structurally defective LES as well as a structurally normal LES that is functionally defective. The simple measure of esophageal exposure time to pH <4 does not pick up the pattern of reflux commonly associated with a structurally normal but functionally defective LES. It is for this reason that it falls out as a significant predictor of outcome.

We conclude that the presence of an abnormal 24-hour pH score, a typical primary symptom, and a significant response to acid suppression therapy predict a successful outcome after laparoscopic Nissen fundoplication. Twenty-four-hour pH monitoring provides the strongest predictor of outcome, which is based more on the correct identification of the disease than on its severity.

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Discussion

Dr. D. Rattner (Boston, Mass.). Would you elucidate for us the 29 patients who had normal pH probe studies and made up approximately 15% of your series? Were these persons who responded to medical therapy or failed to respond to medical therapy? How did you justify an antireflux operation in these patients?

Dr. G. Campos. In the 29 patients who had normal 24-hour pH studies, we had other combined evidence for the presence of disease, such as typical symptoms in 70%, motility studies showing a defective LES, endoscopic evidence of inflammation including erosive esophagitis, metaplasia of the cardia, and Barrett's esophagus, leading us to the clinical judgment that the pH test was falsely negative.

Dr. R. Hinder (Jacksonville, Fla.). You analyzed manometry and I presume that this showed no influence on the outcome. Perhaps you could comment on the LES and on the body motility and how that influenced your outcome. Did poor body motility or normal LES pressure make any difference?

Dr. Campos. Regarding the LES, the stationary pull-through technique can identify whether the sphincter is structurally defective. It cannot determine whether the sphincter is functionally defective. In 30% of the patients who had a structurally normal sphincter on manometry, they would probably be shown to have a defective sphincter if the stomach was challenged by a meal or other factors. Most of those patients had reflux disease as determined by pH monitoring. Regarding esophageal body motility, we defined poor esophageal body motility as the presence in the distal esophagus of amplitude of contractions below the fifth percentile of normal. This occurred in only six of the patients in this series. Interestingly, all of the six patients had an excellent outcome, but we cannot reach any definitive conclusions with only six patients.

Dr. M. Callery (Worcester, Mass.). What about delayed gastric emptying? How does that predict outcome following this procedure? Do you test for it preoperatively? Do you do anything for it intraoperatively? And does it make a difference?

Dr. Campos. Those are interesting questions and they point out the added problem of a normal LES on manometry. When that occurs, one always needs to assess if the patient could have a gastric abnormality that is associated with or causing the reflux. In this study we did not look specifically into gastric emptying.

Gadolinium Chloride Inhibits Lipopolysaccharide-Induced Mortality and In Vivo Prostaglandin E_2 Release By Splenic Macrophages

Claude R. Roland, M.D., Yuji Nakafusa, M.D., M. Wayne Flye, M.D., Ph.D.

The monocytic phagocytic system, consisting primarily of tissue macrophages of the liver and spleen, produces prostaglandin E_2 (PGE_2), a modulator of the septic response. Macrophages are known to internalize gadolinium chloride (GD), a lanthanide metal, which inhibits phagocytic function. Thus we studied the effect of in vivo GD on lipopolysacchride (LPS)-induced mortality and on LPS-stimulated PGE_2 release by cultured splenic macrophages. GD (7 mg/kg intravenously) given on the two days prior to LPS challenge (30 mg/kg intravenously) completely prevented the uniform mortality in rats. This protective effect was transient since rechallenge with LPS 10 days later was uniformly lethal. Previous work in this laboratory has established a critical role of arginine concentration on macrophage behavior in vitro. Therefore, to establish culture conditions reflective of the milieu within the portal venous system, alanine and arginine levels were measured in the portal and hepatic veins of normal and endotoxemic (LPS, 10 mg/kg intraperitoneally) rats. In contrast to alanine levels, which were not altered by endotoxemia, there was a reduction of arginine concentrations from a range of 50 to 250 μmol/L in normal rats to a range of 10 to 50 μmol/L after LPS challenge. Consequently subsequent in vitro assays of splenic macrophage secretory behavior were performed in concentrations of 1200 μmol/L arginine (in standard RPMI-1640), as well as in concentrations reflective of physiologic arginine levels (10 and 100 μmol/L in modified RPMI-1640). Rat splenic macrophages harvested after two consecutive days of either in vivo saline or GD injection (7 mg/kg intravenously) were stimulated with LPS (0.025 to 2.5 μg/ml). At 72 hours of culture, the release of PGE_2 by splenic macrophages from GD-treated rats was significantly ($P < 0.0001$) reduced at all LPS concentrations. Increased PGE_2 production was not present when the splenic macrophages were cultured in the supraphysiologic arginine (1200 μmol/L) concentration. The results demonstrate the relevance of physiologic arginine concentrations in cell culture studies and suggest that the protection conferred by GD against septic mortality may be related to downregulation of the release of immunosuppressive PGE_2 by the monocytic phagocytic system. (J GASTROINTEST SURG 1999;3:301-307.)

KEY WORDS: Splenic macrophages, gadolinium, prostaglandin E_2, arginine, lipopolysaccharide, rat, septic mortality

The macrophages of the monocytic phagocytic system are predominantly concentrated in the spleen and liver. Their diverse functions of phagocytosis,[1,2] antigen presentation,[2] and release of free oxygen radicals and hormonal mediators of inflammation[3] serve to integrate and amplify the host's response to bacterial invasion. The immunosuppressive eicosanoid prostaglandin E_2 (PGE_2) is released by macrophages stimulated by lipopolysaccharides (LPS), the biologically active component of gram-negative bacteria. Monocytes and macrophages retrieved from animals and humans in septic shock release increased amounts of PGE_2 when compared with the cells of healthy subjects.[4,5] Since immunosuppression appears to be an important factor in septic mortality, it has been concluded that PGE_2 may be partly responsible for the poor outcome of sepsis.[4-6]

Both hepatic and, to a lesser degree, splenic macrophages take up rare earth metals of the lanthanide series.[7,8] Gadolinium (GD), one of the lanthanides,

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has recently been shown to reduce the mortality of endotoxemia,[9] bacterial sepsis,[10] and anaphylaxis.[11] It was the purpose of this study, therefore, to determine whether pretreatment with systemic GD could both ameliorate the effects of endotoxemia and alter the PGE_2 secretory response to LPS of cultured rat splenic macrophages.

Since we have previously observed a marked sensitivity of hepatic macrophage synthesis of PGE_2 to arginine concentration,[3] initial experiments to confirm[12] physiologically relevant arginine concentrations across the portal venous system of healthy and septic rats were performed. The arginine concentrations were consequently adjusted in the culture media of subsequent experiments to more accurately reflect the portal venous milieu.

MATERIAL AND METHODS

Gadolinium chloride hexahydrate (Aldrich, Milwaukee, Wisc.) was suspended at 4 mg/ml in normal saline solution. LPS from *Escherichia coli* strain 0111:B4 (Sigma, St. Louis, Mo.) was divided into aliquots and stored at −4° C in phosphate-buffered saline at 200 μg/ml. PGE_2 standard was purchased from Advanced Magnetics, Inc. (Cambridge, Mass.).

Male Sprague-Dawley rats (200 to 300 gm) were purchased from SASCO (Indianapolis, Ind.) and cared for in accordance with National Institutes of Health standards. Rats were fed standard rat chow (Ralston Purina, St. Louis, Mo.) and water ad libitum. Rats were injected with 7 mg/kg GD or normal saline solution via the penile vein under Ketaset (50 mg/kg subcutaneously; Fort Dodge Laboratories, Fort Dodge, Iowa) and inhaled Metofane (Pitman-Moore, Inc., Mundelein, Ill.) anesthesia on the two consecutive days prior to splenic macrophage isolation.

Determination of Serum Arginine Levels

Rats were pretreated with LPS (10 mg/kg intraperitoneally) or saline vehicle. Six hours later the animals were anesthetized with ether, and a 12-gauge catheter attached to a Harvard respirator (Harvard Apparatus, Inc., S. Natick, Mass.) was inserted through a tracheotomy for positive pressure volume-cycled ventilation. Following a ventral midline thoracoabdominal incision, the hepatic vein draining the left lateral lobe was cannulated in a retrograde fashion for aspiration of 1.0 ml blood. Another 1.0 ml of blood was then withdrawn from the portal vein before the rats were killed by exsanguination.

Blood samples were permitted to coagulate at 4° C over 2 hours and centrifuged at 4° C for 20 minutes at 1000 × *g*. Serum was transferred to microcentrifuge tubes for storage at −70° C until assayed. Specimen protein was denatured by the addition of sulfosalicylic acid at a final concentration of 7%. Specimens were diluted by a factor of 5 from the original volume with a lithium citrate sample preparation buffer provided by Beckman Instruments, Inc. (Fullerton, Calif.). Alanine and arginine were analyzed on a Beckman 7300 amino acid analyzer using Agmantine as a standard marker, and ninhydrin detection was employed using the manufacturer's procedure for physiologic fluid analysis.

Splenic Macrophage Isolation

Splenectomy was performed after perfusion through the portal vein with Hanks' balanced salt solution supplemented with 10 mmol/L HEPES buffer, 1×10^5 U/L penicillin, and 1×10^5 μg/L streptomycin. Spleens were forced through a 250 μm metal screen. The homogenate was washed thoroughly with Hanks' balanced salt solution and plated on plastic Petri dishes (Costar Corp., Cambridge, Mass.) in RPMI-1640 medium containing 5% fetal calf serum. After a 3-hour incubation at 37° C in 95% oxygen and 5% carbon dioxide, nonadherent cells were removed using a pipette. The adherent cells were then removed with a rubber policeman. Of the 70% to 90% viable cells (trypan blue exclusion) obtained in this manner, more than 90% phagocytosed Congo red–stained yeast.

Splenic macrophages were cultured in complete medium made from RPMI-1640 (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum (Hyclone Laboratories, Inc., Logan, Utah), 10 mmol/L HEPES buffer, 2 mmol/L L-glutamine, 1×10^5 U/L penicillin, and 1×10^5 μg/L streptomycin. The standard formulation of RPMI-1640 complete medium supplemented with 10% fetal calf serum contains 1200 μmol/L arginine.[13] For medium containing arginine concentrations of 10 or 100 μmol/L, arginine-free RPMI-1640 (Washington University Tissue Culture Center) was supplemented with L-arginine to achieve the desired concentration.

Lipopolysaccharide Stimulation of Splenic Macrophages

After overnight culture, the supernate was replaced with fresh complete medium (10, 100, or 1200 μmol/L arginine). LPS (0.025 to 2.5 μg/ml) was added and cells were returned to the 37° C incubator. Supernates removed from culture wells at 72 hours were frozen to −70° C until assay of PGE_2.

Prostaglandin$_2$ Assay

Splenic macrophage culture supernate was combined with tritiated PGE_2 (New England Nuclear, Boston, Mass.) and a specific rabbit antiserum to PGE_2 (kindly provided by Dr. Aubrey Morrison, Department of Medicine, Washington University). Using a charcoal-dextran mixture, unbound ^{3}H-PGE_2 was removed after a 24-hour incubation period at 4° C. The bound portion of radiolabeled PGE_2 was counted by liquid scintillation spectrometry. Triplicate values were averaged and compared with a standard curve performed with each assay.

Statistics

Parametric data were examined by analysis of variance for multiple comparisons and by unpaired, two-tailed Student's *t* test for single comparisons. Survival data were analyzed by Wilcoxon signed-rank test. Results are representative of at least two experiments. Data are expressed as means ± standard deviation.

RESULTS

Gadolinium Protects Against the Lethality of Endotoxin

Rats injected intravenously with either saline solution (n = 6) or GD (n = 6) on the 2 prior consecutive days were challenged with intravenous LPS (30 mg/kg) on the following day. All animals manifested lethargy, hunched posture, rhinorrhea, and piloerection. However, GD-pretreated rats were completely protected from LPS-induced death, whereas all rats not receiving GD died between 7 and 16 hours after LPS injection (Fig. 1). Surviving GD-treated animals had recovered fully by 72 hours; however, when these same animals were rechallenged with a second dose of LPS 10 days after the initial dose, but without the administration of additional GD, all died within 7 hours.

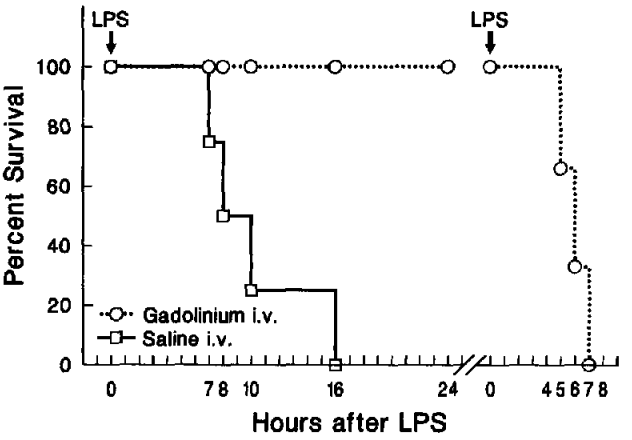

Fig. 1. Effect of gadolinium pretreatment on the mortality of endotoxemia. Rats were injected intravenously with saline (n = 6) or 7 mg/kg gadolinium (n = 6) on two consecutive days before lipopolysaccharide *(LPS)* challenge (30 mg/kg intravenously). Gadolinium-injected rats were completely protected ($P < 0.03$) from the lethal effect of endotoxemia, which occurred in all control animals by 16 hours. A second LPS challenge was given 10 days later in the gadolinium-treated survivors of the first LPS injection. All reinjected rats died by 7 hours.

Physiologic Serum Arginine Levels

We and others have found that macrophage behavior is sensitive to arginine concentrations.[3,13] Therefore we wished to determine the gradient of the arginine concentration across the liver bed by measuring arginine levels in the portal and hepatic veins.[12] In normal animals both serum alanine and arginine concentrations were reduced from the portal to the hepatic veins across the liver (Fig. 2). However, 6 hours of endotoxemia (LPS, 10 mg/kg intraperitoneally) resulted in a further marked ($P < 0.05$) reduction of both portal and hepatic venous arginine levels in four of five rats without significant changes in alanine levels from those measured in normal rats. These results indicate that the in vivo arginine levels to which cells of the monocytic phagocytic system in the liver are exposed range from approximately 10 to 200 μmol/L, well below the 1200 μmol/L arginine concentration available in standard RPMI-1640 supplemented with 10% fetal calf serum. Portal venous arginine levels are further reduced during endotoxemia. The resulting systemic levels of arginine are also lower. The measured portal levels are similar to the in vitro arginine levels we have previously shown to optimally stimulate PGE_2 production.[3]

In Vivo Gadolinium Decreases Lipopolysaccharide-Induced In Vitro Release of Prostaglandin E_2

Since PGE_2 has been implicated as an important factor in the immunosuppression occurring during shock states,[4-6,14] we examined the effect of in vivo GD on the ability of cultured splenic macrophages to synthesize PGE_2 in response to LPS. Splenic macrophages cultured in physiologic arginine concentrations (10 and 100 μmol/L) demonstrated a significantly ($P < 0.0001$) impaired in vitro PGE_2 response to LPS when they had been exposed to GD in vivo (Fig. 3). In fact, PGE_2 release by splenic macrophages

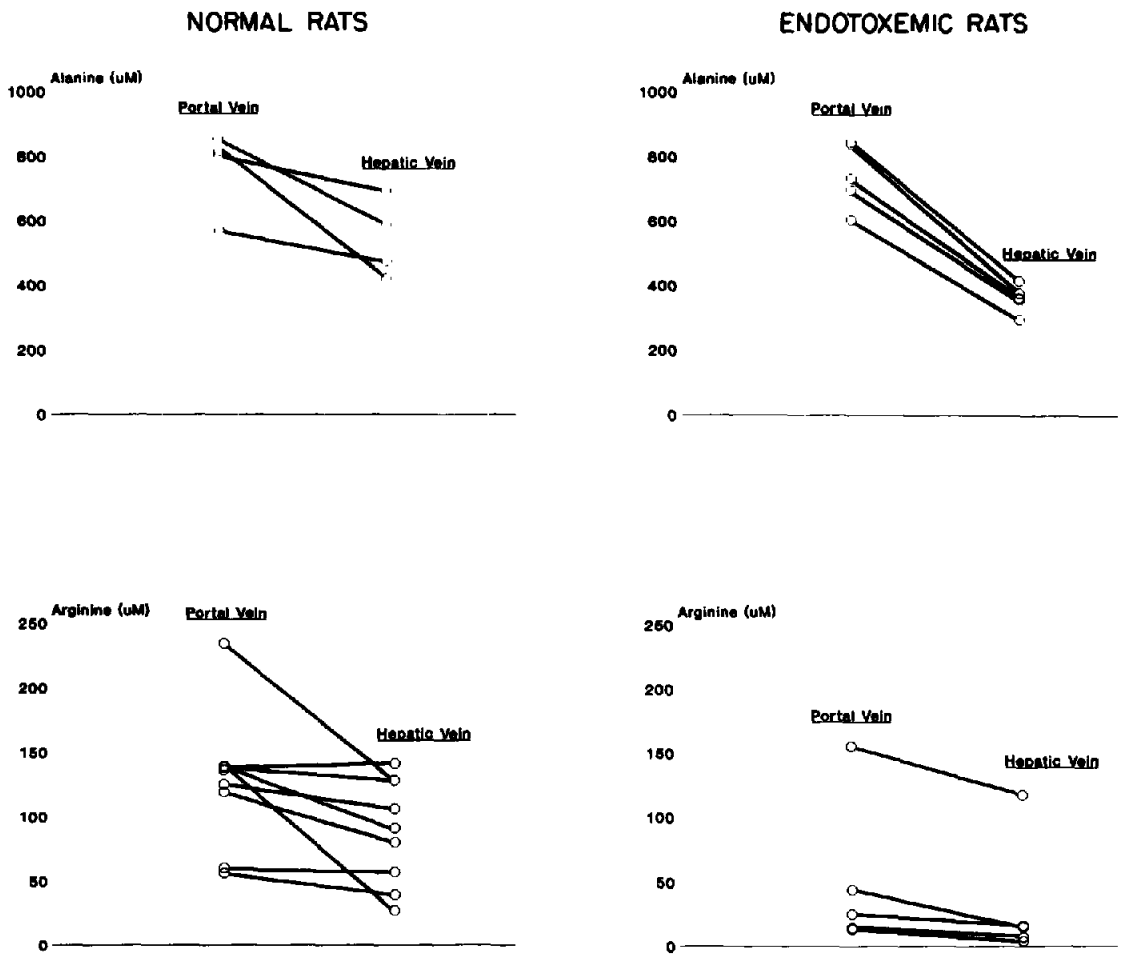

Fig. 2. Both serum alanine and arginine levels were reduced from the portal to the hepatic veins of rats that had received saline solution intraperitoneally *(Normal Rats)*. However, rats that received LPS (10 mg/kg intraperitoneally; *Endotoxemic Rats*) 6 hours before blood was drawn for assay had a significant ($P < 0.05$) reduction in arginine levels in both portal and hepatic veins (4 of 5 rats) when compared against serum levels obtained from normal rats. Alanine levels were not significantly altered by LPS administration.

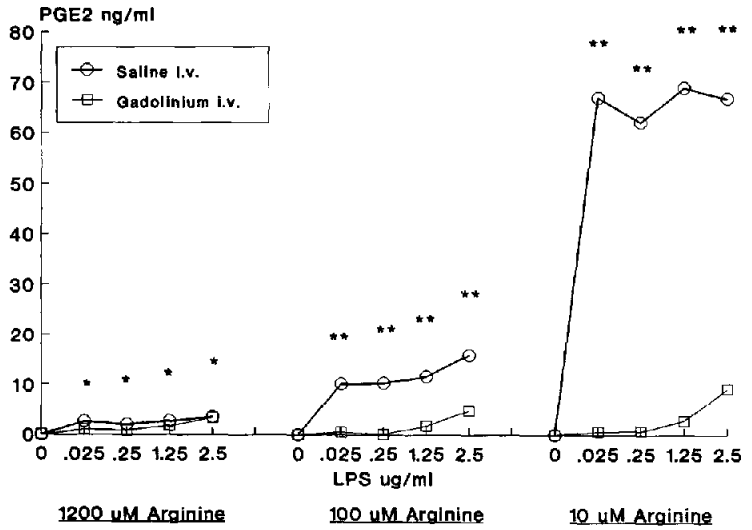

Fig. 3. Splenic macrophage *(PGE_2)* release in response to a 72-hour lipopolysaccharide *(LPS)* dose-response (0.025 to 2.5 μg/ml) exposure as a function of prior in vivo gadolinium exposure and in vitro arginine concentration. Inhibition of PGE_2 release by gadolinium treatment was greatest at the lowest dose of LPS (0.025 μg/ml) and the lowest concentration of arginine (10 μmol/L). * = not significant; ** = $P < 0.0001$. Standard deviations were ≤10% of the mean.

from GD-treated rats was negligible except at the highest LPS dose tested (2.5 μg/ml). As has been shown previously for hepatic macrophages,[3] the splenic macrophage LPS-induced release of PGE_2 reflects a dose response to the arginine concentration in the culture medium. The levels of PGE_2 measured were inversely related to the arginine concentrations, with the physiologically relevant concentrations (10 and 100 μmol/L) of arginine being associated with significant increases in PGE_2 release by normal splenic macrophages in response to LPS (Fig. 3). Of note is the finding that PGE_2 release by splenic macrophages was nearly undetectable when standard RPMI-1640 medium containing a supraphysiologic arginine concentration (1200 μmol/L) was used.

DISCUSSION

In this study we confirmed the completely protective effect of systemic GD pretreatment against a lethal dose of LPS.[9] Although the mechanism of this phenomenon is unknown, multiple effects of GD administration on macrophages have been described. For example, it has been reported that GD reduces hepatic[15] and alveolar[16] macrophage populations. Loss of alveolar macrophages has been shown to occur by apoptosis.[16] A reduction of resident tissue macrophages by GD could ameliorate the response to inflammatory triggers by removing the cells responsible for the initiation and maintenance of the proinflammatory chemokine cascade of the monocytic phagocytic system, since the overexpression of the proinflammatory cascade may promote septic mortality.[3]

However, our observations as well as those of others, support the notion that the effect of GD on the monocytic phagocytic system is more complex than simple depletion of tissue macrophage populations.[2,8,17-19] In fact, splenic macrophages in the white pulp of the spleen are not altered, whereas those in the red pulp only demonstrate a transient and mild loss of staining by ED1 and ED2 macrophage–specific antibodies after GD administration.[15] Consequently GD may instead specifically alter the response of viable macrophages. For example, rat hepatic macrophages incubated in vitro with GD responded to LPS stimulation with a lowered tumor necrosis factor-α production.[20] This effect could improve mortality if tumor necrosis factor-α plays a central role in sepsis.[21] In addition, GD may prevent septic mortality by altering the effector arm of the immune response, as suggested by GD restoration of the suppressed splenocyte interleukin-2 and interferon-γ response to conconavalin A stimulation following cecal ligation and puncture.[22] These lymphokines produced by the T-helper 1 (TH_1) lymphocyte subset are required for competent cell-mediated immune responses. Finally, Iimuro et al.[9] concluded that GD may protect against hepatocyte necrosis and septic mortality of LPS by inhibiting superoxide generation.

No studies have addressed the possibility that GD improves the deranged immune function associated with shock by inhibiting the synthesis of prostaglandins. Such a mechanism would be consistent with the elevated blood PGE_2 levels associated with impaired cell-mediated immunity in severely injured humans.[4,5] Elevated PGE_2 release by monocytes impairs human lymphocyte proliferation[6] and interleukin-2 and interleukin-1 release,[23] as well as murine antigen presentation and Ia expression following hemorrhagic shock.[14] Cyclooxygenase inhibition of PGE_2 production has been shown to improve survival and the physiologic parameters of shock.[24-26]

In addition to the apparent importance of PGE_2 to the septic response, there were several other reasons to assess the effect of GD on PGE_2 release by splenic macrophages. Eicosanoid synthesis by macrophages of the monocytic phagocytic system depends on phospholipase A_2 activation by calcium signaling.[27-29] The potential importance of this calcium dependency resides in the fact that lanthanide metals such as GD act as calcium channel blockers in a variety of cells[30-32] including tissue macrophages (unpublished data). Thus improved endotoxin mortality could partly be accounted for by GD-mediated impaired calcium signaling and phospholipase A_2 activation and in vivo inhibition of immunosuppressive PGE_2 release.

The effect of GD pretreatment on LPS-induced PGE_2 release was most marked when splenic macrophages were cultured in medium containing low arginine concentrations. Hepatic macrophages (Kupffer cells) are normally exposed to lower levels of portal venous blood than are macrophages located systemically, such as those in the spleen. Endotoxemia further lowers not only arginine levels in the intrahepatic environment (see Fig. 2) but also systemic levels, which can affect systemic macrophages in the peritoneal cavity and spleen. For example, peritoneal macrophages activated by *Corynebacterium parvum* produce more PGE_2 with lower arginine concentrations.[13] Sax et al.[12] measured systemic plasma arginine concentrations of 112 ± 11 μmol/L in healthy rats and 63 ± 6 μmol/L in septic rats. In addition, arginine uptake by the liver was significantly enhanced by sepsis.[12] Our results reveal that arginine concentrations as low as 10 μmol/L are achieved during endotoxemia. These data affirm the physiologic relevance of using low arginine concentrations in the study of macrophage responses.

Arginine is a semiessential amino acid intermediary of the hepatic urea cycle.[33] Its role as the sole substrate for nitric oxide synthase[34] explains the extrac-

tion of arginine across the liver, especially in the setting of endotoxemia when inducible nitric oxide synthase activity is upregulated.[35] Arginine enhances the immune responsiveness of lymphocytes[36] and, as the donor of nitrogen,[37] is necessary for nitric oxide–mediated macrophage cytotoxicity against tumor[38] and parasite[39] targets. Thus the inverse relationship between decreased arginine availability and the release of immunosuppressive PGE_2 by LPS-stimulated splenic macrophages correlates with the immunosuppression of sepsis. Although nitric oxide has been shown to induce cyclooxygenase,[40,41] the rate-limiting enzyme of eicosanoid synthesis, our findings of reduced PGE_2 release by LPS-stimulated splenic macrophages in a high-arginine milieu are possibly explained by the known impairment of mitochondrial respiration by nitric oxide.[42]

We conclude that the association between GD-mediated protection against septic mortality and the inhibition of PGE_2 release after in vitro LPS is consistent with other models of septic mortality in which immunosuppression by PGE_2 plays a pivotal role. In addition, we recommend that all experiments conducted on macrophages be performed in medium containing physiologic (10 to 100 μmol/L) concentrations of arginine.

We gratefully acknowledge Dr. Martin Mangino and Michael Murphy for performing the PGE_2 radioimmunoassay. We are indebted to Glenn Hortin and the Metabolic Genetics Laboratory of the Department of Pediatrics at Washington University for providing serum amino acid measurements. We thank Theresa Belgeri for her expert secretarial assistance.

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Discussion

Dr. A. Barbul (Baltimore, Md.). How do you interpret the effect of arginine on PGE_2 release? Second, how would you speculate that gadolinium has such a prolonged effect since calcium has a very short-lived effect in vivo?

Dr. C. Roland. Arginine is the nitrogen donor of nitric oxide, and there are recent data indicating that nitric oxide induces the cyclooxygenase gene, in which case we should have observed an increase in PGE_2 as the arginine levels were increased in the media. Since we observed the opposite effect, it is possible that the nitric oxide we know is generated in the wells by lipopolysaccharide-stimulated macrophages is acting perhaps to alter mitochondrial respiration. As far as the mechanism of gadolinium is concerned, we have shown that it blocks calcium flux in the Kupffer cell. As far as the persistence of its effect, I would hypothesize that this is related to the aggregation of gadolinium in the cytosol, as was originally demonstrated by electron microscopy. Although I have no data to support this, the gadolinium aggregates in the cytosol may slowly release gadolinium, which may then block both the cell membrane calcium channels and those of intracellular calcium storage organelles such as the endoplasmic reticulum.

Calcium Accentuates Injury Induced By Ethanol in Human Gastric Cells

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The mechanism(s) whereby ethanol induces cellular injury remains poorly understood. Furthermore, the role of calcium in gastric mucosal injury under in vitro conditions is poorly defined. The major objectives of this study were to (1) define the temporal relationship between intracellular calcium accumulation induced by ethanol and cellular injury, (2) characterize the mechanism(s) whereby ethanol increases cellular calcium content, and (3) determine whether calcium removal would attenuate ethanol-induced cellular injury. Human gastric cells (AGS) were used for all experiments. Sustained intracellular calcium accumulation induced by ethanol, but not transient changes, preceded and directly correlated with cellular injury. Cells exposed to damaging concentrations of ethanol demonstrated an initial calcium surge that appeared to be a consequence of inositol 1,4,5-triphosphate (IP_3) generation and subsequent internal store release followed by a sustained plateau resulting from extracellular calcium influx through store-operated calcium channels. Finally, both morphologic (cellular injury) and functional (clearance of bovine serum albumin) changes induced by ethanol were significantly attenuated when extracellular Ca^{++} influx was prevented, and further decreased when intracellular Ca^{++} stores were depleted. These data indicate that calcium plays a significant role in cellular injury induced by ethanol.(J GASTROINTEST SURG 1999;3:308-318.)

KEY WORDS: AGS cells, calcium, inositol triphosphate, ethanol, store-operated calcium influx

Over the past several decades, ethanol (EtOH) has been used as a necrotizing agent in experimental models assessing gastroduodenal injury and/or defense, undoubtedly because such models have clinical relevance. However, despite extensive investigation, the mechanism(s) by which EtOH induces cellular cytotoxicity remains poorly understood. Proposed mechanisms of EtOH-induced injury include disruption of the gastic mucosal barrier, decreased mucosal blood flow, and inhibition of mucus and/or bicarbonate secretion.[1,2]

Since the report by Schanne et al.[3] in the late 1970s that cellular accumulation of calcium (Ca^{++}) may be the "final common pathway" of toxic cell death, the relationship between Ca^{++} and cellular injury has been investigated in numerous cell types under many different conditions. However, groups assessing Ca^{++} and EtOH-induced gastric injury under in vivo conditions have reported varying results. The addition of luminal Ca^{++} has been shown to decrease EtOH-induced lesions and to enhance the recovery of mucosal damage.[4,5] In contrast, others have noted that chelating luminal Ca^{++} decreases the extent of EtOH-induced injury.[6] Pretreatment with selective Ca^{++} channel antagonists has been reported to either worsen[6,7] or attenuate[1,8] EtOH-induced lesions.

One explanation for the aforementioned discrepancies is the fact that the damaging effects of EtOH under in vivo conditions are undoubtedly complex and multifactorial. Ca^{++} is necessary for numerous cellular functions and processes; thus the role of Ca^{++} as it relates to cellular integrity is difficult to delineate. Moreover, specific Ca^{++} channel blockers have been shown to have alternate effects including decreased acid secretion and enhanced gastric blood flow.[1,8]

Our objective was to determine the role of Ca^{++} in gastric mucosal injury elicited by EtOH under in

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vitro conditions, thus eliminating the effect of Ca^{++} on blood vessels, nerves, and hormone-secreting cells. We examined the mechanism(s) whereby EtOH increases intracellular Ca^{++} content, assessed the temporal relationship between intracellular Ca^{++} accumulation and cellular injury, and investigated the effect of both extracellular and intracellular Ca^{++} removal on EtOH-induced injury in human gastric cells.

METHOD

Cells

The human gastric cell line known as AGS (CRL-1739) was obtained from American Type Culture Collection (Rockville, Md.) at passage 49. We have previously characterized this cell line and determined AGS cells to be morphologically similar to gastric mucous cells (periodic acid–Schiff and Alcian blue stains) with an ability to differentiate when post-confluent.[9] Cells were maintained at 37° C in an atmosphere of 5% carbon dioxide and 100% relative humidity, and were split on a weekly basis at a ratio of 1:6 on reaching confluence. Cells were detached using 0.5 g porcine trypsin and 0.2 g EDTA tetrasodium per liter of Hank's balanced salt solution (HBSS), and then placed onto either 48-well plates or 35 mm dishes (Costar, Cambridge, Mass.) for experiments or put into 150 cm^2 flasks for propagation. Cells grown for permeability studies were split at a ratio of 1:2 into 3 μmol/L Biocoat Collagen I Cell Culture Inserts (0.3 cm^2 growth area; Becton Dickinson Labware, Bedford, Mass.), and experiments were performed 7 days post confluence. All other experiments were performed at 90% to 100% confluence. Cell passage was maintained between 50 and 65 and medium was changed every 2 to 3 days. AGS medium consisted of Ham's F12 supplemented with 10% fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B.

Solutions

Prior to all experiments, medium was aspirated and replaced with Hank's balanced salt solution (HBSS) plus 10 mmol/L HEPES (H 8264, Sigma, St. Louis, Mo.—137 mmol/L NaCl, 5.7 mmol/L $NaHCO_3$, 5.3 mmol/L KCl, 1.26 mmol/L $CaCl_2$, and 0.8 mmol/L $MgSO_4$). Experiments involving calcium-free buffer utilized HBSS plus 10 mmol/L HEPES and 2 mmol/L BAPTA (H 6648, Sigma—137 mmol/L NaCl, 5.7 mmol/L $NaHCO_3$, and 5.3 mmol/L KCl). All test compounds were dissolved in either HBSS or HBSS (-Ca^{++}). Lanthanum chloride (La^{+++}), verapamil, and nifedipine were obtained from Sigma, and treatment involved a 15-minute preincubation period followed by the addition of the respective inhibitor to all subsequent solutions within treatment groups. Thapsigargin was purchased from Molecular Probes (Eugene, Ore.) in 50 μg aliquots. Experiments were performed at 37° C in a humidified incubator.

Measurement of $[Ca^{++}]_i$ and Extracellular Ca^{++} Influx

Changes in intracellular Ca^{++} concentration ($[Ca^{++}]_i$) were quantitated using the single wavelength Ca^{++} indicator Fluo-3 (Fluo-3, AM; Molecular Probes). Fluo-3 was chosen as a Ca^{++} indicator because it exhibits a large fluorescent enhancement on Ca^{++} binding (40-fold) and exhibits an enhanced resistance to autobleaching.[10]

Fluo-3 was initially dissolved in Pluronic F-127 (20% solution in dimethylsulfoxide [DMSO]; Molecular Probes) to make a 1 mmol/L working solution, and was subsequently added to HBSS plus 1% fetal bovine serum for a final loading concentration of 4 μmol/L. Cells were washed twice with HBSS and subsequently loaded with Fluo-3 for 50 minutes at 25° C in an atmosphere of 5% carbon dioxide and 100% relative humidity. Loading at a lower temperature significantly decreases indicator compartmentalization into the endoplasmic reticulum or mitochondria.[11]

AGS cells were then washed three times to ensure removal of all unloaded Fluo-3, and control and test solutions were added to the respective wells. At each time point, the intracellular Ca^{++} concentration was calculated using the following equation:

$$[Ca^{++}]_i \text{ (nmol/L)} = K_d \frac{(F - F_{min})}{(F_{max} - F)}$$

where $F_{min} = 1.25\ F_{MnCl2} - 0.25\ F_{max}$ and $K_d = 400$ nmol/L.[12]

The maximum Fluo-3 signal, or F_{max}, was determined by permeabilizing AGS cells with 50 μmol/L digitonin (Sigma). The Fluo-3 signal was quenched to obtain F_{MnCl2} using 2 mmol/L $MnCl_2$ and 50 μmol/L digitonin. Tetrakis (2-pyridylmethyl) ethylenediamine (TPEN; 50 μmol/L, Molecular Probes) was used in all solutions as a heavy metal scavenger.[11]

It is well accepted that manganese (Mn^{++}) can be used as a Ca^{++} surrogate to estimate extracellular Ca^{++} influx through the plasma membrane.[13] In separate experiments, Mn^{++} uptake was monitored by quenching Fluo-3 fluorescence with the addition of 2 mmol/L $MnCl_2$ to all solutions (control and experimental). Data are presented as mean fluorescence.

Continuous fluorescent signals during both protocols were quantitated using a CYTOFLUOR II fluo-

rescent multiwell plate reader (PerSeptive Biosystems, Framingham, Mass.) employing 485 nm and 530 nm as the excitation and emission spectra, respectively. Cells were maintained throughout the experiments at a temperature of 37° C with a heated stage.

Measurement of Cell Injury

Cellular injury was quantitated employing the fluorescent agent ethidium homodimer-1 (4 μmol/L; Molecular Probes) to monitor plasma membrane integrity. Ethidium homodimer-1 (Et) enters cells through damaged membranes and undergoes enhanced fluorescence on binding to nucleic acids. This fluorescent probe produces a bright red fluorescence (at 600 nm) in dead cells[14] and was measured with a fluorescent multiwell plate reader at 485 nm excitation and 620 nm emission wavelengths. Injury is reported as relative fluorescence.

Inositol 1,4,5-Triphosphate Production

Inositol 1,4,5-triphosphate (IP_3) generation in AGS cells following EtOH exposure was quantitated by radioreceptor assay. Prior to EtOH treatment, AGS cells grown in 35 mm dishes were rinsed twice with HBSS and preincubated with 20 mmol/L lithium chloride (30 minutes) to inhibit inositol-1-phosphatase. Cells were then treated with either 750 μl of HBSS or 12% (volume/volume) EtOH. At the indicated time points, 200 μl of ice cold 100% trichloroacetic acid was added to the dishes. Cellular homogenates were scraped off the dishes, incubated on ice for 20 minutes, and subsequently centrifuged at 2400 rpm (15 minutes, 4° C). The supernatants were removed and pellets discarded. Trichloroacetic acid was removed from the samples by vortexing vigorously with 2 ml of a mixture of 1,1,2-trichloro-1,2,2-1-trifluoroethane (TCTFE) and triethylamine in a 3:1 ratio. The mixture was allowed to remain at room temperature for 3 minutes and subsequently centrifuged at 2400 rpm (5 minutes). The aqueous portion containing water-soluble IP_3 was removed.

Levels of IP_3 were quantitated with an inositol-1,4,5-triphosphate [^{3}H] radioreceptor assay kit (NEN Life Science Products, Boston, Mass.) as previously described.[15] Briefly, either prepared samples or standard IP_3 (0.12 to 12 pmoles) were incubated (30 minutes, 4° C) with a calf cerebellar IP_3–binding protein preparation in the presence of 10 μCi D-myo[^{3}H]IP_3 in a 50 mmol/L sodium TAPS buffer containing 0.05% sodium azide, 5 mmol/L EDTA, and 5 mmol/L EGTA (pH 8.6). Bound and free labeled IP_3 were then separated by centrifugation at 3300 rpm (18 minutes, 4° C). After the supernatant was aspirated, the pellet was dissolved in 50 μl of 0.15 mol/L NaOH, and radioactivity was determined by liquid scintillation counting (Beckman LS 5000CE, Beckman Instruments, Inc., Irvine, Calif.). IP_3 content of each sample was determined by comparison to a standard curve generated under identical conditions. Total protein concentration was quantitated in separate untreated dishes with a BCA protein assay kit (Pierce Chemical Co., Rockford, Ill.), and data are presented as pmoles/mg protein.

Permeability

Cellular permeability was quantified by measuring the apical to basolateral flux of Texas red conjugated bovine serum albumin (BSA; Molecular Probes). After the respective pretreatments, fresh HBSS (750 μl) was pipetted into the basolateral chamber and experimental HBSS solutions (300 μl) containing BSA (50 μg/ml, 66 kd) were pipetted into the apical chamber. Fifty microliter aliquots were subsequently obtained from the apical and basolateral chambers at baseline and at 10 minutes, and pipetted into 96-well plates (fluorescent clear bottom plate; Costar Corp., Cambridge, Mass.). Fluorescent signals were quantitated using a CYTOFLUOR II fluorescent multiwell plate reader (PerSeptive Biosystems) employing 530 nm and 620 nm as the excitation and emission spectra, respectively. Clearance (Cl) was calculated according to the following equation:

$$Cl\ (nl/hr/cm^2) = \frac{F_{ab}}{[BSA]_a \times S},$$

where F_{ab} = apical to basolateral flux of BSA (light units/hr), $[BSA]_a$ = concentration at baseline (light units/nl), and S = surface area (0.3 cm^2).[16]

Experimental Design

We initially investigated the temporal relationship between intracellular Ca^{++} accumulation induced by graded concentrations of EtOH (0% to 16%) and cell injury. This was achieved with the simultaneous measurement of changes in intracellular Ca^{++} content and Et uptake. To determine whether the initial increase in intracellular Ca^{++} concentration may involve the release of internal Ca^{++} stores through phosphoinositide-specific phospholipase C activation and subsequent IP_3 generation, we measured IP_3 and, in separate experiments, intracellular Ca^{++} content in cells exposed to 12% EtOH. The mechanism of sustained influx of extracellular Ca^{++} was investigated with Ca^{++}-free buffer, the store-operated Ca^{++} channel (SOCC) blocker La^{+++},[13] and the voltage-operated Ca^{+-} channel (VOCC) antagonists verapamil or nifedepine.

Finally we examined the effect of calcium removal on both Et uptake and, in separate studies, changes in BSA clearance induced by 12% EtOH. Calcium-free buffer or La^{+++} pretreatment was used to determine the role of extracellular calcium. To investigate the role of intracellular calcium, separate cells were pretreated with thapsigargin and subsequently exposed to 12% EtOH in calcium-free buffer. Thapsigargin, a microsomal Ca^{++}-ATPase inhibitor, rapidly depletes intracellular calcium stores.[17]

Statistics

Statistical evaluation was performed by analysis of variance with a Scheffe post hoc test. Data ($n = 6$ to 12 per group) are reported as mean ± standard error of the mean. A P value less than 0.05 was taken to represent statistical significance.

RESULTS

Intracellular Ca^{++} Accumulation and Cellular Injury Induced by Ethanol

Both intracellular Ca^{++} concentration and cellular injury (Et uptake) within control cells remained stable throughout the 10-minute period of observation. EtOH exposure elicited a rapid concentration-dependent rise in Ca^{++} accumulation in AGS cells. A large initial surge of intracellular Ca^{++} was observed within the first minute following EtOH exposure (5% to 16%) followed by a slightly lower sustained elevation. However, cells exposed to the lowest EtOH concentration, 1.5%, demonstrated an initial intracellular Ca^{++} elevation followed by a return to resting Ca^{++} levels. AGS cells exposed to 5% to 16% EtOH demonstrated initial signs of Et uptake at 3 minutes after exposure followed by significant concentration-dependent Et uptake at 5 minutes. However, 1.5% EtOH did not appear to induce cellular injury at the later time points when compared to control cells. These data are depicted in Fig. 1. Neither intracellular Ca^{++} content nor Et uptake in both treated and control cells increased significantly beyond the 10-minute time point, suggesting that cellular injury induced by EtOH is extremely rapid and self-limiting (data not shown). In a separate experiment the relationship between intracellular Ca^{++} content at 10 minutes following EtOH exposure and Et uptake was investigated by plotting individual data points as a scatter diagram. There was a strong correlation between intracellular Ca^{++} accumulation and Et uptake (Fig. 2; $R^2 = 0.821$, $P = 0.001$). These data suggested that sustained intracellular Ca^{++} accumulation induced by EtOH, but not transient Ca^{++} changes, as observed in cells exposed to 1.5% EtOH, preceded and directly correlated with cellular injury.

Ethanol-Induced Ca^{++} Accumulation: Role of Internal Ca^{++} Stores

Preliminary experiments investigating the mechanism of EtOH-induced Ca^{++} mobilization showed that the initial increase in intracellular Ca^{++} content was not dependent on extracellular Ca^{++}, suggesting a role for Ca^{++} release from internal stores. We therefore measured generation of IP_3 and, in separate experiments, intracellular Ca^{++} content in AGS cells exposed to EtOH. IP_3 levels remained stable in control cells throughout the experimental time period (data not shown). Following exposure to 12% EtOH, cells demonstrated a large increase in IP_3 content (10 to 20 seconds) followed by a return to baseline. The elevation in IP_3 preceded intracellular Ca^{++} accumulation. These data, shown in Fig. 3, suggest that the initial Ca^{++} elevation elicited by EtOH may be caused by the release of Ca^{++} from IP_3-sensitive internal stores.

Ethanol-Induced Ca^{++} Accumulation: Role of Extracellular Ca^{++} Influx

Sustained intracellular Ca^{++} elevations (5 to 10 minutes) were not observed in cells treated with 12% EtOH in the absence of extracellular Ca^{++}. Quenching of Fluo-3 fluorescence by Mn^{++} was clearly evident 10 minutes after 12% EtOH exposure, suggesting the influx of extracellular Ca^{++} during the latter time period. Pretreatment of AGS cells with either La^{+++} (25 μmol/L; 15 minutes), verapamil (20 μmol/L; 15 minutes), or nifedipine (10 μmol/L; 15 minutes), alone, did not significantly affect intracellular Ca^{++} levels (data not shown). The SOCC blocker La^{+++}, but not the VOCC antagonists verapamil or nifedipine, inhibited both the sustained intracellular Ca^{++} plateau and Mn^{++} uptake following 12% EtOH exposure. These data are shown in Fig. 4 and suggest that the sustained intracellular Ca^{++} elevation, as induced by 12% EtOH, is mediated by the influx of extracellular Ca^{++} through SOCCs, observations that are consistent with store-operated Ca^{++} influx (SOCI).

Removal of Extracellular and Intracellular Ca^{++}

Pilot studies in AGS cells indicated that thapsigargin (500 nmol/L), in Ca^{++}-containing buffer, elicited SOCI (or capacitive Ca^{++} entry). However, cells treated with thapsigargin in Ca^{++}-free buffer elicited an initial increase in intracellular Ca^{++} concentration followed by a decline (at 5 minutes post exposure) to baseline values, suggesting that intracellular Ca^{++} stores were effectively depleted. AGS cells were exposed to 12% EtOH in (1) Ca^{++}-containing buffer,

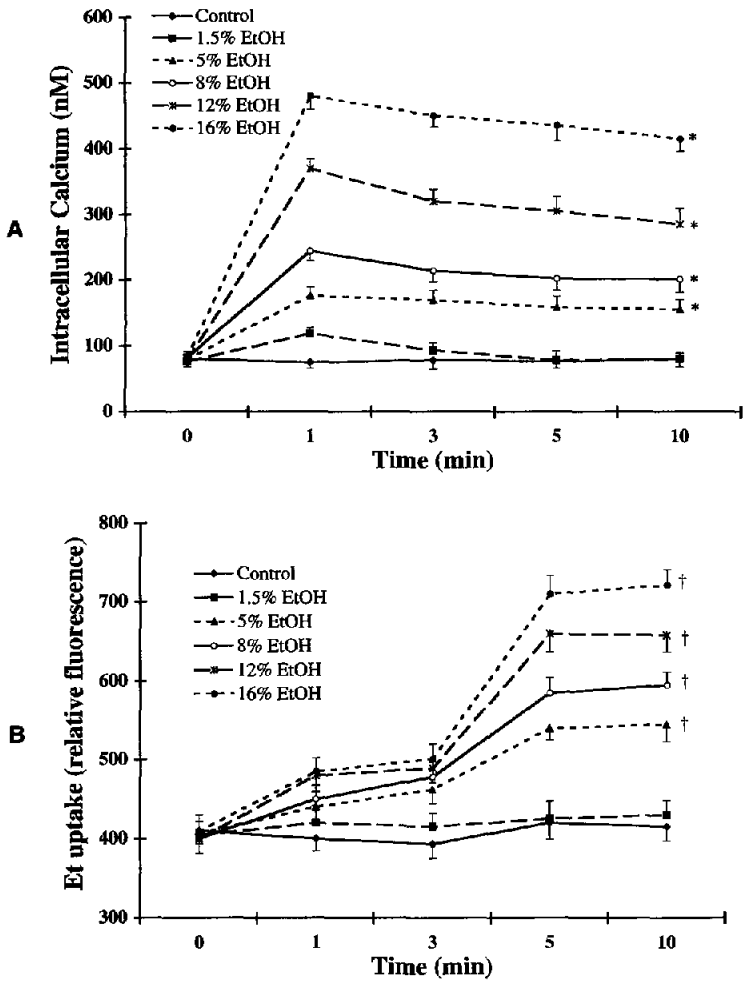

Fig. 1. Effect of graded concentrations of ethanol *(EtOH)* on changes in intracellular calcium concentration **(A)** and ethidium homodimer-1 *(Et)* uptake **(B)** (* = $P < 0.01$ vs. control; † = $P < 0.01$ vs. control; n = 6 to 12 per group).

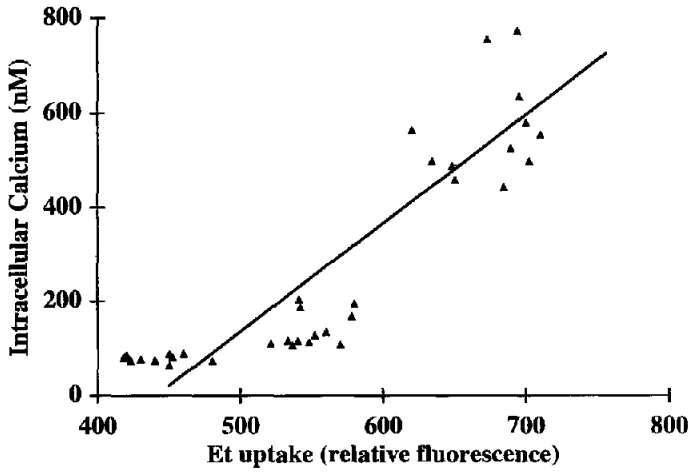

Fig. 2. Correlation between intracellular calcium content and ethidium homodimer-1 *(Et)* uptake in AGS cells exposed to varying concentrations of ethanol for 10 minutes. $R^2 = 0.821$; $P = 0.00$.

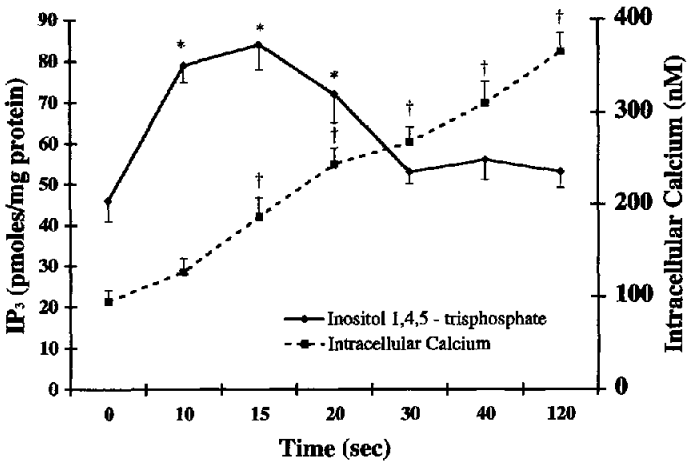

Fig. 3. Effect of 12% (volume/volume) ethanol on inositol 1,4,5-triphosphate *(IP_3)* generation and, in a separate experiment under identical conditions, changes in intracellular calcium content (* = $P < 0.01$ vs. control; † = $P < 0.01$ vs. control; n = 6 to 12 per group). Note: triphosphate = trisphosphate.

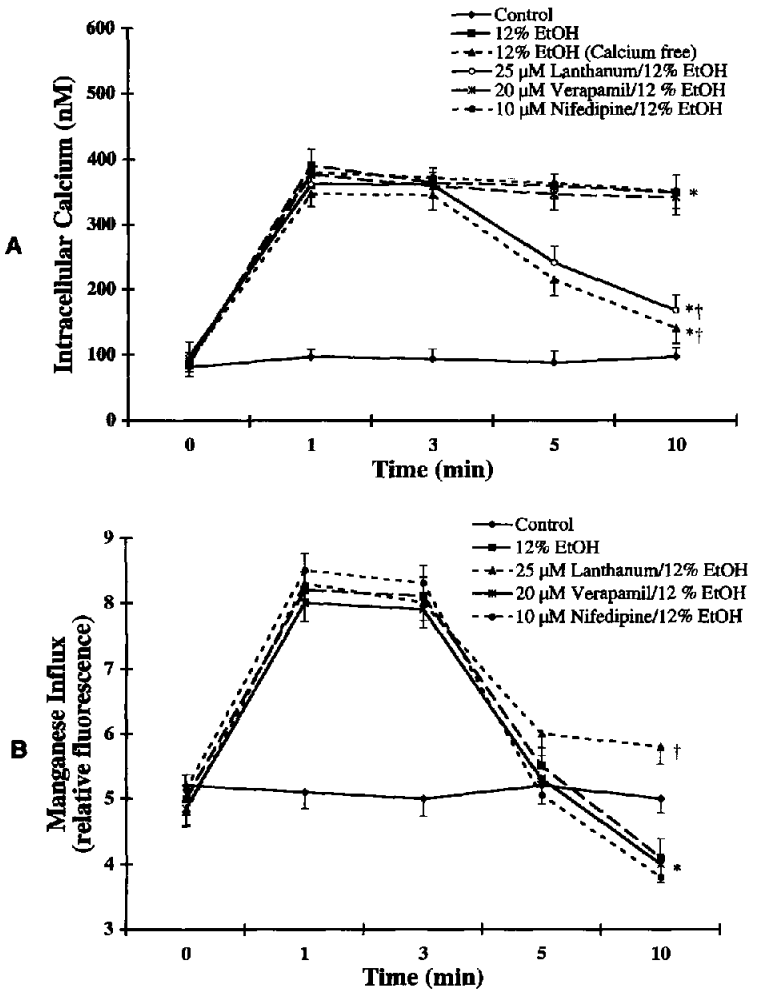

Fig. 4. Effect of lanthanum, verapamil, or nifedipine pretreatment (15 minutes) on changes in intracellular calcium concentration **(A)** or manganese influx **(B)** induced subsequently by 12% ethanol *(EtOH)* (* = $P < 0.01$ vs. control; † = $P < 0.01$ vs. cells exposed to 12% EtOH in calcium-containing buffer; n = 6 to 12 per group).

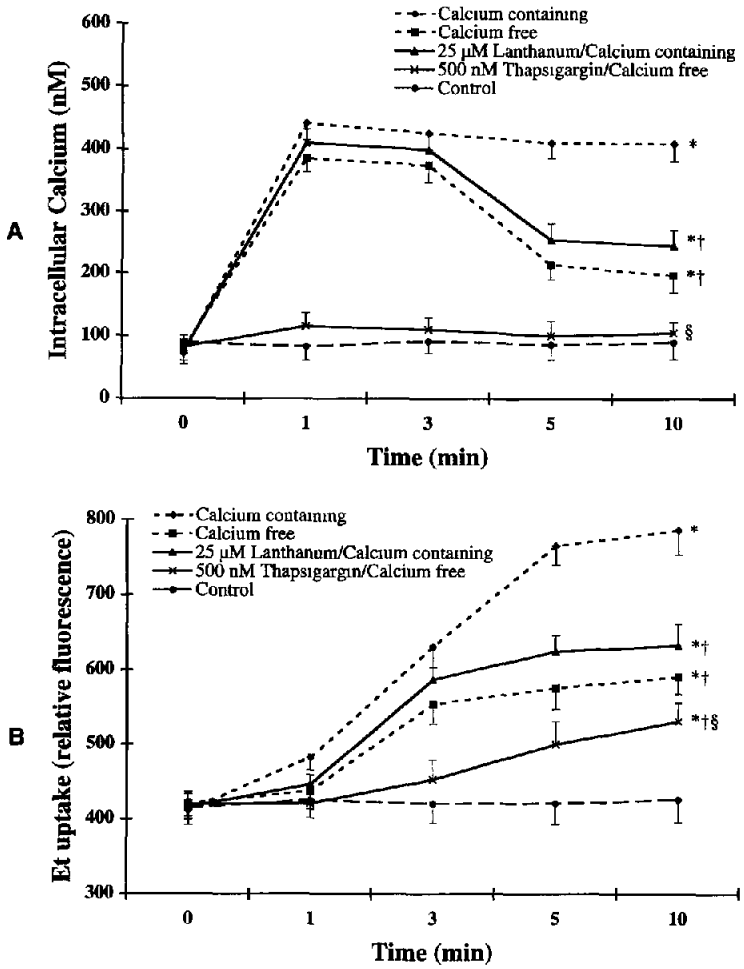

Fig. 5. Effect of calcium removal on changes in intracellular calcium content **(A)** or ethidium homodimer-1 *(Et)* uptake **(B)** induced by exposure to 12% ethanol. The role of extracellular calcium was assessed with either calcium-free buffer or lanthanum pretreatment (15 minutes), whereas separate cells were pretreated with thapsigargin (10 minutes) to investigate the role of intracellular calcium (* = $P < 0.01$ vs. control; † = $P < 0.01$ vs. cells exposed to 12% EtOH in calcium-containing buffer; § = $P < 0.01$ vs. cells exposed to 12% EtOH in calcium-free buffer; n = 6 to 12 per group).

(2) Ca^{++}-free buffer, (3) Ca^{++}-containing buffer following 25 μmol/L La^{+++} pretreatment (15 minutes), and (4) Ca^{++}-free buffer following pretreatment with 500 nmol/L thapsigargin (10 minutes). The aforementioned treatments, alone and under control conditions, did not elicit significant cellular injury (data not shown). In the presence of extracellular Ca^{++}, 12% EtOH exposure caused a large initial increase in intracellular Ca^{++} content followed by a sustained elevation. In the absence of extracellular Ca^{++} or following La^{+++} pretreatment, sustained intracellular Ca^{++} accumulation, as observed at the later time points, was attenuated. In AGS cells pretreated with thapsigargin, subsequent exposure to 12% EtOH in Ca^{++}-free buffer did not appear to evoke any changes in intracellular Ca^{++} content. Cellular injury (Et uptake) induced by 12% EtOH was attenuated when extracellular Ca^{++} influx was prevented with either Ca^{++}-free buffer or La^{+++} pretreatment and further decreased when intracellular Ca^{++} stores were depleted. These data are depicted in Fig. 5.

In separate studies transepithelial clearance of BSA was then quantitated 10 minutes after the aforementioned treatments. Clearance of BSA in control monolayers was considerably less than clearance through the insert alone (563 vs. 25 pl/min/cm^2), implying significant resistance to movement of the fluorescent marker through intact cell monolayers. In control cells the absence of extracellular Ca^{++}, with or without thapsigargin pretreatment or La^{+++} pretreatment, did not appear to alter BSA clearance. Cell monolayers exposed to 12% EtOH demonstrated sig-

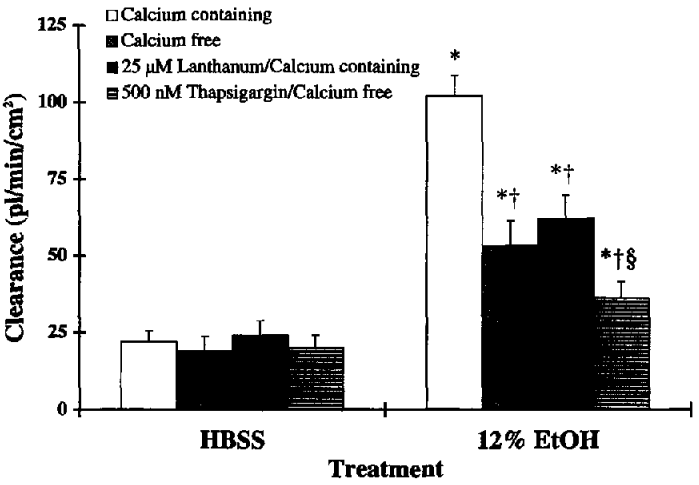

Fig. 6. Effect of calcium removal on changes in bovine serum albumin clearance induced by exposure to 12% ethanol. The role of extracellular calcium was assessed with either calcium-free buffer or lanthanum pretreatment (15 minutes), whereas separate cells were pretreated with thapsigargin (10 minutes) to investigate the role of intracellular calcium (* = $P < 0.01$ vs. control; † = $P < 0.01$ vs. cells exposed to 12% EtOH in calcium-containing buffer; § = $P < 0.01$ vs. cells exposed to 12% EtOH in calcium-free buffer; n = 6 to 8 per group).

nificantly increased transepithelial BSA clearance, an effect that was significantly decreased with removal of extracellular Ca^{++} or La^{+++} pretreatment and further attenuated when intracellular Ca^{++} stores were depleted with thapsigargin (Fig. 6). Thus BSA clearance measurements paralleled findings measuring Et uptake and further supported the observation that Ca^{++} removal blunts EtOH-induced cellular injury.

DISCUSSION

Our results suggest that EtOH, under in vitro conditions in human gastric cells, elicits a rapid increase in intracellular Ca^{++} content through IP_3 generation and internal store release followed by sustained extracellular Ca^{++} influx through SOCCs. Sustained intracellular Ca^{++} elevations appear to precede and directly correlate with cellular injury, whereas transient Ca^{++} changes do not affect cellular integrity. Finally both morphologic (cellular injury) and functional (BSA clearance) changes induced by EtOH were significantly attenuated when extracellular Ca^{++} influx was prevented, and further decreased when intracellular Ca^{++} stores were depleted. Taken together these data strongly suggest that EtOH, at concentrations that are clinically relevant,[18,19] but under conditions independent of blood flow, hormone secretion, and neural innervation, elicits cellular injury, at least in part, through Ca^{++} accumulation.

Most previous studies investigating EtOH-induced gastric mucosal injury have involved in vivo models. Glavin and Szabo,[6] assessing EtOH-induced injury in rats, reported that luminal Ca^{++} chelation with either EGTA or EDTA significantly decreased the extent of gastric mucosal lesions. This protection was lost in vagotomized or adrenalectomized rats, suggesting that EGTA and EDTA attenuate injury through vagal and glucocorticoid and/or catecholamine mechanisms. In contrast, other in vivo studies have shown that luminal Ca^{++} is critical for the recovery of mucosal damage.[4] Our data confirm the work of Arakawa et al.[20] and suggest that the presence of extracellular Ca^{++} significantly worsens EtOH-induced cellular injury. One explanation for the aforementioned discrepancies is that, although Ca^{++} may be acutely injurious, this cation may also be important during gastric epithelial restitution and repair.

Both Ghanayem et al.[1] and Hertz and Cloarec[8] reported that pretreatment of rats with the VOCC antagonists verapamil or diltiazem provided significant protection against both EtOH- and indomethacin-induced gastric lesions. Suggested mechanisms whereby selective Ca^{++} channel blockade attenuates EtOH-induced injury include enhanced gastric blood flow, decreased vasoconstriction and platelet aggregation, and decreased gastric smooth muscle contraction.[1,21] These actions may thereby prevent intravascular thrombogenesis and prevent focal ischemia. Furthermore, VOCC antagonists also appear to have an inhibitory effect on histamine-, gastrin-, and carbachol-induced gastric acid secretion.[1] Our data indicate that EtOH-induced Ca^{++} mobilization under in vitro con-

ditions is inhibited by La^{+++}, an SOCC blocker, but not conventional VOCC antagonists. This observation is consistent with other reports[22] and suggests that nonexcitable gastric mucosal cells do not have VOCCs. Thus the current study supports the premise that verapamil and diltiazem reduce EtOH-induced injury under in vivo conditions through mechanisms involving excitable (smooth muscle, nerves, etc.) but not gastric epithelial cells.

EtOH is an organic solvent which, at high concentrations, interacts with cellular phospholipids leading to fluidization of biologic membranes. It has been suggested that Ca^{++} mobilization induced by EtOH may be related to a nonspecific interaction with cellular membranes and/or interference with normal mechanisms of Ca^{++} homeostasis.[23-25] Our data indicate that the mechanism whereby EtOH increases intracellular Ca^{++} content may involve a specific pathway. EtOH appeared to initially deplete internal Ca^{++} stores, an effect that was preceded by a significant increase in IP_3 content. Following internal store release, cells exposed to EtOH demonstrated sustained extracellular Ca^{++} influx, which was attenuated with the SOCC blocker La^{+++}. Our data thus suggest that EtOH may be comparable to other hormones or agonists that increase intracellular Ca^{++} concentration through phospholipase C activation, IP_3 generation, and internal store release, and subsequent SOCI. Hoek et al.[26] also noted that the activity of EtOH is similar to that of other Ca^{++}-mobilizing hormones. They reported that isolated hepatocytes exposed to EtOH demonstrated phospholipase C activation and a transient IP_3 elevation that preceded a rise in intracellular Ca^{++} content.

Mechanisms whereby sustained Ca^{++} elevations may induce cellular injury include the activation of various phospholipases, proteases, and endonucleases with subsequent disruption of the cytoskeleton (e.g., actin) and plasma membrane.[27] EtOH-induced intracellular Ca^{++} elevation may also cause cellular injury through the generation of oxygen-derived radicals such as superoxide anion, hydrogen peroxide, and hydroxyl radicals. Separate in vitro studies have suggested that both EtOH[28,29] and sustained intracellular Ca^{++} accumulation[30] cause a reduction in cellular glutathione content. Glutathione appears to protect against oxidant injury by acting as a superoxide anion scavenger and a cofactor for the reduction of hydrogen peroxide.[31]

We have also investigated the role of Ca^{++} in deoxycholate-induced injury in AGS cells,[32] and there are several similarities between this necrotizing agent and EtOH. Cells exposed to damaging concentrations of both EtOH and deoxycholate demonstrate an initial Ca^{++} surge caused by the release of Ca^{++} from internal stores followed by extracellular Ca^{++} influx. Cellular injury is preceded by sustained intracellular Ca^{++} accumulation and is attenuated by Ca^{++} removal. One clear difference between these two agents is that both Ca^{-+} accumulation and cellular injury induced by EtOH occur at a much earlier time point. Regardless, these similarities suggest that there may be a common mechanism whereby necrotizing agents induce gastric mucosal injury. Further investigation with other injurious agents in different cellular models is necessary to determine whether Ca^{++} accumulation is, in fact, the "final common pathway" of toxic cell death.

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Discussion

Dr. B. Bass (Baltimore, Md.). This calcium story has been in evolution for many years, and we are still trying to find out whether the observed changes in calcium are early markers of, or the actual cause of, injury. This work allows us to conclude that elevated intracellular calcium is a very early event, and it is likely a cause of subsequent cell injury. I have two questions. First, at what point does this injury become irreversible? At what point does the intracellular calcium become so high that recovery is not possible? Second, what is the mechanism by which calcium damages the cells? Your laboratory has done some very exciting work with microtubules and calcium in the recent past.

Dr. Kokoska. With regard to the reversibility of this phenomenon, I think it is dependent on two things. First is the magnitude of the increase in intracellular calcium and second is the duration, and I think it really does depend on both of these factors. For instance, when you look at agonist stimulation of cells, such as epidermal growth factor, we see changes in intracellular calcium that are maybe two- or threefold, and can last 5 to 10 minutes. We do not observe cellular injury. However, with higher concentrations of ethanol, in which we see a five or six times increase, over 5 to 10 minutes we do see significant cellular injury. With regard to this model, I think it is irreversible by about 10 minutes. When we look at cellular injury induced by 12% ethanol much further out than 10 minutes, it is the same at 60 minutes or 2 hours. So I think that injury occurs within that 10-minute time period, beyond which it is not reversible. With regard to how calcium injures cells, I think it is multifactorial; our bias is that the cytoskeleton is very much involved. I believe that phospholipase activation is involved as well with the breakdown of the phospholipid membrane. So I think those are the critical elements—activation of phospholipases, proteases, and nucleases.

Dr. J. Matthews (Boston, Mass.). I wonder about the time course experiment. Concentrations in your lanthanum/thapsagargin pretreatment group are still increasing at the end of 10 minutes, so I wonder if you extended the time further, would the lines actually converge? In other words, does calcium accelerate an injury that would have occurred anyway? The second part of that question is whether your assay of cell viability or cell injury using membrane integrity and junctional permeability is in itself calcium dependent. In other words, I am wondering if by taking the calcium out, cells are actually just as injured as they would have been, but the manifestation of that injury as a change in junctional permeability or change in membrane permeability requires the calcium to be there.

Dr. Kokoska. With regard to the first question, we have not conduced those types of experiments, but that is an excellent idea. What you are saying is that if we took the calcium-free data and extended the time to 30 minutes, we would see the same magnitude of injury as we saw at 10

minutes with calcium-containing medium; that could very well be the case. In answer to your second question, in the ethanol studies we have not used lactate dehydrogenase (LDH), but we have experimented with LDH using deoxycholate as the damaging agent and we have seen very similar trends. The LDH data complements and correlates very nicely with the ethidium data in deoxycholate damage. Because of that, I think we are okay in the present study with ethidium alone being our marker of damage.

Dr. A. Tarnawski (Huntington Beach, Calif.). I think these findings demonstrate for the first time the dynamic of calcium influx. For the past 25 years, there has been a constant battle between those who believe that calcium is the ultimate killer of the cells and investigators from Sweden who oppose this view. I think Dr. Kokoska has shown the dynamic of calcium influx and demonstrated the major mechanism, which is mediated through IP_3.

Dr. Kokoska. In regard to the last comment, we looked at IP_3 and it increases very nicely before we see the release of intracellular calcium stores, suggesting that this is not a nonspecific action of ethanol on the plasma membrane, but this is stimulating a specific signaling pathway.

Extracellular Matrix Modulates Enterocyte Growth via Downregulation of c-*jun* But Is Independent of p21 and p27 Expression

Seth I. Wolpert, M.D., Kathleen M. Lally, B.S., Ji Li, B.S., Jian-Ying Wang, Ph.D., Barbara Lee Bass, M.D.

Regulation of the intestinal crypt-villus axis is multifactorial and involves growth factors and extracellular matrix composition. Laminin, a component of the enterocyte basement membrane, induces enterocyte differentiation and inhibits proliferation. To investigate the mechanism of this observation, we examined the expression of cell cycle modulators in enterocytes cultured on laminin. IEC-6 enterocytes were cultured on collagen I or laminin for 24 hours in media with serum followed by 48 hours of culture in serum-free media. Cells were then stimulated with epidermal growth factor, and RNA and protein were extracted before and up to 18 hours after stimulation. c-*jun* mRNA expression and p21 and p27 protein expression were analyzed. Expression of c-*jun* was inhibited in cells grown on laminin as compared to collagen I. Expression of p21 and p27 was no different between cells grown on laminin or collagen I. The mechanism of enterocyte growth inhibition mediated by laminin involves downregulation of c-*jun* expression. In contrast, p21 and p27 levels were unaffected by extracellular matrix indicating that the changes in expression of these cyclin-dependent kinase inhibitors do not contribute to the effect of laminin on enterocyte proliferation. (J GASTROINTEST SURG 1999;3:319-324.)

KEY WORDS: Enterocyte, growth, basement membrane, oncogene, cyclin-dependent kinase inhibitor

The small bowel epithelium undergoes rapid turnover. Intestinal crypt cells proliferate to yield the terminally differentiated cells of the villus tip, a process that is completed every 3 to 5 days. It is the cells along the villus that carry out the absorptive function of the bowel. The regulatory mechanisms responsible for maintaining the balance between these populations of enterocytes are poorly understood and likely multifactorial. Peptide growth factors are operative in this process as epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) have been shown to stimulate enterocyte proliferation,[1-3] whereas transforming growth factor beta (TGF-β) inhibits growth and stimulates differentiation.[4]

The basement membrane of the intestinal epithelium has also been shown to be active in enterocyte phenotype regulation. The extracellular matrix is composed of a number of proteins and proteoglycans including laminin, collagen IV, fibronectin, entactin, and heparan sulfate. Although laminin is present throughout the crypt-villus axis,[5] isoforms of laminin have been found to predominate in either the crypts or villus tips.[6] Enterocytes cultured on laminin exhibit a differentiated phenotype as assessed by ultrastructural changes including microvilli, increased mitochondria, and rough endoplasmic reticulum, as well as expression of brush-border enzymes.[7,8]

Although these features of differentiation induced by laminin have been well described, the effect of matrix on enterocyte growth is just beginning to be explored. We have previously demonstrated that laminin inhibits EGF- and IGF-I–induced entero-

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cyte proliferation.[9] We have also identified that one mechanism of this altered growth response is downregulation of both the EGF receptor and the IGF receptor.[10]

The initiation of cell cycle progression is dependent on the transcription of immediate-early genes including c-*jun*. Stimulation of quiescent fibroblasts with serum or EGF induces c-*jun* transcription at the G0 to G1 transition.[11,12] Furthermore, microinjection of antibody directed against c-*jun* in proliferating cells has demonstrated that c-*jun* expression is required for entry into the S phase.[13] The products of the c-*jun* and c-*fos* genes encode the components of the transcription factor AP-1.[14]

Cyclin-dependent kinases (CDKs) are responsible for the transition of cells through the cell cycle. Activation of CDK requires association with cyclin and phosphorylation by CDK-activating kinase.[15] Regulation of CDK activity is mediated through changes in cyclin expression. Proliferating cell nuclear antigen (PCNA) is also required for DNA replication.[16] PCNA forms a quaternary complex with cyclin, CDK, and p21.[17] p21 has been demonstrated to function as a CDK inhibitor,[15] thereby preventing cell cycle progression. Other additional CDK inhibitors have recently been identified including p27, which blocks cyclin E/CDK2 activation preventing cell cycle progression.[18]

Given our consistent observation that enterocytes cultured on laminin have a diminished proliferative response to peptide mitogens, we hypothesized that enterocytes grown on laminin would have downregulated expression of c-*jun* in response to EGF. To test this hypothesis, we measured c-*jun* expression in synchronized enterocytes grown on laminin and collagen I. We also evaluated p21 and p27 to determine if expression of these CDK inhibitors is influenced by laminin.

METHODS

Cell Culture

IEC-6 cells, a rat enterocyte cell line with characteristics similar to crypt cells,[19] were obtained at passage 13 (American Type Culture Collection [ATCC], Rockville, Md.) and used within six passages. Cells were maintained at 37° C in a humidified incubator with 5% carbon dioxide. Experiments were conducted using Dulbecco's modified Eagle medium (DMEM) with 4.5 g/L glucose (Life Technologies, Grand Island, N.Y.), 5% fetal bovine serum (Hyclone, Logan, Utah), and 1% antibiotic/antimycotic (Life Technologies, Inc., Gaithersburg, Md.).

Cells were plated onto flasks coated with either laminin (5 $\mu g/cm^2$) or collagen I (10 $\mu g/cm^2$) (Collaborative Research, Bedford, Mass.). For Northern blot experiments, 2×10^6 cells were plated onto 175 cm^2 flasks and for Western blot experiments, 1×10^6 cells were plated on 75 cm^2 flasks. Cells were allowed 24 hours of growth in media, following which they were incubated in serum-free media to achieve quiescence for 48 hours. Cells were then stimulated with EGF (20 ng/ml) (Boehringer Mannheim Corp., Indianapolis, Ind.). RNA for Northern blot analysis was extracted from cells prior to EGF stimulation (time 0) and 0.5, 1, 2, and 4 hours after EGF stimulation. Protein was extracted from cells prior to EGF stimulation (time 0) and 4, 6, 8, 10, and 18 hours after EGF stimulation. Collagen I was chosen as a matrix control because it is not found in the intestinal basement membrane[20] and does not alter cell growth relative to plastic. Cell populations were preconfluent when harvested.

RNA Isolation and Northern Blot Analysis

Total RNA was extracted from cells using the method of Chirgwin et al.[21] with guanidinium isothiocyanate solution and purified using CsCl density gradient ultracentrifugation. Briefly, cells were washed in D-phosphate-buffered saline, lysed in 4 mol/L guanidinium isothiocyanate, brought to a 2.4 mol/L CsCl concentration, and centrifuged through a 5.7 mol/L CsCl cushion at 150,000 *g* for 24 hours at 20° C. The RNA pellet was dissolved in Tris-HCl (pH 7.5), 1 mmol/L EDTA, 5% sodium laurylsarcosine, and 5% phenol. Purified RNA was precipitated with the addition of 0.1 volume of 3 mol/L sodium acetate and 2.5 volumes of ethanol. RNA was then dissolved in water and concentrations were estimated using an ultraviolet spectrophotometer.

Ten micrograms of RNA from each sample was loaded into a 1.2% agarose gel with 3% formaldehyde, separated by electrophoresis, and transferred by blotting to a nitrocellulose filter. Blots were prehybridized for 24 hours at 42° C with 5× Denhardt's solution–5× standard salmon sperm DNA. cDNA probes for glyceraldehyde phosphate dehydrogenase (GAPDH) (ATCC No. 57090) and c-*jun* (ATCC No. 63026) were labeled with [α-^{32}P]dCTP using a standard nick translation procedure as per manufacturer's instructions (New England Nuclear, Boston, Mass.). Blots were hybridized overnight at 42° C in the prehybridization solution containing 10% dextran sulfate and the labeled cDNA probe. Blots were washed with two changes of 1× standard sodium cit-

rate (SSC)–0.1% sodium dodecyl sulfate (SDS) followed with a final wash in 0.25× SSC–0.1% SDS. Filters were autoradiographed using intensifying screens at −70° C. Relative levels of mRNA expression were determined using an optical densitometer and c-*jun* levels were normalized using GAPDH values. Three Northern blots were performed of which one representative blot is shown.

Western Blot Analysis

Cells were scraped from flasks and protein extracted using a solution containing Na_2HPO_4, NaCl, Triton X-100, sodium deoxycholate, SDS, azide, and NaF. The suspension was sonicated and protein separated by centrifugation at 12,000 *g* for 10 minutes. Protein levels were quantitated using the Pierce BCA assay kit (Pierce Chemical Co., Rockford, Ill.) as per manufacturer's instructions. Five micrograms of protein from each sample was loaded into a gel, separated using SDS-PAGE, and electrophoretically transferred to a nitrocellulose membrane with 80 volts for 1 hour in transfer buffer containing 192 mmol/L glycine, 25 mmol/L Tris, 0.01% SDS, and 20% methanol. Blots were blocked with TBS-T (10 mmol/L Tris-HCl, 150 mmol/L NaCl, and 0.1% Tween) plus 5% nonfat dry milk and then incubated with the primary antibody to either p21 (Oncogene, Cambridge, Mass.) or p27 (Transduction Labs, Lexington, Ky.) in TBS-T for 1 hour at room temperature. Blots were washed and incubated with a secondary antibody conjugated with peroxidase followed by a final wash with TBS-T. Chemiluminescent detection was achieved by saturating the blot with ECL reagent (Amersham, Arlington Heights, Ill.) and exposing the blot to Hyperfilm (Amersham). Three Western blots were performed for each experiment. A representative blot for p21 and p27 is shown.

RESULTS

Fig. 1 shows the results of a typical experiment on the effect of extracellular matrix on c-*jun* mRNA levels in synchronized IEC-6 cells before and with EGF stimulation. EGF stimulation of quiescent IEC-6 cells induces c-*jun* expression in cells grown either on collagen I or laminin. c-*jun* Expression is diminished in cells grown on laminin relative to those cells cultured on collagen I after 48 hours of serum starvation. Following EGF stimulation, cells cultured on laminin show a blunted response to EGF with less c-*jun* expression than cells grown on collagen I. Fig. 2 shows optical densitometry measurements of c-*jun* expression values normalized using GAPDH.

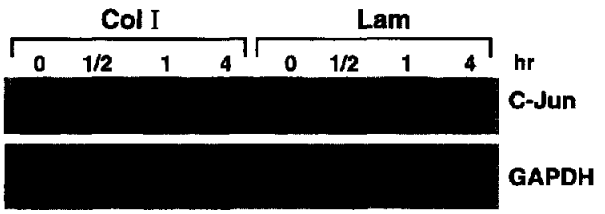

Fig. 1. IEC-6 cells cultured on collagen I *(Col I)* or laminin *(Lam)* for 24 hours in DMEM with serum followed by 48 hours of serum-free culture. Cells were stimulated with EGF and RNA extracted before EGF stimulation *(0)* and 0.5, 1, and 4 hours after EGF stimulation. c-*jun* mRNA levels are shown with corresponding glyceraldehyde phosphate dehydrogenase *(GAPDH)*.

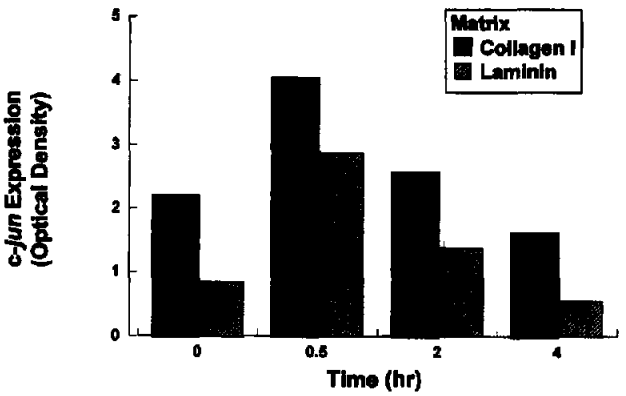

Fig. 2. Arbitrary optical densitometery measurements of mRNA from quiescent IEC-6 cells cultured on laminin or collagen I before *(0)* and 0.5, 2, and 4 hours after stimulation with EGF. Results are normalized to GAPDH.

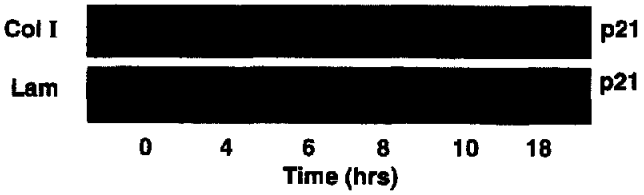

Fig. 3. Western blot of p21 from synchronized IEC-6 cells cultured on collagen I *(Col I)* or laminin *(Lam)* before (0) and 4, 6, 8, 10, and 18 hours after stimulation with EGF.

The effect of p21 expression in cells cultured on laminin as compared to collagen I is shown in Fig. 3. Expression of p21 by Western blot is unchanged be-

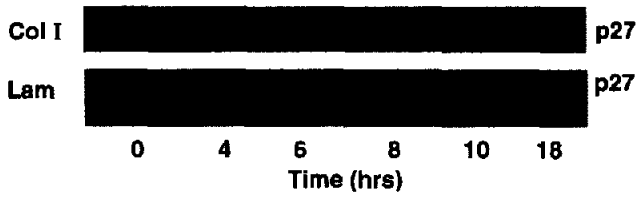

Fig. 4. Protein expression of p27 in quiescent IEC-6 cells grown on collagen I *(Col I)* or laminin *(Lam)* before *(0)* and 4, 6, 8, 10, and 18 hours after EGF stimulation.

tween the collagen I and laminin groups. This is true for quiescent cells at time 0 prior to EGF stimulation as well as the time points out to 18 hours following EGF stimulation.

Also observed is no difference in p27 expression (Fig. 4) between IEC-6 cells cultured on laminin vs. those cells cultured on collagen I. Both quiescent enterocytes and those stimulated with EGF fail to show a difference in p27 levels on either of the two different basement membrane components.

DISCUSSION

IEC-6 enterocytes respond to EGF stimulation with increased expression of the early immediate genes c-*jun*, *junB*, and c-*myc*.[22] We demonstrate in this study that growth of IEC-6 cells on laminin blunts the response of c-*jun* to EGF stimulation. Without induction of c-*jun* expression, enterocytes are unable to enter the S phase and proliferation cannot take place. These data reveal that a matrix component found in the basement membrane can alter the enterocyte's proliferating potential in response to the mitogens present in the crypt-villus milieu.

These results reveal that this unresponsiveness occurs early in the cell cycle. Cell cycle progression also requires a number of nuclear components including the CDKs. In general, the expression of CDKs remains relatively constant throughout the cell cycle,[23] although CDK4 levels have been shown to decrease with exposure to TGF-β.[24] CDK2 and CDK4 protein levels have also been found to be decreased in density-arrested postconfluent Caco-2 cells.[25] Cyclin, CDK, PCNA, and p21 together form the active catalytic unit necessary for cell cycle progression.[26] When p21 expression is increased, it serves as a CDK inhibitor and cell cycle progression is inhibited.[15] Thus it is the stoichiometric relationship in p21 levels that is important in regulating CDK activity.[27] TGF-β growth inhibition has been associated with increased p21 expression in enterocytes.[28]

The CDK inhibitor p27 is structurally similar to p21 and serves to prevent CDK activation and the kinase activity of cyclin-CDK complexes. Levels of p27 mRNA have been found to be the same in both proliferating and contact-inhibited Mv1Lu cells. p27 levels also do not change when contact-inhibited cells are released and allowed to proliferate.[29] p27 protein levels have also been shown not to change with serum stimulation of quiescent Swiss 3T3 cells.[30] Consistent with a posttranslational mechanism for p27 regulation of cellular proliferation, Morisaki et al.[31] have shown that p27 phosphorylation is cell cycle dependent. The inhibitory effect of p27 may be blocked by cyclin D/CDK complex sequestration of p27, thereby allowing CDK 2 activation.[32] Hence cell cycle factors may be either increased, decreased, or unchanged in absolute amount. As p21 and p27 are cell cycle inhibitors, our preliminary investigation questioned whether absolute levels of these CDK inhibitors would be regulated by laminin.

In this study we have shown that both p21 and p27 protein expression remains unchanged in enterocytes cultured on laminin as compared to control. These findings may suggest that either these pathways of cell cycle regulation are unrelated to the regulatory mechanism employed by laminin-induced growth inhibition or that the levels do not reflect the functional effect of these proteins. Beauchamp et al.[33] have demonstrated an absolute decrease in p21 levels in IEC-6 cells that have undergone density growth arrest but an increase in the association of p21 with cyclin D1/CDK 4 consistent with the stoichiometric mechanism of p21.

Our finding that laminin does not alter p27 expression is consistent with other studies that have identified stable levels of this factor during growth arrest. Further studies will be required to determine if inhibition of cyclin by p27 is operative in laminin-induced growth inhibition.

Alternatively, the failure of laminin to influence the expression of these CDK inhibitors may allow

for different regulatory systems to influence the enterocyte by different mechanisms. TGF-β has been shown to inhibit enterocyte proliferation through a decrease in cyclin D1 expression[34] but without changes in transcription of the early immediate genes c-*myc*, *jun*-B, or *zif*268.[35] This contrasts with our findings where laminin does inhibit c-*jun* expression, thereby allowing two different modulators of enterocyte growth to regulate cell cycle progression through separate pathways. Matrix may have a dominant effect on mitogen growth factor receptor expression and signaling, whereas other systems such as TGF-β pathways may more directly influence cell cycle progression.

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Cumulative Risk of Developing Polyps or Malignancy at the Ileal Pouch–Anal Anastomosis in Patients With Familial Adenomatous Polyposis

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Restorative proctocolectomy with an ileal pouch–anal anastomosis is performed in an increasing number of patients with familial adenomatous polyposis (FAP). Two techniques are currently used to construct an ileal pouch–anal anastomosis: (1) a double-stapled anastomosis between the pouch and the anal canal and (2) mucosectomy with a hand-sewn ileoanal anastomosis at the dentate line. Although this procedure is thought to abolish the risk of colorectal adenoma, an increasing number of case reports have been published concerning the development of adenoma at the anastomotic site. The purpose of this study was to evaluate the overall cumulative risk of developing adenomatous polyps after ileal pouch–anal anastomosis and to compare the cumulative risk after either anastomotic technique. A total of 126 consecutive FAP patients undergoing a restorative proctocolectomy were identified from polyposis registries in The Netherlands, Denmark, Italy, Germany, and New York. Life-table analysis was used to calculate the cumulative risk of developing polyps in 97 patients with at least 1 year of endoscopic follow-up (median 66 months, range 12 to 188 months). A double-stapled anastomosis was used in 35 patients, whereas in 62 patients a hand-sewn anastomosis with a mucosectomy was performed. In 13 patients polyps developed at the anastomotic site, four with severe and four with moderate dysplasia. None of the patients developed a carcinoma at the anastomotic site. The cumulative risk of developing a polyp at the anastomotic site was 8% (95% confidence interval 2% to 14%) at 3.5 years and 18% (95% confidence interval 8% to 28%) at 7 years, respectively. The risk of developing a polyp at the anastomotic site within 7 years was 31% for patients with a double-stapled vs. 10% for patients with a hand-sewn anastomosis with mucosectomy ($P = 0.03$ [log-rank test]). Because FAP patients undergoing a restorative proctocolectomy with either a double-stapled or hand-sewn anastomosis have a substantial risk of developing adenomatous polyps at the anastomotic site, lifelong endoscopic surveillance is mandatory in both groups. (J GASTROINTEST SURG 1999;3:325-330.)

KEY WORDS: Familial adenomatous polyposis, adenomatous polyps, ileal pouch–anal anastomosis, anastomosis, surgical, intestinal mucosa

Familial adenomatous polyposis (FAP) is an autosomal dominantly inherited disease caused by mutations in the adenomatous polyposis coli (APC) gene on chromosome 5. The disease is characterized by hundreds of adenomatous polyps throughout the colon and rectum. Most patients develop polyps during the second and third decade of life. Without timely surgical intervention, virtually all patients will develop colorectal cancer, often by the fourth decade.[1]

Until recently, the most frequently used procedure for patients with FAP was a colectomy and ileorectal anastomosis.[2] This is a technically simple procedure to

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perform, with a low complication rate and good functional outcome.[3] However, there are some disadvantages to this procedure because the cumulative risk of cancer evolving in the rectal stump is reported to be 15% after 25 years of follow-up,[4-7] and more than 40% of patients with an ileorectal anastomosis will need a secondary proctocolectomy after 20 years because of uncontrollable polyps.[7] The alternative to an ileorectal anastomosis in patients with FAP is a proctocolectomy with an ileal pouch–anal anastomosis (IPAA). This procedure provides removal of virtually all the rectal mucosa, thereby reducing the risk of recurrence of adenomatous polyps or adenocarcinoma. The IPAA can be constructed with either hand sutures or a stapling device. The hand-sewn anastomosis is generally preceded by a mucosectomy down to the dentate line to eliminate all mucosa at risk. The double-stapling technique can potentially leave rectal mucosa behind but reduces operating time and is reported to achieve better functional results.[8]

Although an IPAA is thought to abolish the risk of colorectal adenoma, an increasing number of case reports have been published on adenomatous polyps or carcinoma developing at or distal to the IPAA.[9-13] These findings raise concern about the at-risk mucosa left behind at the anastomotic site. The present study was therefore undertaken to evaluate the risk of developing adenomas or cancer at the anastomosis after IPAA. In addition, the risk was compared between patients with a double-stapled anastomosis and those with a hand-sewn anastomosis with a mucosectomy. The possible impact of a coexistent colorectal carcinoma at the time of the proctocolectomy on the recurrence of polyps was also assessed.

PATIENTS AND METHODS

The following six centers or registries where IPAA procedures for FAP are regularly performed and recorded agreed to participate in the study: Academic Medical Center Amsterdam, University Hospital Rotterdam, University Hospital Nijmegen, Leiden University Medical Center, Memorial Sloan-Kettering Cancer Center, and the Italian and Danish polyposis registries. At each center or registry, all FAP patients who had undergone an IPAA were selected. A questionnaire was mailed to all participants requesting the following information: date of IPAA, presence of synchronous cancer at the time of surgery, type of anastomotic procedure, postoperative complications, type of follow-up examination (endoscopic or digital), presence of polyps (location, grade of dysplasia) or cancer, interval between IPAA and diagnosis of adenomas, treatment, need for pouch excision, and date of last follow-up examination.

Statistical Analysis

Proportions of events were compared by means of chi-square tests with P values <0.05 considered statistically significant. Cumulative incidence of polyps was analyzed by Kaplan-Meier survival analysis, and differences between the two anastomotic techniques were compared by log-rank test. All reported P values are two tailed. Cox regression analysis was used to quantify the effect of the presence of synchronous cancer at the time of surgery and the two different anastomotic techniques on the recurrence of polyps. Possible confounding effects of the presence of synchronous cancer at the time of surgery and the two anastomotic techniques on recurrence of adenomas at the anastomotic site was studied using a Cox proportional hazard model.

RESULTS

Between 1981 and 1997, 126 consecutive FAP patients underwent a restorative proctocolectomy with IPAA at six different centers or registries. In 97 patients follow-up examinations were performed endoscopically for at least 1 year (median 66 months, range 12 to 188 months), whereas 29 patients were screened by means of rectal digital examination or were surveyed for less than 1 year. In the endoscopically surveyed group, there were 41 women and 56 men with a median age of 35 years (range 16 to 60 years) and a median age at the time of surgery of 30 years (range 10 to 55 years). Of the 97 endoscopically surveyed patients, 62 had a hand-sewn anastomosis with mucosectomy and 35 patients had a double-stapled anastomosis. The number of control endoscopies per year varied among the different study participants. The median number was one endoscopy per year, ranging from three time per year to once every 2 years.

In five patients a pouch excision followed by construction of a permanent ileostomy was performed. A permanent ileostomy was constructed in one patient because of recurrent distal obstruction due to a nonresectable desmoid tumor. Two patients had a permanent ileostomy constructed because of anastomotic pouch complications. In one patient the pouch had to be excised because of recurrent cancer, and in one patient the pouch was removed because of unmanageable polyp formation in the pouch. The median interval between restorative proctocolectomy and pouch excision was 17 months (range 2 to 131 months). The date of pouch excision was taken as date of last follow-up in these five patients.

With a median follow-up of 78 months (range 25 to 137 months), 13 of the 97 endoscopically surveyed patients developed adenomatous polyps at the anasto-

motic site. The interval between IPAA and diagnosis and the histologic stage of adenomas are outlined in Table I. The cumulative risk of developing an adenoma at the anastomotic site was 8% (95% confidence interval [CI] = 2% to 14%) at 3.5 years and 18% (95% CI = 8% to 28%) at 7 years, respectively (Fig. 1).

Six of 13 patients who developed polyps at the anastomotic site had a hand-sewn anastomosis and mucosectomy, whereas the other seven patients had a double-stapled anastomosis. The risk of developing adenomatous polyps at the anastomotic site within 7 years was 10% for patients with a hand-sewn anastomosis and mucosectomy. This is significantly lower than the risk for patients with a double-stapled anastomosis, which is 31% ($P = 0.03$ [log-rank test]) (Fig. 2).

The relative risk of developing polyps at the double-stapled anastomotic site was 1.9 times (95% CI 1.2 to 3.2) that of patients with a hand-sewn anasto-

Table I. Presence of adenomatous polyps in 13 of 97 FAP patients at ileal pouch–anal anastomosis

Patient	Age (yr)	Sex	Interval* (mo)	Histologic findings	Type of anastomosis	Pouch excised (reason)
1	23	F	41	Moderate dysplasia	DS	–
2	33	M	23	Severe dysplasia	DS	–
3	41	M	40	Moderate dysplasia	DS	–
4	25	F	55	Severe dysplasia	DS	–
5	25	F	25	Moderate dysplasia	DS	–
6	34	M	52	Mild dysplasia	DS	–
7	60	F	43	Mild dysplasia	DS	–
8	35	M	74	Severe dysplasia	HS	–
9	49	F	81	Mild dysplasia	HS	–
10	47	M	95	Mild dysplasia	HS	–
11	56	M	131	Moderate dysplasia	HS	+ (pouch dysfunction)
12	57	F	24	Severe dysplasia	HS	+ (multiple polyps in the pouch)
13	22	M	15	Mild dysplasia	HS	–

FAP = familial adenomatous polyposis; IPAA = ileal pouch–anal anastomosis; HS = hand sewn; DS = double stapled.
*Time from IPAA to development of adenomatous polyps.

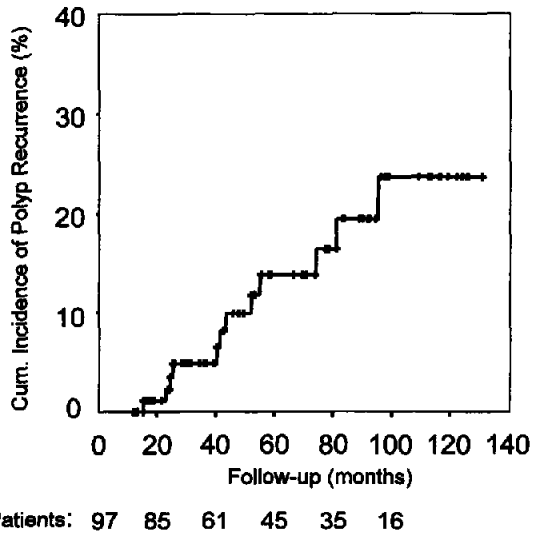

Fig. 1. Hazard curve for the cumulative incidence of polyp recurrence at the anastomotic site in FAP patients who underwent proctocolectomy with IPAA. + = Censored cases; numbers shown at the bottom of the graph indicate patients at risk.

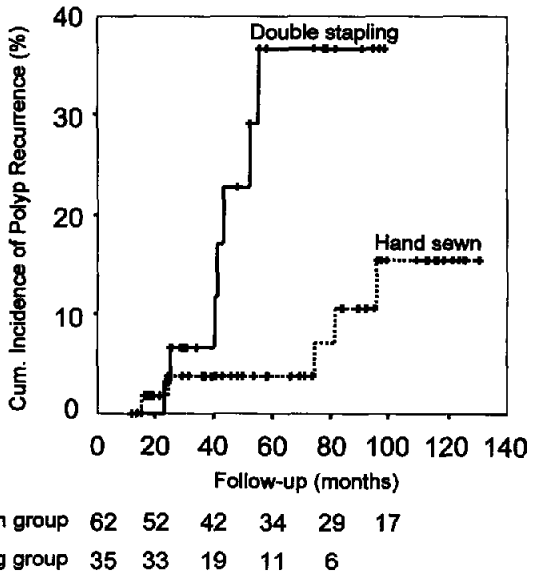

Fig. 2. Hazard curves for the cumulative incidence of polyp recurrence at the anastomotic site in FAP patients who underwent proctocolectomy with a hand-sewn IPAA and mucosectomy *(dotted line)* and in FAP patients who underwent proctocolectomy with a double-stapled IPAA *(solid line)* (log rank: $P = 0.03$). + = Censored cases; numbers shown at the bottom of the graph indicate patients at risk.

mosis and mucosectomy ($P < 0.01$ [univariate Cox regression]).

A coexisting colorectal carcinoma at the time of surgery was present in 20 patients; 11 of them had a hand-sewn anastomosis with a mucosectomy and nine had a double-stapled anastomosis. The relative risk (RR) for developing polyps at the anastomotic site in case of a synchronous colorectal carcinoma was not increased (RR = 1.2 [95% CI = 0.7 to 2.1]). The inclusion of the variable "presence of synchronous cancer at the time of surgery" did not change the relative risk for the two different anastomotic techniques in any substantial way.

DISCUSSION

Restorative proctocolectomy with an IPAA, as compared to conventional colectomy and ileorectal anastomosis, has the advantage that theoretically all rectal mucosa at risk is eliminated.[14] The functional results of this procedure are improving as morbidity and the incidence of pouch failure are reported to be decreasing.[15] The incidence of sexual and bladder dysfunction is very low.[16,17] Finally, quality of life is rated by IPAA patients to be satisfying or very satisfying, although this has been assessed by nonvalidated questionnaires.[18-20] These advantages might explain why IPAA is being performed in an increasing number of patients with FAP.

Recent studies have shown that restorative proctocolectomy with an IPAA does not seem to provide a total risk reduction, as illustrated by case reports on adenomatous polyps[9-11] and cancer[12,13] arising at the anastomosis after IPAA. There are some reports indicating that histologically examined anorectal mucosal strippings, taken at the time of the proctocolectomy, contained dysplasia in from 85.7% up to even 100% of cases.[21,22] Two other studies showed the presence of polyps or dysplasia in stapled distal "doughnuts" or biopsies taken just distal to the IPAA in patients who underwent a restorative proctocolectomy for FAP.[10,23] Investigators at the Mayo Clinic reported that small islets of residual rectal mucosa were found buried in the fibrous tissue between the rectal muscular cuff and the ileal serosa in 4 of 26 ileoanal specimens excised on average 17 months after construction of the ileoanal anastomosis.[24] Moreover, Hoehner and Metcalf[12] described an adenocarcinoma arising at a straight ileoanal anastomosis in a 34-year-old woman 20 years after proctocolectomy. Pathologic review of the posterior exenteration specimen revealed an invasive adenocarcinoma arising at the junction of the dentate line. Von Herbay et al.[13] described an adenocarcinoma arising at the anastomotic site in a 33-year-old woman 8 years after a hand-sewn pouch procedure with a mucosectomy. Histopathologic examination of the excised anal canal showed some islets of residual rectal-type mucosa situated between the ileal pouch and the anal transitional zone with a moderately differentiated adenocarcinoma infiltrating into the internal anal sphincter.

Despite this information, the absolute risk estimate for development of polyps or even adenocarcinoma at the anastomotic site is still unknown. The present study showed that patients who underwent a restorative proctocolectomy with an IPAA still have a substantial risk of developing adenomas.

There is little agreement among surgeons as to whether or not a mucosectomy should be performed. Mucosectomy is believed to eliminate the risk for the development of polyps. Nevertheless, it has been reported that small islets of rectal mucosa may remain after a proctocolectomy with a hand-sewn IPAA and a mucosectomy down to the dentate line.[24,25] Furthermore, these mucosal islands remaining between the pouch and the rectal muscular cuff might contribute to postoperative pelvic sepsis.[26] It is also reported that after such a mucosectomy, up to 77.5% of the patients have symptoms of soiling,[27] especially at night.[15] Others report no such difference in the incidence of soiling between hand-sewn and double-stapled techniques.[28,29]

A proctocolectomy with a double-stapled IPAA without a mucosectomy is claimed to be safer and technically less difficult than the hand-sewn technique in that the mucosectomy is omitted, thereby reducing anal canal manipulation and keeping operating time to a minimum. This may result in better postoperative manometric and functional results.[29] In addition, the mucosa of the anal transitional zone just above the dentate line is preserved, which may be important for fine control of continence and the ability to discriminate between gas and stools.[30] However, there is always a substantial risk that rectal mucosa will be retained because of the formation of bilateral "dog ears" at the time of stapling of the anastomosis.[10]

Our findings indicate that after both procedures a substantial risk of polyp formation remains, even if a mucosectomy is performed. Therefore patients who have undergone either one of these surgical procedures require strict surveillance. However, if polyps do recur at the anastomotic site, they can be treated by local excision or an additional local mucosectomy and pouch advancement.[11] Nonetheless, concern remains that islets of mucosa between the pouch and the rectal muscular cuff after a mucosectomy and hand-sewn anastomosis cannot be checked for dysplastic changes.

A history of bowel cancer at the time of the colectomy is one of the significant risk factors for the de-

velopment of rectal carcinoma in the rectal stump in FAP patients who have undergone a rectum-saving procedure with an ileorectal anastomosis.[8] The data presented in this report show that the presence of a synchronous colorectal carcinoma at the time of a restorative proctocolectomy with an IPAA did not significantly influence the relative risk for developing polyps at the anastomotic site.

Although our study is limited because of the varying extent of follow-up, we can conclude that there is a substantial risk for development of polyps at the anastomotic site, which can be partially but not totally reduced by a hand-sewn anastomosis with mucosectomy. All FAP patients who undergo an IPAA procedure, irrespective of the applied surgical technique, should undergo endoscopic IPAA surveillance at regular intervals of at least once a year.

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Discussion

Dr. J. Becker (Boston, Mass.). In the group of patients who had mucosectomy, did you review the histologic specimens to assess whether or not the dissection was begun at or distal to the dentate line?

Dr. P. van Duijvendijk. No. Unfortunately we did not ask for that information since this was a retrospective study. These details were not available for all patients.

Dr. M. Dayton (Salt Lake City, Utah). When we began using the double-stapling technique a few years ago, the concern was polyp recurrence and the eventual development of malignancy, so your study is a very important one. Did you as a group decide where the distalmost portion of the perineal mucosectomy would start? Was it distal to the transition zone? Would you also clarify how you handled both groups of patients when polyps did recur? I noticed that a total of five pouches were excised, but I did not infer that they were excised because of the recurrence of polyps.

Dr. van Duijvendijk. None of the pouches was excised because of polyp formation at the anastomotic site. Concerning the onset of the mucosectomy, that is a very difficult question and it is not up to our group to decide where to start. If we find polyps at the anastomosis after a double-stapled procedure, we find it easy to perform an additional mucosectomy afterward as an outpatient procedure. But I agree that there is a higher risk for development of polyps at the anastomotic site after a double-stapled anastomosis.

Dr. Dayton. So you did not decide in advance whether you had to remove all of the epithelium? I noticed that 8% of your patients who had a mucosectomy developed polyps. That is disturbing if the dissection extended all the way down to the transition zone.

Dr. van Duijvendijk. A report from the Mayo Clinic (Dis Colon Rectum 1987;30:1-5) found that small mucosal islands were left behind between the muscular cuff and the pouch in 4 of 26 excised pouches. You can imagine that if there are mucosal islands between the pouch and the rectal cuff, there can also be small mucosal islands left behind between the transition zone and the point where the pouch begins.

Dr. K. Kelly (Scottsdale, Ariz.). Did all of your patients have polyps in the rectum to start with? If so, a strong case can be made for removing all the rectal mucosa. In patients with polyposis who have no polyps in the rectum and no dysplasia, what should be done? Also, on follow-up were any polyps found in the pouch itself away from the suture line?

Dr. van Duijvendijk. Starting with your last question, we did not ask the centers about polyps in the pouches, so I do not have any information on that. Not all of our patients had multiple polyps in the rectum at the time of the initial procedure, but that is also an item that is fairly difficult to assess in a retrospective study such as this.

Dr. T. Schrock (San Francisco, Calif.). What information do you have concerning the height of the anastomosis above the dentate in the double-stapled anastomoses? Were some of these in fact ileorectal rather than ileoanal anastomoses?

Dr. van Duijvendijk. I cannot answer that question, but these pouch constructions were all performed at specialized centers where several are done every year, so we assumed that all anatomoses were performed at the top of the anal canal.

Dr. L. Way (San Francisco, Calif.). Was there an association between hospital or surgeon and recurrence, suggesting that specific technical features might have influenced the results?

Dr. van Duijvendijk. That is a very interesting question. However, our aim was just to study the recurrence rate and we specifically told all of the participating centers that we did not want to compare them with one another; that is the only reason why we got such good cooperation.