Volumetric Analysis Predicts Hepatic Dysfunction in Patients Undergoing Major Liver Resection

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Liver-enhancing modalities, such as portal vein embolization, are increasingly employed prior to major liver resection to prevent postoperative liver dysfunction. Selection criteria for such techniques are not well described. This study uses CT-based volumetric analysis as a tool to identify patients at highest risk for postoperative hepatic dysfunction. Between July 1999 and December 2000, a total of 126 consecutive patients who were undergoing liver resection for colorectal metastasis and had CT scans at our institution were included in the analysis. Volume of resection was determined by semiautomated contouring of the liver on preoperative volumetrically (helical) acquired CT scans. Hepatic dysfunction was defined as prothrombin time greater than 18 seconds or serum bilirubin level greater than 3 mg/dl. Marginal regression was used to compare the predictive ability of volumetric analysis and the extent of resection. The percentage of liver remaining was closely correlated with increasing prothrombin time and bilirubin level ($P < 0.001$). After trisegmentectomy, 90% of patients with ≤25% of liver remaining developed hepatic dysfunction, compared with none of the patients with more than 25% of liver remaining after trisegmentectomy ($P < 0.0001$). The percentage of liver remaining was more specific in predicting hepatic dysfunction than was the anatomic extent of resection ($P = 0.003$). Male sex nearly doubled the risk of hepatic dysfunction (odds ratio = 1.89, $P = 0.027$), and having ≤25% of liver remaining more than tripled the risk (odds ratio = 3.09, $P < 0.0001$). Hepatic dysfunction and ≤25% of liver remaining were associated with increased complications and length of hospital stay ($P < 0.0001$ and $P = 0.0003$, respectively). Preoperative assessment of future liver volume remaining distinguishes which patients undergoing liver resection will most likely benefit from preoperative liver enhancement techniques such as portal vein embolization. (J GASTROINTEST SURG 2003;7:325–330.)

KEY WORDS: Hepatic dysfunction, liver resection, metastasis

Major liver resection for metastatic disease is being performed with increasing frequency. Perioperative mortality in noncirrhotic patients undergoing resection for colorectal metastasis is less than 5% in major centers.[1–4] As the safety of hepatic resections improves, more patients with extensive disease requiring extended lobectomy for complete resection are considered operative candidates. Although the mortality rate is low, patients undergoing large resections continue to be at increased risk for postoperative liver dysfunction and complications. In an attempt to prevent postoperative liver dysfunction, many centers are now employing preoperative liver-enhancing techniques such as portal vein embolization.[5–8] Although this technique has been shown to result in hypertrophy of the nonembolized lobe,[9] the selection criteria for this technique have not been well described.

Exactly how much remaining liver is necessary to minimize the likelihood of liver dysfunction with normal hepatic parenchyma postoperatively is unclear. Some centers have demonstrated that resection of as much as 75% to 80% of the liver can be performed safely.[1,2] Others recommend that patients with normal liver parenchyma undergo portal vein embolization if the planned resection is either a trisegmentectomy or resection of 75% or more.[10] The goal of this study

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was to employ CT-based volumetric analysis as a tool to identify those patients with colorectal hepatic metastasis, and otherwise normal liver parenchyma, at highest risk for postoperative liver dysfunction. This would, in turn, help to predict which patients would most likely benefit from preoperative liver-enhancing modalities such as portal vein embolization.

METHODS

From a prospective database maintained by the Department of Surgery, 186 patients undergoing hepatic resection between July 1999 and December 2000 for colorectal metastasis were identified. Of these, 126 underwent preoperative CT scanning at our institution and were included in the analysis. All CT scanning was performed with a helical scanner, and the CT data were transferred to an independent workstation for assessment. Retrospectively, total liver volume and the volume of liver resected were calculated. Total liver volume and volume of resection were determined by semiautomated contouring of the liver on the preoperative CT scans. This was performed on serial transverse scans at 1.0 cm intervals including the entire liver. On each slice, the total liver was outlined and the sum of the slices calculated by integrated software techniques using density threshold. This was repeated for the volume of liver to be resected. The tumor volume was determined and subtracted from the calculation. The difference between total liver volume and resected volume was considered the volume of liver remaining. The percentage of liver remaining was determined for each patient. The actual weight of the resected liver was not routinely documented, and therefore no correlation was made with estimated volume. Anatomic resection was noted to be trisegmentectomy, lobectomy, or segmentectomy. Extensive resection was defined as having $\leq$25% of liver remaining.

Peak bilirubin levels and prothrombin times were recorded for all patients within 30 days of surgery. Results of preoperative liver functions tests were normal in all patients, and all were considered to have normal liver parenchyma. Postoperative hepatic dysfunction was considered when the prothrombin time was greater than 18 seconds or serum bilirubin was greater than 3 mg/dl. These parameters were established on the basis of previous literature suggesting an association with these complications.[9,10] Patients were not routinely given fresh-frozen plasma for extended resections. All in-hospital and 30-day complications were recorded. These were subsequently graded on the basis of the severity of complication as evaluated at Memorial Sloan-Kettering Cancer Center. Briefly, this system places complications into five categories with the more severe complications being graded III to V. The grading system is as follows: grade I = requiring treatment with oral antibiotics; grade II = requiring intravenous treatment; grade III = requiring operative or radiologic intervention; grade IV = resulting in significant chronic disability; grade V = death as a result of the complication. Grades III to V were considered major complications, and grades I and II were considered minor. Length of hospital stay was recorded as postoperative days in the hospital prior to discharge.

Statistical Analysis

Specificity, sensitivity, positive predictive value, and negative predictive value of extended lobectomy and remnant liver volume as predictors of hepatic dysfunction are estimated by the sample proportions and compared using marginal regression.[11] Confidence intervals were computed using exact methods.[12] Comparisons within the group of patients who did and did not undergo trisegmentectomy were performed using an exact binomial test. Fisher's exact test was used to investigate the association between complications, hepatic dysfunction, and $\leq$25% of liver remaining. The Wilcoxon test was used for length of stay, hepatic dysfunction, and $\leq$25% of liver remaining. Logistic regression was used for multivariate modeling of hepatic dysfunction.

RESULTS

Of the 126 patients included in the study, there were 59 men and 67 women whose median age was 62 years (range 28 to 84 years). The most common procedure was less than a lobectomy (n = 49) followed by right lobectomy (n = 34), right trisegmentectomy (n = 30), left lobectomy (n = 10), and left trisegmentectomy (n = 3). The median volume of liver remaining for the group was 850 ml (range 190 to 2170 ml). The median percentage of liver remaining was 52% (range 12% to 95%). A trisegmentectomy was performed in 33 patients (26%), and 20 patients (16%) had less than 25% of liver remaining after resection.

Hepatic dysfunction, defined as prothrombin time greater than 18 seconds or serum bilirubin level greater than 3 mg/dl occurred in 38 patients (30%). Some patients had elevations in prothrombin time or bilirubin level, but not both. Prothrombin time was greater than 18 seconds in 14 patients (11%), and the bilirubin level was greater than 3 mg/dl in 33 pa-

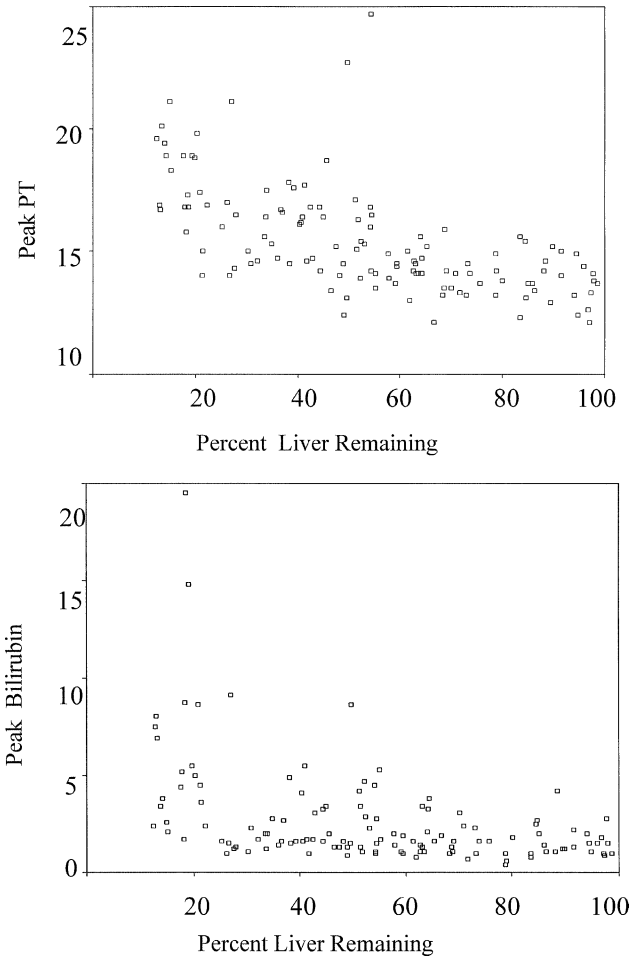

Fig. 1. Percentage of liver remaining after resection as it correlates with (**A**) peak prothrombin time (*PT*) ($P < .001$) and (**B**) peak bilirubin level ($P < 0.001$).

tients (26%). Volumetric analysis of the percentage of liver remaining after resection correlated with the peak bilirubin level ($P < 0.001$) and peak prothrombin time ($P < 0.001$) measured postoperatively (Fig. 1). The percentage of liver remaining also correlated with hepatic dysfunction, as shown in Fig. 2.

Of the 33 patients who underwent an extended lobectomy, 17 (52%) developed postoperative hepatic dysfunction. Of the 20 patients with ≤25% of liver remaining, 18 (90%) developed hepatic dysfunction. This is shown in Table 1. Table 2 outlines the sensitivity, specificity, positive predictive value, and negative predictive value for hepatic dysfunction based on either trisegmentectomy or with ≤25% of liver remaining. The sensitivities are similar, but volumetric analysis estimating ≤25% of liver remaining is significantly more specific in predicting hepatic dysfunction than trisegmentectomy alone. None of the patients undergoing trisegmentectomy with >25% of liver remaining developed postoperative hepatic dysfunction, compared to 90% of those with ≤25% of liver remaining after trisegmentectomy ($P < 0.0001$). This is outlined in Table 3.

Using a logistic regression model, sex and ≤25% of liver remaining were identified as independent predictors of hepatic dysfunction. Blood loss, age, and operative time were not significant. Male sex nearly doubled the risk of hepatic dysfunction (odds ratio = 1.89, $P = 0.027$) and having ≤25% of liver remaining more than tripled the risk (odds ratio = 3.09, $P < 0.0001$).

Patients who developed hepatic dysfunction had a higher incidence of perioperative complications (61%) and severe complications (39%) than those who did not have postoperative hepatic dysfunction (25% and 10%, respectively; $P < 0.0001$). Major resection with ≤25% of liver remaining was significantly associated with postoperative complications and major complications ($P = 0.01$). Subsequently, the length of hospital stay was longer in patients with hepatic dysfunction and those with ≤25% of liver remaining. These are outlined in Table 4.

DISCUSSION

This retrospective study examines the utility of preoperative volumetric analysis in predicting liver

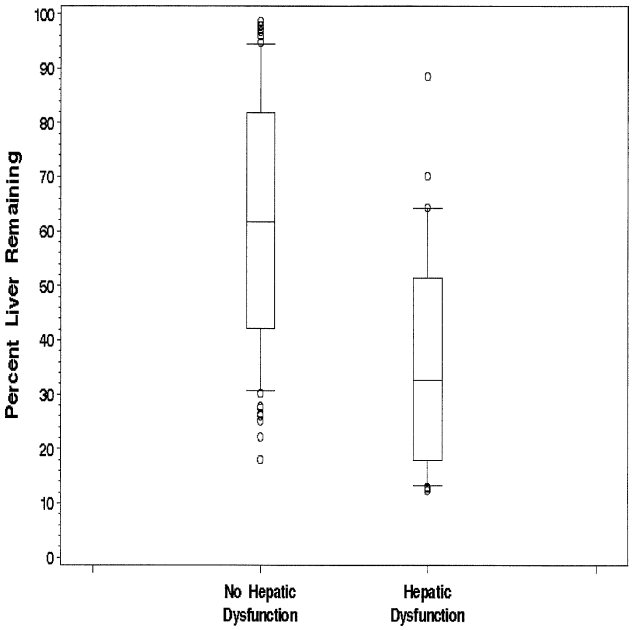

Fig. 2. Box plot of liver remaining vs. hepatic dysfunction. Boxes represent lower and upper quartiles with the median as the horizontal line in the middle. Whiskers at both ends are tenth and ninetieth percentiles with outlying data represented as circles.

Table 1. Prediction of hepatic dysfunction by trisegmentectomy and by liver remaining

No hepatic dysfunction				Hepatic dysfunction			
	>25% LR	≤25% LR	Total		>25% LR	≤25% LR	Total
<Triseg	72	0	72	<Triseg	20	1	21
Triseg	14	2	16	Triseg	0	17	17
TOTAL	86	2	88	TOTAL	20	18	38

LR = liver remaining; Triseg = trisegmentectomy.

dysfunction after resection in patients with normal liver parenchyma. We demonstrated that the percentage of liver remaining as measured on 126 preoperative CT scans significantly correlates with the postoperative peak bilirubin level and prothrombin time. These observations are similar to those of Vauthey et al.[10] However, their review of 20 patients included those with primary liver tumors as well as metastatic colorectal cancer, whereas the current study is specific for metastatic disease, and all patients had normal liver parenchyma.

Volumetric analysis has been described as a tool to predict postoperative liver failure and death in patients with hepatocellular carcinoma.[13] In the current study there were no deaths and no liver failures, but 20 patients undergoing trisegmentectomy with ≤25% of liver remaining were at considerable risk for postoperative hepatic dysfunction, defined as a bilirubin level greater than 3 mg/dl or a prothrombin time greater than 18 seconds. In addition, patients with ≤25% of liver remaining had significantly more postoperative complications, major complications, and subsequently a longer hospital stay.

Because extensive liver resection is being used with increasing frequency for patients with metastatic colorectal cancer, liver-enhancing modalities such as portal vein embolization have been described as an effort to minimize postoperative hepatic dysfunction and complications.[5–8] The current data support efforts to reduce the incidence of postoperative hepatic dysfunction, because the 38 patients in this study with elevated prothrombin times and bilirubin levels experienced significantly more postoperative complications, more major complications, and an increased length of hospital stay. The role of portal vein embolization prior to resection is still under scrutiny, but preliminary results are encouraging, as some centers have documented significant hypertrophy of the nonembolized lobe.[7,8] Others claim that this technique allows select patients with extensive disease in whom complete resection was previously thought to be unsafe to become candidates for hepatic resection.[5] Regardless, guidelines for identifying patients most likely to benefit from this invasive technique have not yet been clearly defined.

The first step in establishing such guidelines is the identification of parameters that predict liver failure without portal vein embolization. The current data suggest that preoperative volumetric analysis of the percentage of liver remaining after resection is 98% specific in predicting hepatic dysfunction. This method of identifying the percentage of normal remaining parenchyma is more specific in predicting hepatic dysfunction than extensive resection alone. More precisely, patients undergoing trisegmentectomy with ≤25% of liver remaining demonstrated a 90% incidence of developing hepatic dysfunction. None of the patients undergoing trisegmentectomy with more than 25% of liver remaining had postoperative hepatic dysfunction. The former group would therefore likely benefit from portal vein embolization, whereas the latter group would not.

Table 2. Parameters to predict hepatic dysfunction based on either ≤25% of liver remaining or trisegmentectomy (95% confidence intervals are given in parentheses)

	Specificity	Sensitivity	PPV	NPV
≤25% LR	86/88	18/38	18/20	85/105
	98%	47%	90%	81%
	(92%, 100%)	(31%, 64%)	(68%, 99%)	(72%, 88%)
Trisegmentectomy	72/88	17/38	17/33	72/93
	82%	43%	52%	77%
	(72%, 89%)	(29%, 62%)	(34%, 69%)	(68%, 85%)
P value*	0.003	NS	0.02	NS

LR = liver remaining; PPV = positive predictive value; NPV = negative predictive value; NS = not significant.
*Wald test from a marginal regression model for paired binary data.

Table 3. Comparison of postoperative hepatic dysfunction after trisegmentectomy

Trisegmentectomy	N	PT >18 sec	Bili >3 mg/dl	PT >18 sec or Bili >3 mg/dl
≤25% LR	20	50%	75%	90%
>25% LR	14	0%	0%	0%
P value*		0.002	0.0001	<0.0001

PT = prothrombin time; Bili = bilirubin.
*Binomial test with exact reference distribution.

Although the high positive predictive value of the volumetric analysis is encouraging, the sensitivity in predicting hepatic dysfunction is disappointing. Therefore volumetric analysis is limited because it will not capture all of the patients who will develop liver dysfunction. Performing embolization in a patient who will be expected to have ≤25% of liver remaining is likely to be beneficial, but some patients will continue to have postoperative hepatic dysfunction despite what appears to be adequate remaining liver. In this study, 20 patients undergoing resection with more than 25% of liver remaining still developed hepatic dysfunction, as did a handful of patients with more than 40% of liver remaining. No intraoperative variables were identified as an explanation for this; however, it is clear that other factors must play a major role in determining hepatic recovery after resection. This may be due to other parenchymal, metabolic, or vascular factors, or it may be the result of a more technically challenging resection. In our multivariate analysis, only ≤25% of liver remaining and male sex were associated with hepatic dysfunction, whereas the duration of the operation and the estimated blood loss were not. It is unclear why male sex was associated with hepatic dysfunction, but this is consistent with our recent findings in a review of more than 1800 liver resections for all types of disease.[14] This suggests that more men may be candidates for adjunctive therapy with a greater percentage of liver remaining, as compared to women.

CONCLUSION

Based on these data we believe that patients undergoing trisegmentectomy or other extensive liver resection for colorectal metastasis should be analyzed volumetrically in a prospective fashion. Major anatomic resection alone is not a predictor of postoperative hepatic dysfunction or complications. However, patients with normal parenchyma and an anticipated liver remnant of less than 25% of total liver volume are at significant risk for hepatic dysfunction and postoperative complications. These patients represent the ideal candidates for trials of preoperative liver-enhancing techniques such as portal vein embolization.

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Table 4. Complications and length of hospital stay based on hepatic dysfunction and percentage of liver remaining

	N	Complications	Grade III-V complications*	Median length of hospital stay
Hepatic dysfunction	38	23/38 (61%)	15/38 (39%)	9 days
No hepatic dysfunction	88	22/88 (25%)	9/88 (10%)	7 days
P value†		<0.0001	<0.0001	0.0003
≤25% LR	21	12/20 (60%)	8/20 (40%)	8 days
>25% LR	105	33/106 (31%)	16/106 (15%)	7 days
P value†		0.01	0.01	0.005

LR = liver remaining.
*Grade III = requiring operative or interventional radiologic treatment; grade IV = resulting in chronic disability; grade V = death due to complication.
†Binomial test with exact reference distribution.

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Discussion

Dr. G.V. Aranha (Maywood, IL): This was a very nice presentation. Were you able to correlate your preoperative assessment of normal liver that was remaining with an intraoperative biopsy of the remaining segment?

Dr. M. Shoup: We went back and looked at the results of all the pathologic examinations of the resected specimens and found that none showed cirrhosis or fibrosis. Of the 20 patients whose liver function tests were abnormal postoperatively who did not have less than 25% of the liver remaining, only four had steatosis. So, at this point, we really do not have a good reason why some patients develop liver dysfunction and others do not, if they still have an adequate amount of liver remaining. To answer this question, we are currently analyzing all of the normal remaining liver specimens with tissue array techniques. Hopefully this will shed some light on the matter.

Dr. G.G. Tsiotos (Athens, Greece): Based on this work, the next question that comes to mind—and it is an intriguing one—is, have you duplicated the same quantitative method in patients with hepatocellular carcinoma, especially the subgroup with Child-Pugh class C liver function, at your institution?

Dr. Shoup: We have not, but others have, looking at hepatocellular carcinoma, and I think this is very easily duplicated. The technique we use in our studies is simple, but there is no question that it is time consuming, and if we were to begin this study today, I would recommend starting over using the more advanced CT scans that we now have that are three-dimensional images; it makes the whole process much easier and quicker.

Dr. A. Sauvanet (Clichy, France): In the patients who developed postoperative liver dysfunction, did you analyze the influence of preoperative chemotherapy?

Dr. Shoup: No, we did not look, but that is a good point. We are currently going back, as I said, and looking at the liver parenchyma in those patients, as well.

Dr. R. Deshpande (London, UK): When you do volumetric analysis, you are looking for functional liver mass. Do you discount the amount of tumor mass as a nonfunctional liver mass? Second, in patients in whom you expect less than 25% of the liver to remain, do you perform a preoperative biopsy, not just to rule out steatosis, but also to rule out other subclinical conditions or disorders that could lead to "small-for-size" syndrome postoperatively?

Dr. Shoup: To answer your first question, we did factor in tumor volume and we subtracted it from our analysis. When we considered the percentage of normal liver remaining, that is exactly what it was. We did not consider tumor volume as normal liver parenchyma, but subtracted that out ahead of time. With regard to the second question, we do not routinely perform biopsies of the liver preoperatively. These patients were all thought to have normal liver parenchyma simply because they had normal liver function tests, and their underlying disease was metastatic colorectal cancer, not hepatocellular carcinoma. At this time we do not carry out any other functional studies, either preoperatively or intraoperatively.

Dr. T.M. Young-Fadok (Rochester, MN): You used the remaining percentage of liver as a predictive factor for determining how patients did, but it appeared that you actually picked your percentage ahead of time, as either less than 25% or more than 25%. Can you explain your rationale for looking at it as an either/or variable rather than as a continuous factor in your multivariate analysis?

Dr. Shoup: We originally looked at it as a continuous variable, and because of that it seemed that it was at the 25% level where everything seemed to factor out. Other studies looking at hepatic dysfunction tend to think of 25% of liver remaining as an extensive liver resection. Several portal vein embolization protocols use this percentage, as well.

Endothelin A Receptor Blockade Reduces Hepatic Ischemia/Reperfusion Injury After Warm Ischemia in a Pig Model

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It is well established that endothelin-1 (ET-1) is a very potent mediator of vasoconstriction that leads to microcirculatory disturbances. The aim of the study was to evaluate the effect of a selective endothelin A receptor antagonist on severe ischemia/reperfusion injury in a pig model. Fourteen pigs were subjected to 120 minutes of complete vascular exclusion of the liver with a passive bypass. The animals were randomized into two groups: a control group, which was given isotonic saline solution, and a therapy group, which received the selective endothelin A receptor antagonist BSF 208075 at the beginning of reperfusion. On postoperative days 4 and 7, animals were relaparotomized to obtain tissue specimens. Blood monitoring included aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamate dehydrogenase (GLDH), alkaline phosphatase, and ET-1. Partial oxygen tension ($p_{ti}O_2$) was measured by a Clarke-type electrode and blood flow by laser Doppler. A semiquantitative scoring index was used for assessment of histologic injury and for immunohistochemical analysis of ET-1. Treatment with the endothelin A receptor antagonist significantly reduced the severity of the ischemia/reperfusion injury, as evidenced by lower levels of AST, ALT, and GLDH. The dramatic increase in plasma ET-1 in the therapy group is clear evidence of effective receptor blockade. Analysis of $p_{ti}O_2$ and blood flow revealed a significant improvement in capillary perfusion and blood flow in the treated group and was associated with relevant reduction of tissue injury. In summary, in the control group we observed serious microcirculatory disturbances and severe histologic damage in the liver after reperfusion. Treatment with a selective endothelin A receptor antagonist attenuated the ischemia/reperfusion injury in a porcine model of severe ischemia/reperfusion, as demonstrated by improved microcirculation, a reduction in histologic damage, and an decrease in liver enzymes. (J GASTROINTEST SURG 2003;7:331–339.)

KEY WORDS: Ischemia, reperfusion, pig, endothelin, receptor antagonist

Total hepatic vascular exclusion causing severe warm ischemia/reperfusion injury is an often-used technical option, particularly in cases of extended liver resection and severe hepatic trauma.[1] This technique helps reduce the risk of massive hemorrhage or air embolism during surgery. Total vascular exclusion is carried out by completely mobilizing the liver and occluding the portal triad (Pringle maneuver) and the inferior vena cava above and below the liver.

Ischemia and reperfusion of the liver is associated with transient hepatic dysfunction. A large variety of pathophysiologic events have been identified as being involved in postischemic organ malfunction. Based on the concept of microcirculatory impairment as a major determinant of hepatic ischemia/reperfusion injury, improving microcirculation appears to be a logical therapeutic approach. Novel strategies tend to inhibit vasoactive mediators that cause vasoconstriction and sinusoidal constriction

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followed by a reduction in perfusion and pathologic leukocyte-endothelium interactions.[2–5] Endothelin-1 (ET-1) is thought to be one of the most important mediators involved in the pathophysiology of microcirculatory disturbances induced by ischemia/reperfusion.[6–9] ET-1 is the most abundant and potent member within the endothelin family. It was originally isolated from cultured porcine endothelial cells and was found to be a powerful vasoconstrictor.[10] It is a peptide comprising 21 amino acids and is produced by ischemic or injured endothelial cells. The effects of ET-1, which acts mainly as a paracrine factor, are mediated by two receptor subtypes, designated endothelin A and endothelin B.[10–12] Endothelin A receptors mediate vasoconstriction, and endothelin B receptors mediate primarily nitric oxide–dependent vasodilation and endothelin degradation. In the liver, endothelin A receptors are found only in hepatocytes and hepatic stellate cells, whereas endothelin B receptors are expressed by all types of liver cells. A strong constricting action targets contractile hepatic stellate cells.[13]

Several previous studies have shown a beneficial effect of endothelin receptor antagonists in the treatment of warm ischemia/reperfusion injury.[14–16] These studies, all of which used a mixed endothelin A/endothelin B receptor antagonist mimicked the clinical situation of partial warm liver ischemia (occlusion of the portal triad only), which is routinely performed during liver resection. Until now, no study has assessed the effectiveness of an endothelin receptor antagonist in preventing warm ischemia/reperfusion injury in the context of total vascular exclusion causing severe hepatic ischemic injury. Furthermore, administration of a selective endothelin A receptor antagonist (ETA-RA), which we used in our study, is the current therapeutic standard. Most of the postischemic damage induced by endothelin is mediated via the endothelin A receptor subtype, including vasoconstriction, upregulation of adhesion molecules, and triggering the formation of other mediators.[15,17]

The aim of this study was to investigate whether the administration of a selective ETA-RA at the beginning of reperfusion after total vascular exclusion of the liver has an effect on microcirculation, histologic findings, and immunohistochemical and biochemical alterations.

MATERIAL AND METHODS

Experimental Protocol

The investigation was performed in accordance with the German legislation on protection of animals, and the experiments were approved by the Committee on Animal Care (Regierungspräsidium Leipzig, Germany; No. 02/00). Total vascular exclusion was performed for 2 hours in 14 female German Landrace pigs (20 to 25 kg). The control group (n = 7) was given 50 ml of isotonic saline solution, and the therapy group (n = 7) received 5 mg/kg of body weight intravenously of the specific endothelin A receptor antagonist BSF 208075 (Knoll AG, Ludwigshafen, Germany) in 50 ml of isotonic saline solution. The ETA-RA was administered as a continuous intravenous infusion during the first 30 minutes of reperfusion. On days 4 and 7 after surgery, the animals underwent relaparotomy to obtain tissue specimens for determination of histologic damage and were then killed.

Anesthesia and Surgical Procedure

Animals were premedicated with the following: azaperone (15 mg/kg i.v.; Stresnil; Cilag-Janssen, Neuss, Germany), atropine (0.2 mg/kg i.v.; Atropinsulfat; Braun, Melsungen, Germany), and ketanest (3 mg/kg i.v.; Ketamin; Ratiopharm, Ulm, Germany). Anesthesia was induced with thiopental (8 mg/kg i.v.; Trapanal; Byk Gulden, Konstanz, Germany) and maintained with isoflurane (Forene; Abbott, Wiesbaden, Germany) and fentanyl (Fentanyl; Janssen, Neuss, Germany). Ventilation was performed with an oxygen/air mixture (respirator "Julian"; Draegerwerk AG, Luebeck, Germany) with an F_iO_2 of 40%. A polyethylene catheter inserted into the right external jugular vein for infusion of anesthetic drugs was then used for the infusion of the ETA-RA or normal saline solution and for collection of blood samples. A catheter in the right carotid artery was used to measure blood pressure.

The animals were subjected to a midline laparotomy. The liver was completely skeletonized from the surrounding ligaments. Total hepatic vascular exclusion was achieved by clamping the suprahepatic and infrahepatic vena cava in addition to the portal triad. During ischemia, blood from the portal vein and femoral vein was bypassed to the right internal jugular vein using a Y-shaped polyethylene tube. After 2 hours of total vascular exclusion, the clamps were serially removed from the suprahepatic vena cava, the portal pedicle, and the infrahepatic vena cava. The right internal jugular and left femoral veins were ligated. Antibiotic prophylaxis with mezlocillin (Baypen; Bayer, Germany) was given intravenously after anesthesia was induced.

Partial Oxygen Tension in Tissue

Continuous measurement of partial oxygen tension in the hepatic tissue ($p_{ti}O_2$) was performed by implantation of a Clarke-type electrode (Licox; Gesellschaft für medizinische Sondentechnik mbH, Kiel-Mielkendorf, Germany) into the left lobe of the

liver. Tissue $p_{ti}O_2$ was measured after laparotomy for 30 minutes, during the entire period of ischemia (120 minutes), and for 120 minutes after reperfusion.

Laser Doppler Flow Measurement

Blood flow was measured by placing the Doppler flow probe (DP 1) at three different points in the liver: the left lobe, the middle lobe, and the right lobe. Measurements were obtained after laparotomy before vascular exclusion, and at 30, 60, and 120 minutes after reperfusion; blood flow was monitored on a DRT4 monitor (Moor Instruments, Devon, UK). The principle of the probe is that light generated by a laser diode (780 nm wavelength with a maximum emission energy of 1.0 mW) penetrates the tissue where it is reflected by circulating blood cells. Analog laser Doppler flow signals were digitalized and processed on a personal computer with DRTSOFT V2.9 software (Moor Instruments). Blood flow was recorded for at least 30 seconds after a stable signal was obtained. The values are given as arbitrary units. Post-sampling data processing included pulse wave analysis with an integral under the curve. For integral estimation, the mean of the pulse waves within the 30-second sampling period was calculated.

Histologic Analysis

Specimens were taken from the left lobe of the liver, fixed in 4% formaldehyde, embedded in paraffin, and sectioned and stained with hematoxylin and eosin. Histomorphologic alterations were semiquantitatively assessed using a scoring system that ranged from absent to severe. Impairment before organ manipulation was assessed by means of four parameters; injury during warm ischemia, reperfusion, and follow-up was assessed by 10 parameters (Table 1). Points were assigned according to the importance of each parameter for organ function. Scores were given as percentages of maximal attainable points.

Immunohistochemical Analysis of Endothelin-1

For immumohistochemical analysis of ET-1, specimens were routinely fixed in 4% formaldehyde solution and embedded in paraffin. Sections (4 μm thick) were cut, dewaxed in xylene, and then rehydrated. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in phoshate-buffered saline solution for 20 minutes. After brief rinsing with phosphate-buffered saline, the sections were boiled in citrate buffer for 15 minutes in a microwave oven (600 watts). Because there is much endogenous biotin in liver tissue, which can lead to false positive results, the sections were incubated with avidin and biotin for 15 minutes, respectively. After cooling, the sections were covered with normal goat serum for 20 minutes and then incubated with the primary antibody against ET-1 (DPC Biermann, Bad Nauheim, Germany). Thereafter the sections were washed with phosphate-buffered saline, incubated with biotinylated goat antirabbit immunglobulin G (BioGenex R, Hamburg, Germany) for 30 minutes, and covered with peroxidase-conjugated streptavidin (BioGenex R). The peroxidase reaction was allowed to proceed for 5 minutes, with 3-amino-9-ethylcarbazole solu-

Table 1. Semiquantitative histologic score

	Slight	Moderate	Severe
Before manipulation			
Activation of Kupffer cells	1	1	1
Portal infiltration	0	1	2
Steatosis	1	3	5
Intralobular necrosis	3 (single cell)	8 (group cell)	15 (mass cell)
Warm ischemia, reperfusion, follow-up			
Interstitial edema	2	4	6
Intracellular swelling	2	4	6
Quality of sinusoidal space	2	4	6
Steatosis	1	3	5
Reaction of Kupffer cells	0	0	2
Intralobular necrosis	3 (single cell)	8 (group cell)	15 (mass cell)
Subcapsular necrosis	1 (single cell)	4 (group cell)	8 (mass cell)
Reaction of liver capsule	0	0	1
Hemorrhage	0	1 (subcapsular)	3 (interstitial)
Only after reperfusion			
Hyperemia	2	8	15

tion as substrate. Slides were counterstained with Meyer's hemalum and finally mounted. The intensity of intracytoplasmic ET-1 immunostaining was graded semiquantitatively on a scale of 0 (no staining) to 3 (maximal intensity of staining). The number of positive cells was counted and scored semiquantitatively from 0 to 4 (0 = no cells; 1 = 1 to 30 cells; 2 = 31 to 60 cells; 3 = 61 to 90 cells; and 4 = >90 stained cells in 1 of 10 observed areas). Negative control specimens were included in each batch replacing the primary antibody. An overall score per animal and the duration of the investigation were calculated by multiplying the value for intensity of immunostaining by the score for positive cells.

Histologic and immunohistochemical analyses were performed independently by three of us (B.A., A.T., and D.U.) who were blinded to the clinical and pathologic information.

Serum Aspartate Aminotransferase, Alanine Aminotransferase, Glutamate Dehydrogenase, Alkaline Phosphatase, and Plasma Endothelin-1

Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamate dehydrogenase (GLDH), and alkaline phosphatase were measured with an automatic analyzer (Hitachi 917; Hitachi, Ltd., Tokyo, Japan) by photometry. ET-1 levels from central venous blood were determined in EDTA-plasma using the QuantiGlo ET-1 Chemiluminescent Immunoassay (R&D Systems, Nivelles, Belgium), which contains synthetic human ET-1 and antibodies raised against the synthetic factor.

Statistical Analysis

Data were expressed as means ± standard deviation (SD). Histologic and immunohistochemical scores for injury were represented in the form of "box and whisker" plots showing the median value, the twenty-fifth and seventy-fifth percentiles, and the ninety-fifth percentile. Variables were tested for group differences by means of the Mann-Whitney U test. P values <0.05 were considered significant.

RESULTS

Macrohemodynamic and Blood Gas Changes

No differences in mean arterial pressure were found between the control and therapy groups before sur-

Table 2. Hemodynamic and metabolic data in the control and therapy groups at baseline and 30 minutes and 60 minutes after reperfusion (mean ± SD)

Parameter	Baseline	Reperfusion	
		30 min	60 min
Heart rate (beats/min)			
Control group	90.0 ± 12.1	114.0 ± 19.2	109.0 ± 9.6
Therapy group	88.7 ± 4.6	103.6 ± 16.5	112.1 ± 18.4
Mean arterial pressure (mm Hg)			
Control group	72.4 ± 11.1	63.0 ± 8.3	61.6 ± 7.1
Therapy group	71.3 ± 9.7	59.7 ± 9.8	58.4 ± 3.6
Central venous pressure (mm Hg)			
Control group	9.2 ± 2.6	9.6 ± 1.8	9.6 ± 1.8
Therapy group	9.1 ± 3.2	10.0 ± 3.6	10.0 ± 3.6
a-pH			
Control group	7.45 ± 0.08	7.34 ± 0.01	7.41 ± 0.03
Therapy group	7.41 ± 0.07	7.26 ± 0.09	7.27 ± 0.08
a-pO_2 (kPa)			
Control group	169.9 ± 21.8	175.9 ± 9.0	178.9 ± 16.5
Therapy group	166.2 ± 17.3	182.0 ± 19.5	188.7 ± 6.8
Hemoglobin (g/dl)			
Control group	8.0 ± 1.3	8.8 ± 3.7	7.2 ± 1.6
Therapy group	8.2 ± 1.2	6.9 ± 1.2	6.3 ± 1.1
Hematocrit (%)			
Control group	24.5 ± 4.1	27.4 ± 12.2	21.9 ± 5.4
Therapy group	25.1 ± 3.6	20.8 ± 3.8	19.8 ± 3.6
Arterial oxygen saturation (%)			
Control group	69.8 ± 13.7	88.3 ± 7.6	88.0 ± 9.6
Therapy group	72.7 ± 12.9	84.0 ± 6.4	85.4 ± 8.0

gery (72.4 ± 11.1 mm Hg vs. 71.3 ± 9.7 mm Hg). The values dropped during clamping to 63.2 ± 85.4 mm Hg and 63.1 ± 9.4 mm Hg, respectively, after 120 minutes of vascular exclusion.

After reperfusion, the arterial pressure showed no increase and did not differ significantly between the two groups, despite the use of vasoactive drugs in the therapy group (Table 2). The changes in arterial blood gases are also shown. There were no significant differences between the two groups in any of the measured parameters.

Tissue Oxygenation

Tissue oxygenation values are presented in Fig. 1. Mean $p_{ti}O_2$ levels before surgery were not significantly different between the two groups (control vs. treatment: 38.4 ± 6.48 mm Hg vs. 36.7 ± 15.9 mm Hg). At the end of the period of warm ischemia, the levels dropped to 0 to 1 mm Hg in both groups. After reperfusion, the mean $p_{ti}O_2$ level in the control group increased to 29.5 ± 9.3 mm Hg after 15 minutes and remained at this level until 90 minutes after reperfusion. From 90 to 120 minutes after reperfusion, a slight but nonsignificant increase in the $p_{ti}O_2$ values was observed. In the therapy group the $p_{ti}O_2$ level showed a peak of 45.1 ± 9.8 mm Hg at 15 minutes after reperfusion but decreased again 30 minutes after reperfusion to 39.8 ± 7.6 mm Hg. The pattern in the therapy group was similar to that in the control group, but the corresponding mean $p_{ti}O_2$ values were found to be significantly higher at 30, 45, 60, 75, and 120 minutes after reperfusion in the therapy group ($P < 0.001$). Compared to the preischemia $p_{ti}O_2$ levels, the levels in the control group were significantly lower ($P < 0.01$), whereas the corresponding values in the therapy group did not differ at any of the times measured.

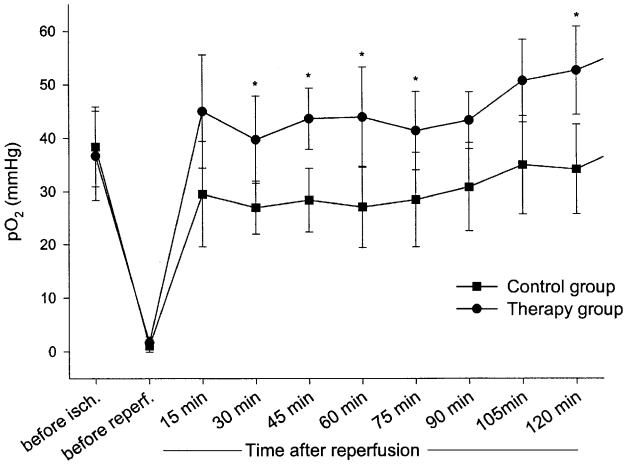

Fig. 1. Partial oxygen tension ($p_{ti}O_2$) in the liver before ischemia, at the end of ischemia, and from 10 to 120 minutes after reperfusion in the pigs given endothelin A receptor antagonist (*therapy group*) or saline solution (*control group*). Values are means ± SD. *Significant difference, control vs. therapy group ($P < 0.001$). pO_2 was significantly higher at 30 to 75 minutes and 120 minutes after reperfusion in the therapy group as compared to the control group.

Laser Doppler Flow Measurements

Laser Doppler flow measurements are presented in Table 3. Before surgery, the integrals under the curve revealed no significant differences between the control and therapy groups at all of the measurement points. Thirty minutes after reperfusion, a significant decrease in blood flow was found at all measurement points in the control group, whereas the corresponding values in the therapy group were comparable to the preischemia values. Sixty minutes after reperfusion, no relevant changes in blood flow were seen within the control group as compared to the 30-minute time point. Within the therapy group, a slight decrease in blood flow was observed except for an increase in the right lobe of the liver. At 120 minutes, the integrals under the curve for the control and therapy groups revealed higher but not significantly different values compared to values at 60 minutes. When the control and therapy groups were compared, a significant difference in blood flow was observed at 30, 90, and 120 minutes after reperfusion in favor of the therapy group at all measurement times ($P < 0.05$).

Histologic Analysis

Before liver manipulation, there was no evidence of relevant morphologic damage in either group. At the end of the period of warm ischemia, a slight increase in injury was seen in the control and therapy groups (interstitial and intracellular edema, single necrotic cells, activation of Kupffer cells) with no difference between the two groups (control = 19%; therapy = 16% injury).

Ten minutes after reperfusion, the histologic injury increased in both groups but was found to be significantly lower in the therapy group ($P = 0.002$). A slight increase in edematous injury (approximately 36%), a reaction of the capsule of the liver (86%) and of the Kupffer cells (71%), was detected in the therapy group, whereas the histomorphologic alterations in the control group included a strongly developed interstitial and intracellular edema (57% and 90%, respectively), irregular trabecular disruption, hemorrhage, invasion of inflammatory cells, dilatation of

Table 3. Laser Doppler flow measurements

	Laser Doppler area under the curve			
	Before ischemia	After reperfusion		
		30 min	60 min	120 min
Control group				
Left lobe	216 ± 15	133 ± 23†	156 ± 15†	183 ± 15
Middle lobe	240 ± 16	127 ± 22†	148 ± 17†	158 ± 23†
Right lobe	243 ± 17	122 ± 19†	151 ± 24†	173 ± 18†
Therapy group				
Left lobe	220 ± 17	210 ± 18*	175 ± 21*	228 ± 14*
Middle lobe	244 ± 17	212 ± 13*	172 ±18*†	198 ± 17*†
Right lobe	244 ± 21	222 ± 24*	245 ± 32*	248 ± 22*

Blood flow measured by laser Doppler was calculated as the integral under the curve (values are given as arbitrary units; for definition, see Material and Methods).

*$P < 0.05$ control group (intergroup comparisons).

†$P < 0.05$ vs. preischemic values (intragroup comparisons).

the sinusoidal space, and sinusoidal congestion. To summarize, the difference between the two groups was found to be significant (55% in the control group vs. 19% in the therapy group; $P < 0.001$).

One and 2 hours after reperfusion, a further increase in morphologic damage was noted in the control group (strong edema, hemorrhagic and inflammatory infiltration of the sinusoidal space, increase in subcapsular and intralobular necrosis), whereas in the therapy group no relevant change in injury was found. To summarize, injury 2 hours after reperfusion was 60% in the control group vs. 15% in the therapy group; $P = 0.002$). During follow up (4 and 7 days postoperatively), an obvious decrease in morphologic-pathologic alterations was observed in both groups for all investigated parameters. However, 7 days postoperatively there was still a significant difference in scores between the two groups (33% in the control group vs. 13% in the therapy group; $P = 0.004$).

Immunohistochemical Analysis of Endothelin-1

Immunohistochemical scores for ET-1 are presented in Fig. 2. Before ischemia, no immunoreactivity or only a focal presence of ET-1 immunoreactivity was found in either group. At the end of the period of warm ischemia, we observed a faint intracytoplasmic immunoreactivity in hepatocytes and vascular endothelial cells that was comparable in the two groups. Ten minutes and 1 hour after reperfusion, the median ET-1 levels showed a relevant increase in the control group only, and these levels were significantly higher than those in the therapy group ($P = 0.04$ and $P < 0.01$, respectively). On the fourth postoperative day, ET-1 expression decreased significantly in the control group. On the seventh postoperative day, similar to the situation before ischemia, very few ET-1–positive cells were seen in either group.

Serum Aspartate Aminotransferase, Alanine Aminotransferase, Glutamate Dehydrogenase, and Alkaline Phosphatase

Serum AST, ALT, GLDH, and alkaline phosphatase levels were within the normal range in both

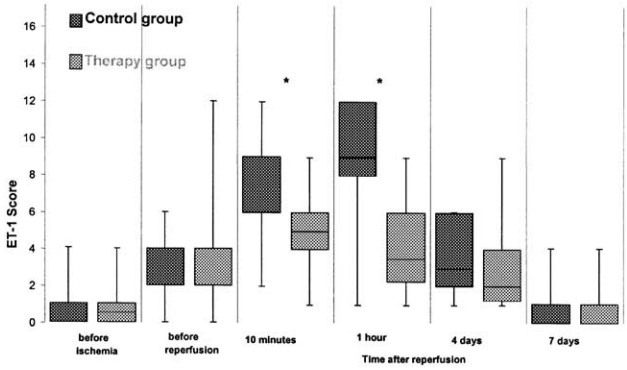

Fig. 2. Immunohistochemical score indexes for endothelin-1 (*ET-1*) in the liver tissue before, during, and after total vascular exclusion. The pigs were given either endotholin A receptor antagonist (*therapy group*) or saline solution (*control group*). Scores were calculated by multiplying mean values for intensity of immunostaining by the mean number of positive cells. The score indexes for each group at each time of investigation are represented in the form of box and whisker plots showing the median (*line in box*), the twenty-fifth and seventy-fifth percentiles (*boxes*), and the ninety-fifth percentile (*outer box*). *Significant difference between control and therapy groups ($P = 0.04$ and $P < 0.01$, respectively). ET-1 expression was significantly higher 10 minutes and 1 hour after reperfusion in the control group as compared to the therapy group.

the control and therapy groups before ischemia (AST = 0.47 ± 0.07 μmol/L and 0.44 ± 0.10 μmol/L; ALT = 0.50 ± 0.10 μmol/L and 0.47 ± 0.16 μmol/L; GLDH = 0.02 ± 0.01 μmol/L and 0.03 ± 0.01 μmol/L; alkaline phosphatase = 2.03 ± 0.48 μmol/L and 1.96 ± 0.49 μmol/L). After reperfusion, serum AST displayed a marked increase with a maximum at 18 hours, which was higher in the control group (control/therapy group = 27.36 ± 3.86 μmol/L/17.21 ± 3.77 μmol/L; $P < 0.001$). On comparison of the control and therapy groups at 2, 6, 18, and 42 hours after reperfusion, significantly lower AST measurements were found in the therapy group ($P < 0.05$ to 0.001). On the seventh postoperative day, AST levels had normalized to baseline values in both groups (Fig. 3). Comparable to AST, ALT levels reached a maximum 18 hours after reperfusion (control/therapy group = 2.2 ± 0.35 μmol/L/1.65 ± 0.37 μmol/L); however, there were no significant differences between the two groups. GLDH levels increased in both groups to a maximum at 6 hours after reperfusion (control/therapy group = 0.7 ± 0.09 μmol/L/0.38 ± 0.08 μmol/L; $P < 0.01$) and remained elevated until 42 hours after reperfusion (control/therapy group = 0.54 ± 0.22 μmol/L/0.17 ± 0.09 μmol/L; $P < 0.01$). Between these times there was a significant difference in GLDH levels in favor of the therapy group. At the end of the observation period, GLDH levels had normalized to baseline values in both groups. Serum alkaline phosphatase levels increased to a maximum of 8.40 ± 0.63 μmol/L 18 hours after reperfusion in the control group. The corresponding value in the therapy group was 6.20 ± 0.45 μmol/L ($P < 0.01$). From 1 hour after reperfusion to postoperative day 4, serum alkaline phosphatase levels were significantly higher in the control group as compared to the therapy group ($P < 0.01$). On the seventh postoperative day, alkaline phosphatase values were within the normal range in both the control and therapy groups.

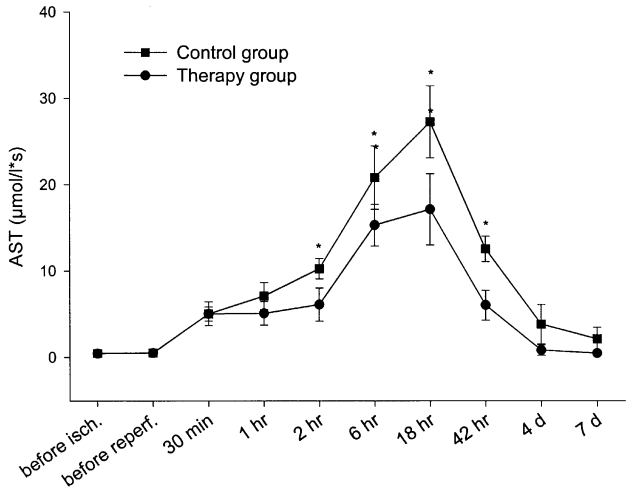

Fig. 3. Serum levels of aspartate aminotransferase (*AST*) before ischemia, at the end of ischemia, and from 30 minutes to 7 days after reperfusion in pigs given endothelin A receptor antagonist (*therapy group*) or saline solution (*control group*). Values are means ± SD. *Significant difference between control and therapy groups ($P < 0.05$ and $P < 0.001$, respectively). Serum levels were significantly lower 2 hours to 42 hours after reperfusion in the therapy group as compared to the control group.

Plasma Endothelin-1

Plasma ET-1 values are presented in Fig. 4. The mean ET-1 values in the central venous blood did not differ between the two groups before surgery. Immediately before reperfusion, the levels in both groups were significantly higher as compared to baseline values, with no difference between groups. After reperfusion, a further significant increase to a maximum level of 3.48 ± 1.09 pg/ml ($P < 0.05$) at 2 hours was measured in the control group. In the therapy group a marked increase to a maximum of 35.2 ± 5.3 pg/ml at 2 hours after reperfusion was seen ($P < 0.001$). On the fourth and seventh postoperative days, ET-1 levels similar to those before reperfusion were observed in the two groups. During the period from 30 minutes to 12 hours, significantly higher ET-1 values were measured in the therapy group compared to the control group ($P < 0.05$ to 0.001).

DISCUSSION

In hepatic ischemia/reperfusion injury, dysfunction of the microcirculation has to be considered as a primary process that triggers the final manifestation of tissue injury. Postischemic microcirculatory dysfunction includes the failure of sinusoidal perfusion (i.e., no-reflow phenomenon), which is caused by endothelial swelling, intravascular hemoconcentration, and an imbalance between the vasoactive mediators endothelin and nitric oxide. Apart from perfusion failure, ischemia/reperfusion injury further promotes a microcirculation-associated inflammatory response (i.e., reflow paradox), which involves the release of aggressive mediators such as oxygen radicals, tumor necrosis factor-alpha, and interleukin-1, the upregulation of leukocytic and endothelial adhesion molecules (selectins, beta-integrins, ICAM-1), and the interaction of platelets and leukocytes with the microvascular endothelium. The ultimate parenchymal cell damage includes both necrotic and apoptotic cell death.

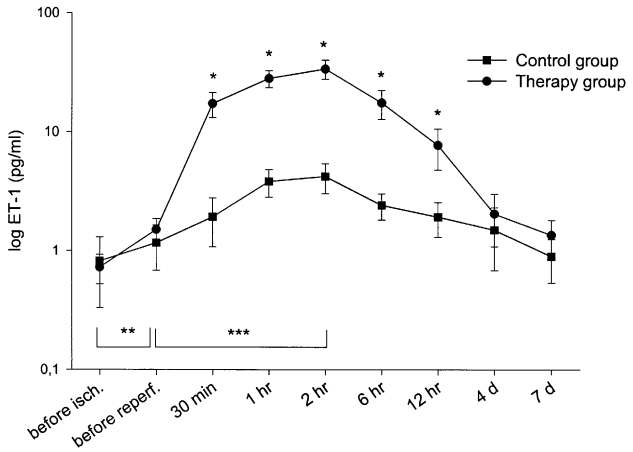

Fig. 4. Mean plasma endothelin-1 (*ET-1*) values for pigs given endothelin A receptor antagonist (*therapy group*) or saline solution (*control group*). Values are means ± SD. Administration of the specific endothelin A receptor antagonist led to significantly higher ET-1 levels as compared to the control group during the period from 30 minutes to 12 hours after reperfusion. *Therapy group vs. control group, $P < 0.05$ to 0.001. **Before reperfusion vs. baseline, $P < 0.05$ (control and therapy groups). ***Two hours after vs. before reperfusion, $P < 0.05$ (control group), $P < 0.001$ (therapy group).

The significant increase in ET-1 levels in our control group indicates its important role in hepatic warm ischemia/reperfusion injury. After hepatic ischemia, elevated plasma endothelin levels in the suprahepatic vena cava have been reported, indicating clearly that endothelin is produced in the liver.[8,19] The significantly higher intrahepatic ET-1 protein expression in the untreated group in our series supports the hypothesis that ET-1 plays an important role in the pathogenesis of hepatic ischemia/reperfusion injury. Cytokines, such as interleukin-1 and tumor necrosis factor, are known to be potent stimulators of ET-1 release,[20] as well as anoxia and wall shear stress.[7] ET-1 induces a specific sinusoidal constriction in isolated liver preparations that disrupts normal acinar flow dynamics, and the sinusoidal constriction is colocalized with Ito cells, which are located mainly in the periportal zone of the sinusoids.[13] ET-1 release induced within the sinusoids by Kupffer cells or endothelial cells may act in a paracrine fashion on hepatic stellate cells. Subsequently, ET-1 released from the stellate cells may constrict sinusoids in an autocrine fashion as well.[21] Furthermore, ET-1 plays a proinflammatory role through its ability to induce neutrophil and eosinophil influxes and to stimulate release of oxygen radicals by macrophages and production of proinflammatory cytokines by monocytes.[22]

The rationale for administration of the ETA-RA at the beginning of reperfusion was the concept that the first minutes after reperfusion seem to be particularly important for the manifestation of ischemia/reperfusion injury.[23] In a rat model of renal warm ischemia/reperfusion injury, preischemic and postischemic treatment with a nonselective endothelin antagonist resulted in improved renal function, but pretreatment administration rendered better results than posttreatment administration.[24] In the postischemic setting, the ETA-RA was given just before reperfusion as was done in our study. We did not observe relevant changes in systemic hemodynamic parameters when the endothelin A receptor antagonist BSF 208075 was given in a dose of 5 mg/kg body weight.

The dramatic increase in ET-1 levels in the therapy group is clear evidence of effective receptor blockade by the endothelin A receptor antagonist BSF 208075, which is in agreement with the findings of Uhlmann et al.[15]

In this study, continuous tissue oxymetry was used to assess the microcirculation. Tissue oxygenation was significantly decreased in the control group as compared to the group treated with ETA-RA during almost the entire period of $p_{ti}O_2$ measurement. Likewise, a significantly better blood flow, as assessed by laser Doppler flowmetry, was seen in the therapy group at all measurement points in the liver. The significantly improved tissue oxygenation and blood flow early after reperfusion in the therapy group points to the vasoconstrictive role of ET-1.

The severity of reperfusion injury, as evidenced by the maximum AST, ALT, and GLDH levels 18 and 12 hours after reperfusion, revealed a clear association with impairment of microcirculation and grade of tissue injury. Katsuramaki et al. showed a significant correlation between the increase in AST and the decrease in hepatic tissue blood flow and histologic alterations.[25] An early sign of microvascular dysfunction is the increase in permeability, which accounts for the development of edema. Subsequently, alterations in parenchymal cells and the inflammatory response characterized by neutrophil infiltration are observed. The results of the present study are in accordance with these histologic observations and indicate that the administration of a selective ETA-RA attenuates morphologic injury by improving microcirculation.

CONCLUSION

ET-1 plays a central role in the hepatic ischemia/reperfusion injury after 2 hours of hepatic vascular exclusion. The present study clearly demonstrates that endothelin A receptor blockade significantly reduces the severity of ischemia/reperfusion injury by improving hepatic capillary perfusion, which is asso-

ciated with less tissue injury. In view of these findings, future studies on the clinical application of endothelin receptor antagonists in liver ischemia and transplantation would seem to be warranted.

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Combination Probe and Dye-Directed Lymphatic Mapping Detects Micrometastases in Early Colorectal Cancer

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Nodal metastasis is the single most important prognostic factor in early colorectal cancer (CRC). Lymphatic mapping can identify sentinel nodes for focused histopathologic examination and thereby improve the nodal staging of CRC; however, the optimal technique for identifying sentinel nodes in CRC is unclear. We hypothesized that a combination of radiotracer and blue dye would more accurately identify tumor-positive sentinel nodes than blue dye alone. Lymphatic mapping was performed in 48 consecutive patients undergoing resection for CRC and in two original patients who underwent sentinel node mapping in 1996. Prior to resection, 1% vital blue dye and radiotracer were injected around the tumor in the subserosal layer. Nodes were designated as sentinel by blue coloration and/or radioactivity. Lymphatic mapping identified at least one sentinel node in 49 patients. Focused examination of multiple sentinel node sections by means of hematoxylin and eosin and immunohistochemical analysis showed that sentinel nodes accurately predicted the status of the nodal basin in 93.8% (46 of 49) of patients. Of the 19 patients with nodal metastases, 11 had macrometastases (>.2 mm), three had micrometastases (between 2 mm and 0.2 mm), and five had isolated tumor cells or clusters (<.2 mm) identified by immunohistochemical analysis only. Patients had significantly fewer blue/radioactive ("hot") nodes than blue-only nodes (1.38 vs. 2.48 per patient; $P = 0.0001$). It is important to note that nodal metastases were more common in blue/hot nodes than in blue-only nodes (27.3% [19 of 68] vs. 8.8% [11 of 124]; $P = 0.005$). Dual-agent lymphatic mapping more accurately identifies sentinel node metastases than blue dye alone. In addition, this technique allows a more focused histopathologic examination of these nodes, in conjunction with the revised American Joint Committee on Cancer guidelines, and thereby offers the potential for significant upstaging of CRC. (J GASTROINTEST SURG 2003;7:340–346.)

KEY WORDS: Lymphatic mapping, colorectal cancer, micrometastasis

Colorectal cancer (CRC) is the second leading cause of cancer-related deaths in the United States. In 2002 there will be an estimated 148,300 new cases and 56,600 deaths secondary to CRC.[1] The most important prognostic indicator of recurrence and survival in CRC is metastases within lymph nodes.[2,3] Adjuvant chemotherapy can decrease cancer-related mortality by up to 33% in patients with node-positive and advanced-stage tumors.[4] However, the benefit of adjuvant chemotherapy in patients with node-negative disease remains controversial, despite a known recurrence and mortality rate of up to 20% to 30%.[5] One explanation for these recurrences is missed nodal metastases at the time of the initial resection.

The importance of ultrastaging, that is, staging beyond standard hematoxylin and eosin–based pathologic methods, is emphasized by the revised

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guidelines of the American Joint Committee on Cancer and the International Union Against Cancer. These guidelines classify nodal tumor deposits into macrometastases (>2 mm), micrometastases (between 2 mm and 0.2 mm), and isolated tumor cells or clusters (ITC) (<0.2 mm).[6,7] This specialized staging nomenclature reflects the growing body of retrospective evidence that micrometastases or ITC deposits have prognostic significance. For instance, Greenson et al.[8] reviewed the lymph nodes of 50 patients with node-negative CRC by means of hemotoxylin and eosin staining and demonstrated cytokeratin-positive tumor cells detected by immunohistochemical analysis (IHC) in 14 patients (28%). Six of these patients (43%) died within 66 months compared with only one IHC-negative patient (3%) over the same period. Liefers et al.[9] used reverse transcription–polymerase chain reaction (RT-PCR) for carcinoembryonic antigen to evaluate node-negative CRC in 26 patients; nodal micrometastases were identified in 54% of these patients. It is important to note that 5-year survival was 50% vs. 91% for patients with vs. without RT-PCR evidence of micrometastases, respectively. Similarly, Hayashi et al.[10] noted decreased survival in patients with p53 or K-ras mutations detected within colonic lymph nodes.

Although immunohistochemical or molecular analysis of all nodal tissue is prohibitively time-consuming and costly, it would be feasible for a few nodes. Sentinel lymph node mapping identifies a limited number of nodes that are most likely to harbor disease. Investigators at the John Wayne Cancer Institute initially pioneered sentinel node mapping in melanoma and breast cancer, and we have extended this technique to CRC.[11] Our group successfully tested sentinel node mapping of CRC by blue dye alone in large prospective studies.[12,13] However, in melanoma the addition of a radioactive tracer to the blue dye can increase the accuracy of sentinel node detection.[14,15]

In this study we hypothesized that dual-agent mapping could accurately identify nodes most likely to contain metastases from CRC, and that examination of these sentinel nodes by means of IHC would identify micrometastases and/or ITC deposits missed by hemotoxylin and eosin staining.

PATIENTS AND METHODS

In March of 2001, the John Wayne Cancer Institute (Santa Monica, CA), Century City Hospital (Los Angeles, CA), and McLaren Regional Medical Center (Flint, MI) began a multicenter, institutional review board–approved trial of sentinel lymph node mapping in patients with early CRC. All patients prospectively enrolled in this study had biopsy-proved primary CRC without clinical or radiographic evidence of distant metastases. Two original patients who underwent sentinel node mapping in 1996 are also included in this study.

Sentinel Node Mapping and Colectomy

Informed, written consent was obtained from all patients. At operation, the colon was carefully mobilized without disrupting the lymphatic channels. A 1% solution of isosulfan blue dye (Lymphazurin; Ben Venue Laboratories, Bedford, OH) was injected into the subserosa circumferentially around the tumor using a tuberculin syringe. In two patients this was performed endoscopically. The total volume of dye was 0.5 to 1 ml. Approximately 500 μCi of technetium-labeled sulfur colloid diluted in 1 ml of saline solution was injected in a similar fashion immediately after the blue dye. For one patient with a low rectal cancer, the technetium sulfur colloid was injected 2 hours before the operation: colloid was injected transanally through a spinal needle in the nuclear medicine suite, and lymphoscintigraphy was performed to localize the drainage basins.

Both tracers traveled along lymphatic channels to adjacent lymph nodes. Visualization of the dye's path occasionally required gentle dissection of the mesentery. After 1 to 5 minutes, blue-stained nodes were identified and tagged as sentinel nodes by a suture. A hand-held gamma probe device (Navigator; U.S. Surgical, Norwalk, CT) was used to record counts in the primary tumor bed, along the lymphatic channels, and in lymph nodes. Counts were reconfirmed approximately 30 minutes later. Radioactive ("hot") nodes were identified as sentinel nodes if their counts were at least twice the background counts (3 neutral sites in the body away from the injection site and mesentery). Sentinel nodes were carefully labeled and designated as hot and/or blue by the operating surgeon. Sentinel node mapping was followed by a standard colectomy that included all sentinel nodes, and the en bloc specimen was sent to the pathologist.

Processing of Colorectal Cancer Specimens

Each tagged sentinel node was excised, measured, and bisected along its longest axis. If the lymph node was smaller than 5 mm, the node was processed whole. Nodal tissue was frozen and prepared for cryostatic sectioning. A face section was cut to a thickness of 5 microns for staining with hematoxylin and

eosin. Next, six to eight additional sections, representing approximately 72 microns of node, were sectioned for molecular analysis. The remaining nodal tissue was placed in 10% formalin and embedded in paraffin. Two additional 4-micron sections spaced by approximately 100-micron intervals were taken for hemotoxylin and eosin staining and cytokeratin-IHC. Cytokeratin-IHC was performed with a pan-specific cocktail of antibodies for human cytokeratin, AE1/AE3 (DAKO, Carpinteria, CA), and processed with an automated immunostainer (Ventana ES 320; Ventana Medical Systems, Tucson, AZ). A cytokeratin-IHC stain was considered positive if it demonstrated strongly positive cell clusters or individual cells with anatomic and cytologic features of tumor cells.

Nonsentinel nodes were fixed in formalin and bivalved; single-face sections were stained with hemotoxylin and eosin.

Statistical Analysis

The Wilcoxon signed-rank test was used to compare the number of blue vs. radioactive sentinel nodes. The exact Mantel-Haenszel test was used to compare the percentage of tumor-positive blue-only vs. blue/hot sentinel nodes. P values of <0.05 were considered statistically significant. Cohen's kappa value was used to test for agreement between blue dye and radioactivity within sentinel nodes.[16] All statistical calculations were performed by a statistician from the John Wayne Cancer Institute on automated statistical software.

RESULTS

Between March 2001 and May 1, 2002, a total of 48 consecutive patients underwent intraoperative sentinel lymph node mapping during primary surgery for early-stage CRC under a protocol approved by the institutional review board. In addition, two original patients who underwent sentinel node mapping in 1996 are also included in this study. There were 27 males and 23 females whose mean age was 71 years (range 46 to 88 years). Tumors were in the right colon (n = 28), transverse colon (n = 3), left colon (n = 6), sigmoid colon (n = 7), and rectum (n = 6). Tumor characteristics and nodal staging are presented in Table 1.

Blue nodes were clearly identified 1 to 5 minutes after injection of the blue dye. Radioactive nodes were concurrently detected by the gamma probe. At least one blue-stained node was identified in 49 (98%) of 50 patients and at least one radioactive node was identified in 47 (94%) of 50 patients. One failure occurred in a patient undergoing laparoscopic-assisted hemicolectomy involving injection of tracers through a trochar port for a T1 lesion in the ascending colon. Although 14 nodes were harvested from the en bloc specimen, none were blue or hot. Two other mapping procedures failed to demonstrate a hot node. One of these was in an in situ cancer and the other was in a T1 lesion; all blue-stained sentinel nodes and all nonsentinel nodes were negative for tumor.

Of the 775 lymph nodes identified in 50 patients, 127 (16%) were sentinel nodes and 648 (84%) were nonsentinel nodes. Of the 19 patients with nodal metastasis, seven (36.8%) had metastatic disease in the blue-only nodes and 16 (84%) had disease in blue/hot nodes ($P = 0.0028$). Eight cases of nodal metastasis were micrometastatic/ITC detected by IHC only. Thus IHC analysis of the sentinel node resulted in upstaging of 8 (20.5%) of 39 hemotoxylin and eosin–stained node-negative tumors.

Each CRC specimen contained an average of 2.54 sentinel nodes and 15.5 total nodes ($P < 0.001$). The sentinel node accurately predicted the tumor status of the nodal basin in 46 (93.8%) of 49 patients. In the patient in whom this technique was first used, a nonsentinel node contained tumor, but all sentinel nodes were negative. A second nonsentinel node metastasis occurred in a patient who had previous radiation to the pelvis for prostate cancer. The third nonsentinel node metastasis occurred in an 83-year-old man who had a bleeding, near-obstructing lesion of the sigmoid colon.

Overall, there were significantly fewer radioactive sentinel nodes per patient than blue sentinel nodes (1.38 vs. 2.48; $P = 0.0001$). The calculated kappa value of 0.662 demonstrated substantial agreement between blue coloration and radioactivity (Table 2). In other words, both tracers tended to travel along the same lymphatic channels to the same sentinel nodes. It is important to note that nodal metastases were more common in blue/hot nodes than in blue-only nodes (27.3% [19 of 68] vs. 8.8% [11 of 124]; $P = 0.005$). Metastases were limited to blue/hot nodes in 5 (26.3%) of 19 node-positive tumors, and these were all micrometastases/ITC found by IHC in lymph nodes averaging less than 1 cm in greatest diameter.

DISCUSSION

One reason for node-negative CRC recurrences may be missed nodal metastases. Recent advances in IHC and RT-PCR methods for evaluating lymph

Table 1. Tumor classification and nodal characteristics

T stage of primary lesion	No. of patients	Positive nodes			Negative nodes
		Macrometastases	Micrometastases	ITC	
Tis	13	—	—	—	13 (100%)
T1	6	1 (17%)	—	—	5 (83%)
T2	9	2 (22%)	1 (11%)	2 (22%)	4 (44%)
T3	21	7 (33%)	2 (9.5%)	3 (14%)	9 (42%)
T4	1	1 (100%)	—	—	—

ITC = isolated tumor cells or clusters.

nodes with possible microscopic tumor deposits have prompted the search for a cost- and time-efficient method to identify nodes that have the highest likelihood of harboring tumor. Equally important has been the introduction of new guidelines by the American Joint Committee on Cancer and International Union Against Cancer for defining microscopic nodal tumor deposits. This is developing in the face of a growing body of retrospective literature suggesting decreased survival of patients whose CRC is associated with microscopic nodal disease. It is, however, impractical to evaluate numerous lymph nodes for microscopic disease. Lymphatic mapping and sentinel lymph node analysis has been shown to be a highly accurate and focused technique for the analysis of lymph nodes in melanoma and breast cancer. In this study we hypothesized that a dual-agent mapping technique using blue dye and technetium-sulfur colloid might improve the accuracy of detecting nodal micrometastases in patients with colon cancer.

Because of its much larger particle size, the colloidal tracer remains in the first draining node for a longer period before traveling to the next node. Furthermore, the radioactivity adds a second signal that complements the blue dye and thereby helps the surgeon identify the sentinel node. We found this quite helpful when a blue-stained sentinel node was hidden deep within the bowel mesentery. The handheld gamma probe could identify the general location of the node by its radioactivity, resulting in a more focused search for the blue node.

In contrast to the stark demarcation of blue staining, radioactivity within a lymph node tends to be much more variable. There can be a spectrum of radioactivity among several lymph nodes within a regional basin. In fact, the definition of a radioactive sentinel node varies among investigators.[17,18] In this study we chose a predefined value of two times the baseline count. This definition avoids the confounding effect of "shine through," a phenomenon in which radioactivity from the primary tumor injection site can interfere with peripheral counts.

Although we demonstrated significantly fewer radioactive/blue sentinel nodes than blue-only nodes (1.38 vs. 2.48), metastases were more frequent in the blue/hot nodes (27.3% [19 of 68] vs. 8.8% [11 of 124]). More important, micrometastatic/ITC tumor deposits were isolated to the blue/hot nodes in 12.8% (5 of 39) of patients whose blue-only sentinel nodes were tumor negative by IHC and whose nonsentinel nodes were negative by hematoxylin and eosin staining. Although we cannot comment on the long-term prognosis of patients with micrometastases in this study, several studies support the prognostic impact of nodal micrometastases in patients with CRC.[8–10]

In the literature on lymphatic mapping for CRC, the rate of sentinel node identification is 70% to 98%, and the rate of false-negative sentinel nodes is 0% to 60%.[12,19–25] These wide ranges reflect a lack of standardized technique for identifying the sentinel node. For instance, Merrie et al.[19] combined blue dye with ^{99m}Tc colloidal antimony to map sentinel nodes in 25 colorectal tumors. In this study the false-negative rate of 45% was unacceptably high and the investigators concluded that lymphatic mapping of the sentinel node was of little clinical value in CRC. However, their study involved eight surgeons and 25 procedures. From our experience in melanoma and breast cancer, there is a definite learning curve that must be overcome prior to successful lymphatic mapping. Also, the sentinel nodes were harvested, on

Table 2. Cohen's kappa value demonstrating substantial agreement between blue dye and radioactive (hot) tracer

Hot	Blue		
	Positive	Negative	Total
Positive	68	2	70
Negative	56	649	705
Total	124	651	775

Kappa = 0.662; 95% confidence intervals (0.583 and 0.741).

average, 100 minutes after dye injection. In our experience, blue-stained nodes can be identified 1 to 5 minutes after dye injection; longer periods allow the dye to move to other nodes along the drainage pathway, making identification of the true sentinel node more difficult. By tagging blue-stained nodes immediately after they are visualized intraoperatively, we have been able to minimize the chance of missing first-tier nodes that the dye has already exited or falsely identifying second- and third-tier nodes as sentinel nodes. This problem was encountered by Joosten et al.,[21] who identified up to 16 blue nodes in the resected specimen and reported a false-negative rate of 60%.

Use of a radioactive tracer for lymphatic mapping can be disappointing if the hand-held gamma probe is not used. Merrie et al.[19] identified radioactive sentinel nodes by an ex vivo lymphoscintigraphic technique in which the surgical specimen was mounted on a photographic plate. We believe this technique is not as accurate as real-time mapping of sentinel nodes using a hand-held gamma probe in conjunction with visualization of blue dye. This may explain why 15 (18.5%) of their 81 sentinel nodes were radioactive and not blue, compared to only 2 (1.6%) of 127 sentinel nodes in our series.

To our knowledge, this is the first reported series that uses a hand-held gamma probe in conjunction with a vital blue dye for intraoperative mapping of the sentinel node in CRC. Our data demonstrate the sensitivity and accuracy of focused examination of the sentinel node in detecting nodal disease. The sentinel node accurately predicted the status of the nodal basin in 93.8% of cases. The three failures clearly identified limitations of sentinel node mapping. As mentioned earlier, the first failure occurred in the first trial case indicating that there is a learning curve, which must be overcome by both the operating surgeon and the pathologist in order to accurately stage tumors. The second failure occurred in a patient who had previous radiation to the abdomen and pelvis. This suggests that sentinel node mapping may not be appropriate after treatments that may have altered the original lymphatic drainage patterns. Finally, large, obstructing or perforated lesions may also alter lymphatic drainage, as seen in our third case of nonsentinel node metastasis. Such patients may not be suitable for sentinel node mapping.

Currently we are conducting prospective studies to determine whether patients with IHC-detected sentinel node micrometastases/ITC from early CRC are at an increased risk for recurrence and therefore suitable for adjuvant chemotherapy clinical trials. However, for these studies to be valid, the sentinel node technique must be defined and standardized. To this end, our published results using blue dye alone for sentinel node mapping in CRC have been promising, and here we extend those results to demonstrate that the addition of radioactive tracer confers specific advantages when rigorously applied. In the current evolution of nodal staging there is clearly an emphasis on using more sophisticated techniques to probe for the presence of smaller metastatic deposits. The combination of blue dye and technetium sulfur colloid for lymphatic mapping of the sentinel node in early CRC appears to be feasible and may prove more accurate and sensitive than blue dye alone.

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Discussion

Dr. K. Mayer (Sacramento, CA): I tried to perform the sentinel lymph node biopsy on a patient who had a metastatic liver lesion, but the node was negative. Do you think that micrometastases are moving through the vascular system and bypassing the lymphatic system? If our hospitals are using only hematoxylin and eosin staining, are we below a standard of care that we should be setting nationwide and are we doing our patients a disservice if we are not performing immunohistochemical analysis?

Dr. D.T. Nora: Clearly, there are a variety of types of metastases, not just lymphatic metastases. We can also have hematogenous metastases. At this point there is no good way of finding patients with hematogenous metastases other than to biopsy the liver or bone marrow with the specific limitations of sampling error. We know for a fact that lymph node status has a great deal of prognostic significance. It just seems to be the easiest way to identify patients who are at high risk for recurrence. To answer your second question, yes, in the future IHC may need to be performed on all lymph nodes for patients with colorectal cancer. We now have specific guidelines for the classifications of patients who have microscopic tumor within lymph nodes. So we must comply with the new American Joint Committee on Cancer staging system to answer the question of whether or not these are prognostically significant. So, yes, I think it will be the standard of care in the future.

Dr. J.P. Hoffman (Philadelphia, PA): I wonder if you have correlated your false-negative results with the location of the tumor, particularly whether or not it is on the mesenteric or the antimesenteric border? I have found that if a small tumor is located on the mesenteric side, sometimes it is very difficult to even determine the edge of that tumor to find an edge into which dye can be injected, and I am wondering if that may contribute to some of the false-negative results. Also, in terms of the radioactive method, doesn't one need to be licensed in order to administer radionuclide in many hospitals, and second, how long do you wait between the injection and your assessment of the nodes?

Dr. Nora: In regard to the first question, we examined our series extensively using blue dye alone. We found that the patients with extremely large tumors causing either perforation or obstruction are the patients who are at high risk for having a "skip" metastasis or a false-negative result. We also found patients who had previous radiation to the pelvis to be at high risk for having a false-negative result, perhaps because the lymphatic pathways have been destroyed by the radiation. In general, these patients with large perforating or obstructing tumors are going to be treated with chemotherapy anyway, so I do not know the importance of finding a positive node in these cases. Yes, you need a license to perform injection

of the nuclear dye, and we have the nuclear medicine physician come into the operating room and do the injection for us. In terms of the time delivery, we scan the nodes immediately after injection, 10 minutes after injection, and at least 30 minutes after injection as well. The technetium sulfur colloid is a much larger particle than the 1% Lymphazurin dye, so it does take longer to travel along the lymph nodes, but because of its larger size, it tends to embed within the lymph node and stay there for a longer period of time. I think that is the reason why we found fewer radioactive nodes as compared to blue nodes.

Dr. Hoffman: What do you do about the tumor on the mesentery?

Dr. Nora: The key is to inject circumferentially around the tumor. Tangential injections may be necessary with precautions taken to avoid injection directly into the mesentery. At times, one may have to inject on both sides of the mesentery. We have also tried colonoscopic injections with good success.

Invited Discussion—Expert Commentator

Robin S. McLeod, M.D. (Toronto, Ontario, Canada): Adjuvant chemotherapy is effective in improving survival in patients with stage III or lymph node–positive colon cancers. Patients who are inaccurately staged as having stage I/II cancers may not receive the benefits of adjuvant therapy. Various methods have been advocated to improve staging accuracy in patients undergoing surgery for colon and rectal cancer. Dr. Nora and his colleagues report their results with the use of the strategy of identification of the sentinal node. These investigators performed lymph node mapping with vital blue dye and a radioactive tracer in 32 patients undergoing surgery for early colorectal cancer. The tumor status of the sentinel nodes accurately predicted the status of the nodal basin in 90% of patients. Thirty-four percent of the patients had nodal metastases: 45% of these had tumor limited to the sentinel node and in 36%, the primary tumor was a T1 or T2 lesion.

Sentinel node examination is another way of maximizing the accuracy of the nodal status. It has less obvious benefit in colorectal cancers than in breast cancers. As a result, it has failed to gain the same acceptance as sentinel node identification in breast cancer surgery. Unlike breast cancer surgery, the extent of resection is rarely, if ever, modified because of the results of the sentinel node examination. Instead it is used to enhance examination of the specimen.

In this study the investigators looked at the added benefit of using a radioactive tracer in addition to mapping with vital blue dye. Unfortunately, they did not provide the data for the individual tests and the combined tests so that the added benefit of the radioactive tracer could be determined. Also, this is a small series of patients and a larger series of patients with long-term prospective follow-up will be necessary before it can be determined whether sentinel lymph node mapping is useful in patients with colorectal cancer and if the combined method is better than mapping with vital blue dye alone.

Small Bowel Extrinsic Denervation Does Not Alter Water and Electrolyte Absorption From the Colon in the Fasting or Early Postprandial State

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Small bowel transplantation (SBT) causes watery diarrhea. The decreases shown previously in absorption of water, electrolytes, and bile salts in the jejunum and ileum, although present, are not dramatic and seem not to be great enough to explain the diarrhea. Our aim was to determine changes in water and electrolyte absorption in the colon during fasting and postprandially in a canine preparation of jejunoileal extrinsic denervation, which serves as a model of jejunoileal autotransplantation. We hypothesized that colonic absorption of water and electrolytes decreases transiently in the colon after SBT. Six dogs had cannulas implanted in the colon to study absorption of an ileal-like basal electrolyte solution with or without 10 mmol/L glucose. Absorption during fasting and postprandially was measured before and 2 and 12 weeks after a validated preparation of jejunoileal extrinsic denervation. All dogs developed diarrhea after SBT. Net colonic absorptive fluxes of water and electrolytes in the colon did not change from baseline values at 2 or 12 weeks after extrinsic denervation, either during fasting or postprandially; glucose in the infusate did not alter absorptive fluxes during fasting or postprandially. Extrinsic denervation of the small intestine does not appear to alter colonic absorption of water or electrolytes during fasting or postprandially. These observations suggest that the neurally intact colon has a minimal role in the diarrhea after SBT. (J GASTROINTEST SURG 2003;7:347–353.)

KEY WORDS: Colon, intestinal transplantation, colonic absorption, extrinsic denervation, diarrhea

Small bowel transplantation (SBT) continues to evolve as a viable option for patients with intestinal failure.[1–5] However, SBT results in a poorly understood diarrhea. We have shown previously that transient decreases in net absorption of water, electrolytes, and bile salts occur in the canine small bowel after complete extrinsic denervation of the jejunoileum, a model of jejunoileal autotransplantation.[6,7] This decrease in net absorptive function resolves by 12 weeks after denervation. However, the magnitude of these decreases in net absorption from the small bowel do not appear to be large enough to explain completely the prominent diarrhea that occurs in the early postdenervation period (first few weeks), raising the question of the role of the colon in the pathogenesis and/or resolution of this postdenervation diarrhea.

Another consideration in absorptive physiology of the gut is the effect of feeding on absorptive capacity. Postprandial augmentation of absorption in the small bowel is a well-documented phenomenon.[8,9] However, changes in absorption postprandially in the colon are less well defined. Although some evidence suggests that augmentation of absorption occurs in the colon postprandially,[10,11] this effect appears to require intraluminal nutrients within the colon segment studied.[12] We were interested in determining changes in absorption in the colon in both the fasted and postprandial states after our canine model of SBT both in the absence and presence of glucose in the luminal infusate.

The aims of this study were (1) to evaluate changes over time in the absorption of water and electrolytes in the neurally intact colon after selec-

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tive extrinsic denervation of the small bowel both under baseline (fasting) conditions and postprandially, and (2) to determine whether intraluminal glucose would increase water absorption in the colon before or after small bowel extrinsic denervation. Our hypotheses were the following: (1) there would be a transient decrease in the absorptive flux of water and electrolytes in the neurally intact colon early after extrinsic denervation of the small bowel that would resolve by 12 weeks after denervation; (2) net absorption would be increased postprandially both before and after extrinsic denervation, but this effect would require the presence of glucose in the infusate solution; and (3) morphometric changes in the colonic mucosa reflecting an adaptation to the denervation would occur.

METHODS

Experimental Design

Six adult female mongrel dogs weighing 14 to 20 kg had two cannulas implanted in the colon; none was placed 2 cm distal to the ileocecal junction, and another was 50 cm distal to the first cannula. Colonic absorption was measured infusing an isosmolar, ileal-like, balanced electrolyte solution. After baseline experiments in the neurally intact state, each dog underwent extrinsic denervation of the jejunoileum, which serves as a model of autotransplantation of the jejunoileum, with repetition of the absorptive experiments 2 and 12 weeks later. Colonic biopsies were taken for morphometric analysis at the time of cannula implantation and after completion of the 12 weeks of experiments.

Animal Preparation

Our protocol was approved by the Institutional Animal Care and Use Committee of the Mayo Foundation in accordance with guidelines set forth by the National Institutes of Health and the Public Health Service Policy on humane care and use of laboratory animals. Anesthesia was induced with intravenous sodium methohexital (12.5 mg/kg) and maintained with inhaled isoflurane. A midline celiotomy was used to access the abdominal cavity. Transmural biopies were taken from the proximal and distal colon for morphometric analysis at the site of cannula placement. Each dog had two cannulas (inner diameter 1.2 cm) placed into the colonic lumen, one just distal to the ileal-cecal junction and another 50 cm distal to the proximal cannula. The cannulas were exteriorized through the abdominal wall. The animals were given intravenous fluids for 2 to 3 days postoperatively and allowed to begin a regular diet on the third day. Scheduled analgesics were administered for 2 days from the time of each operation. After a 2-week recovery period, baseline absorptive experiments were performed on each dog.

After completion of the baseline (neurally intact) experiments, each dog underwent reoperation and was subjected to in situ neural isolation of the jejunoileum, our preparation of complete (selective) extrinsic denervation of the small intestine. This preparation of extrinsic denervation has been described and validated previously in our laboratory by demonstrating the loss of adrenergic innervation to the small intestine postoperatively; because the only adrenergic innervation to the gut comes via the extrinsic nerves, this preparation is a very good model for studying the effects of extrinsic denervation.[13] This preparation provides a complete, in situ neural isolation, both extrinsic and intrinsic, of the small intestine without transection or occlusion of the superior mesenteric artery and vein, thereby avoiding the potentially confounding factor of ischemia/reperfusion injury to the bowel, and maintaining extrinsic innervation to the colon. This preparation of complete, but selective, extrinsic denervation of the jejunoileum is a model of *auto*transplantation and does not require immune suppression or the risk of immune rejection that would also potentially confound interpretation of the results. In brief, the base of the small bowel mesentery was transected radially up to the proximal jejunum and distal ileum, including all nerves and lymphatics, so that only the superior mesenteric artery and vein, just distal to the middle colic vessels (and extrinsic nerves traveling with these vessels to innervate the proximal colon), remained in continuity with the small intestine. These vessels were stripped of investing adventitia using optical magnification to complete the extrinsic denvervation of the small intestine. Then the bowel was transected just distal to the ligament of Treitz and 5 cm proximal to the ileocecal junction, completing the disruption of intrinsic (enteric) continuity of the jejunoileum with the duodenum proximally and with the large intestine distally. End-to-end jejunojejunostomy and ileoileostomy, respectively, at the sites of intestinal transection restored intestinal continuity. The dogs were managed in a similar manner postoperatively, as described earlier, for the cannula placement.

Conduct of Experiments

Each experiment was performed while the dog rested comfortably in a Pavlov sling. The dogs were fasted overnight before each experiment but were allowed access to water ad libitum. Only one experiment was performed per day on an individual dog.

To evaluate absorption, a catheter assembly consisting of a short infusion port and a 15 cm aspiration catheter was inserted distally into the colon via the proximal cannula; the distal cannula was opened to collect the effluent from the distal end of the study segment. This modification of a triple-lumen perfusion technique allowed measurement of absorption in a 35 cm in situ colonic segment. Water (100 ml) was flushed into both the proximal and distal cannulas to clear the cannulas and colonic study segment of stool. A warmed (39° C) isosmolar, ileal-like basal solution (all in mEq/L; $Na^+ = 130$, $K^+ = 10$, $Cl^- = 115$, and $HCO3^- = 25$) without and with glucose (10 mmol/L) and containing the nonabsorbable marker polyethylene glycol (PEG; 5 g/L) labeled with ^{14}C-PEG was then infused at 5 ml/min. The (proximal) aspiration catheter withdrew intraluminal contents at 1 ml/min, leaving approximately 4 ml/min of test solution and small bowel contents entering the colon from the small intestine. The benefit of this technique is that it allowed us to determine accurately the volume and electrolyte concentration of the luminal fluid actually entering the 35 cm colonic test segment in a dynamic fashion, which would take into consideration changes in proximal inflow that might occur throughout the test period. Samples were collected at 1-hour intervals throughout the 5-hour experiment. A steady state was established over the first hour; the remaining 4 hours were considered experimental time and were divided equally into 2 hours of fasting measurements and 2 hours of postprandial experiments. Steady state was confirmed by a relatively constant concentration of PEG at the proximal aspiration catheter. After completion of the 2-hour fasting experiment, the dogs were fed a 550 Kcal meal consisting of pork liver and cream that they consumed rapidly within 3 minutes; the subsequent 2 hours comprised the postprandial experimental period. Experiments were repeated four times in each dog for each of the infusates without and with glucose. Each dog was restudied at 2 and 12 weeks after extrinsic denervation.

Morphometric Analysis

Two 1×1 cm transmural biopsy samples of the colon were taken at the time of cannula implantation in the proximal and distal colon from the sites where the two cannulas were placed into the bowel. After completion of the 12-week postdenervation experiments, biopsies were again taken from the colonic study segment both proximally and distally for comparison. The biopsy segments were pinned with the mucosa exposed and fixed in formalin. Three sections were cut from each biopsy specimen and stained with hematoxylin and eosin. Crypt depth was determined by means of a light microscope with an ocular reticule. Using methods described previously,[14] three areas of each of the three cut sections from an individual biopsy specimen were evaluated giving a total 27 measurements per biopsy; the values were averaged for each dog at each site in both the baseline and postdenervation biopsy specimens. Poorly oriented biopsies were excluded.

Analysis of Data

Concentrations of the nonabsorbable marker ^{14}C-PEG in the infusate, proximal aspirate, and effluent were measured using liquid scintillation techniques (Beckman LS 7800; Beckman Instruments, Fullerton, CA). Sodium and potassium concentrations were determined by means of flame photometry (Beckman Kline Flame; Beckman Instruments), and chloride concentrations by a chloridometer (Corning 920 M Chloridometer; Corning Scientific Instruments, Medfield, MA). Absorptive fluxes of water (μl/cm/min) and electrolytes (μEq/cm/min) were calculated in the test segment using the corrections of absorption based on the concentration changes of the nonabsorbable marker, as described previously.[15,16] The mean values of the individual test results from each dog were used to calculate a mean value for each time period (baseline, 2 weeks, 12 weeks).

Statistical Analysis

The six dogs served as their own controls with measurements at 2 and 12 weeks after extrinsic denervation compared to baseline measurements (0 weeks). Comparisons of net absorptive fluxes for water and electrolytes across the three time points were performed using analysis of variance (ANOVA) separately both for the infusion solutions without and with glucose and for the fasted and postprandial states. At each time point, comparisons between the mean values pooled during fasting and during the postprandial states were compared using Student's *t* test for paired data separately for the infusion solutions without and with glucose. Comparisons were also made between the first and second postprandial hour. In addition, similar comparisons between the solutions without and with glucose were made at each time point during the postprandial periods to determine whether the glucose in the solution altered the postprandial response. Values in the text are presented as the mean ± standard error of the mean with significance accepted at the 0.02 level (Bonferonni correction for three related comparisons).

Table 1. Net absorptive flux of water in fasting and postprandial (PP) states using basal and glucose solutions

	Neurally intact		Time after in situ neural isolation of jejunoileum			
	Baseline		2 wk		12 wk	
Solution	Fasting	PP	Fasting	PP	Fasting	PP
Basal	6.1 ± 1.1	6.4 ± 1.3	6.2 ± 1.9	6.6 ± 2.3	7.4 ± 2.1	8.4 ± 1.7
Glucose	7.2 ± 1.4	7.5 ± 1.5	8.8 ± 1.5	9.5 ± 1.8	8.4 ± 2.0	7.1 ± 2.0

Water flux in μl/cm/min, mean ± SEM, n = 6 dogs; no significant differences noted across time points or between fasting and postprandial values at any time point; $P > 0.02$.

RESULTS

General Health of the Dogs

All dogs tolerated placement of the proximal and distal colonic cannulas, as well as the in situ extrinsic denervation of the small bowel. Placement of cannulas did not appear to alter the appetite of the dogs or cause weight loss in any dog. After neural isolation of the small bowel, three of the dogs lost weight (mean weight loss 0.7 kg among the dogs that lost weight), but all three dogs regained the weight lost by the 12-week time point. Three dogs required operative replacement of one cannula each between the 2-week and 12-week time points; this reoperation did not interfere with the conduct of any experiments, and the length of the test colonic segment did not change.

Absorptive Experiments: Effect of Denervation

Net colonic absorptive fluxes of water in the fasted state and in the first and second hours of the postprandial states (in μl/cm/min) are shown in Table 1 without and with glucose in the infusate. There were no differences from baseline in the net fasting absorptive fluxes of water in the proximal colon at 2 weeks or 12 weeks after extrinsic denervation of the small bowel either with or without glucose in the infusate solution. The addition of 10 mmol/L glucose to the basal solution also did not change the net absorptive flux of water in the colon at baseline or 2 or 12 weeks after extrinsic denervation of the small intestine. Similar patterns occurred in the postprandial state for both infusate solutions. The net colonic absorptive fluxes of electrolytes followed similar patterns as for water (Table 2). Fluxes of Na^+, K^+, and Cl^- did not differ across time points either during fasting.

We also examined the changes in the concentration of nonabsorbable marker in the proximal aspiration catheter as a monitor of proximal small bowel enteric inflow. There were no important changes in PEG concentrations either during fasting or after feeding (data not shown). Because we had shown no changes in absorptive flux in the proximal colon, this observation also shows that there were no dramatic or important changes in the volume of small bowel contents entering the colon postprandially.

Absorptive Experiments: Fasting vs. Postprandial State

As shown in Table 1, water flux in the postprandial state did not change from values in the fasted state either at baseline or at 2 or 12 weeks after extrinsic denervation of the small bowel. Similarly, when glucose was added to the infusate solution, again no differences in net absorptive flux of water at any time point between the fasted and postprandial states could be demonstrated. Also, there were no changes in the net absorptive flux of electrolytes between the fasting and postprandial states using the basal solution (Table 2).

Table 2. Net absorptive flux of electrolytes in fasted and postprandial (PP) states using basal solution

	Neurally intact		Time after in situ neural isolation of jejunoileum			
	Baseline		2 wk		12 wk	
Infusate	Fasting	PP	Fasting	PP	Fasting	PP
Na^+	0.73 ± 0.15	0.73 ± 0.19	0.78 ± 0.27	0.79 ± 0.30	0.96 ± 0.30	1.05 ± 0.22
K^+	0.03 ± 0.01	0.04 ± 0.01	0.05 ± 0.02	0.04 ± 0.03	0.05 ± 0.02	0.04 ± 0.02
Cl^-	0.85 ± 0.14	0.82 ± 0.17	0.84 ± 0.24	0.94 ± 0.31	0.94 ± 0.28	1.17 ± 0.23

Na^+, K^+, and Cl^- flux in μEq/cm/min, mean ± SEM, n = 6 dogs; no significant differences noted across time points or between fasting and PP values at any time point; $P > 0.02$.

Postprandial Absorption: Comparison of the First and Second Hours

Figure 1, *A* shows the net absorptive flux of water with the basal solution (without glucose) in the fasting state along with the postprandial state divided into first and second hours. At baseline and at 2 and 12 weeks after extrinsic denervation, there were no differences in the colonic absorptive flux of water between the first and second hours postprandially. There were also no differences in the flux of water between the fasting state and either the first or second postprandial hour when compared separately at any time point. When glucose was added to the infusate, a similar pattern was found (Fig. 1, *B*).

Morphometric Analysis

Table 3 shows crypt depth from the proximal and distal colon before and 12 weeks after extrinsic denervation of the jejunoileum. The proximal biopsy specimens showed no difference in crypt depth between baseline and 12 weeks after denervation. After denervation, there was an increase in the crypt depth in the distal colon compared to baseline ($P < 0.016$). Data from two dogs were excluded from analysis because of poor orientation of the biopsies; thus n = 4 dogs for this comparison.

DISCUSSION

In an enterically intact, extrinsically innervated, in situ canine colonic segment, we found no change in the net absorptive flux of water or electrolytes after our preparation of complete extrinsic denervation of the jejunoileum. Also, the net absorptive flux of water in this segment of colon did not increase in the early postprandial state either before or after extrinsic denervation, whether or not the infusate contained glucose. Interestingly, crypt depth in the distal colon was increased 12 weeks after extrinsic denervation of the je-

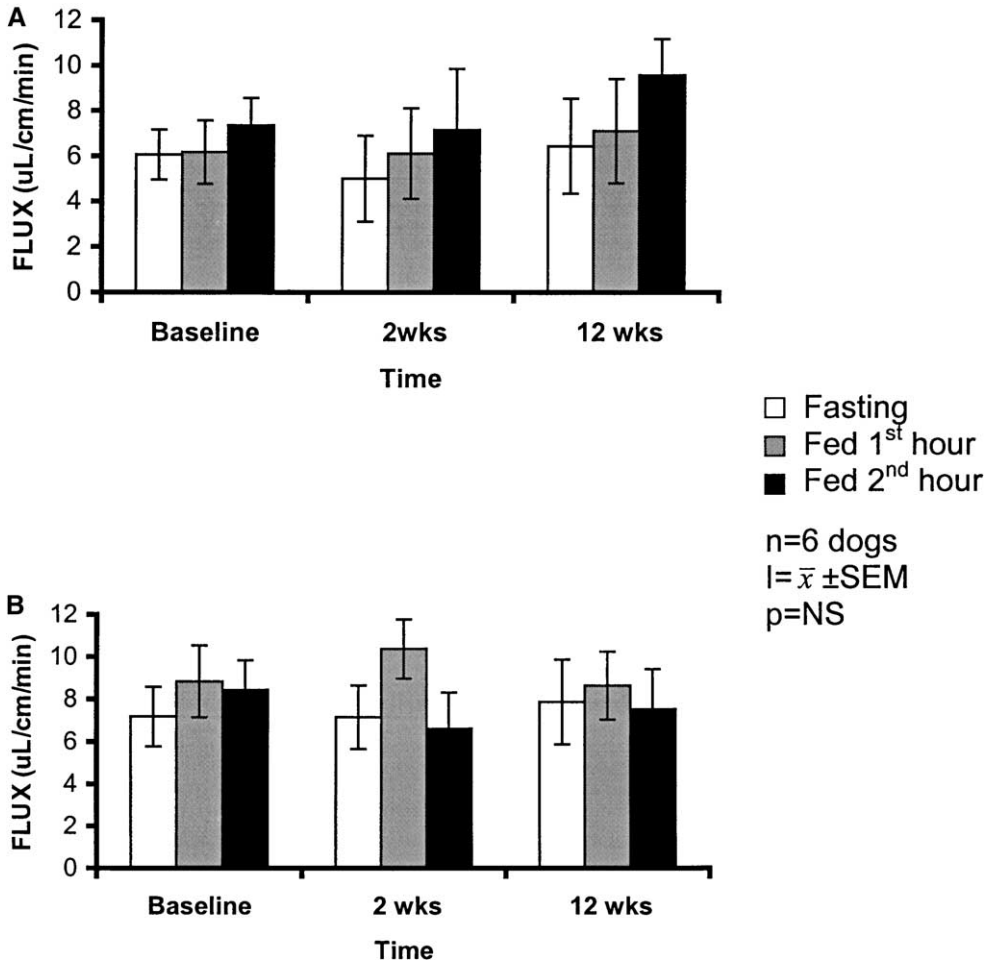

Fig. 1. Net absorptive flux of water comparing the fasted state to the first and second postprandial hours using basal solution (0 glucose) (**A**) and basal solution containing 10 mmol/L glucose (**B**).

Table 3. Morphometric measurement of crypt depth from proximal and distal colon before and 12 weeks after in situ neural isolation of the jejunoileum

Colonic site	Baseline	12 wk
Proximal	700 ± 25	815 ± 50
Distal	660 ± 15	865 ± 45*

Values are in μm, mean ± SEM, n = 4.
*Differs from baseline; $P = 0.016$.

junoileum compared to baseline. These findings suggest that changes in the net absorption of water and electrolytes in the colon do not play a major role in the diarrhea that complicates clinical SBT. Moreover, we could not demonstrate a postprandial augmentation of colonic absorptive function, thereby challenging the importance of previous work with enterically isolated colonic loops.

A poorly understood diarrhea occurs after SBT both in the clinical setting and in experimental models in large animals.[13,15,16] Using a canine model of autotransplantation of the small bowel, we have shown previously that transient decreases in the net absorptive fluxes of water and electrolytes occur in the jejunum[6] and in the ileum.[7] These relatively small changes in absorption of water and electrolytes induced by extrinsic denervation of the jejunoileum do not seem to be of a magnitude large enough to explain the profuse diarrhea that occurs after this procedure. Previous work[7] also demonstrated a decrease in ileal absorption of bile salts after this canine model of jejunoileal autotransplantation, but there was no marked increase in secretion into the lumen suggestive of a secretory diarrhea of small bowel origin. No previous studies have evaluated the effect of small bowel denervation on absorption in the colon. We hypothesized that the transient decrease in bile salt absorption from the ileum and subsequent presence of bile salts in the colon might cause a secretory diarrhea and therefore decrease the net absorptive fluxes of water and electrolytes from the colon after autotransplantation of the small bowel. However, contrary to this hypothesis, we found no change in the net absorptive fluxes of water and electrolytes from the neurally intact colon after this model of jejunoileal autotransplantation either during fasting or early after feeding, suggesting that the colon is not contributing to the diarrhea via either a bile salt–induced colonic secretion or some other neurally mediated mechanism secondary to small bowel denervation. Similarly, lack of change in concentration of the nonabsorbable marker at the site of proximal colonic aspiration demonstrates the lack of a marked change in ileal contents entering the colon. Previous work has shown a transient decrease in the absorptive fluxes of water and electrolytes from an enterically isolated proximal segment of colon after selective extrinsic denervation of this colonic segment.[17] Thus, although extrinsic denervation of the proximal colon decreases net absorptive flux of water in the colon, denervation of the small intestine with preservation of extrinsic innervation to the colon does not change net absorption of water or electrolytes in the colon.

To further evaluate colonic absorptive function, we measured absorption in the fasted and early postprandial states before and after this preparation of selective extrinsic denervation of the entire jejunoileum. Postprandial augmentation of net absorption is well characterized in the small intestine,[18–20] but postprandial absorptive changes in the colon are less well established.[10,11] Our laboratory has demonstrated an increase in absorption from an enterically isolated proximal canine colonic segment, but only when glucose was present intraluminally.[12] This augmentation occurred rapidly within the first 2 hours after ingestion of a meal, which is consistent with prior studies of isolated loops of proximal[21] and distal canine colon[11] and enterically intact pig colon.[10] The present study failed to demonstrate any postprandial augmentation of absorption in the first 2 hours after a high-calorie meal ingested orally either before or after extrinsic denervation of the small bowel and with or without glucose in the infusate.

In our previous work and that of others in the canine colon, an enterically isolated colonic loop was used for study; the current work specifically used an in situ, enterically intact segment of colon. Previous findings suggest that enteric content or enteric neural continuity may be important in determining absorptive function in the colon and may affect the occurrence of postprandial augmentation of colonic absorption as well. Postprandial augmentation of absorption has been shown previously to be stimulated, at least in part, by a neurohormonal mechanism involving peptide YY.[21,22] However, this augmentation of absorption seems to depend on other factors (possibly enteroendocrine) that may be influenced by proximal enteric content that would be absent chronically in enterically isolated segments; in addition, chronic enteric isolation also leads to a disuse atrophy that itself may alter normal physiologic function.[23] We monitored postprandial absorption for only 2 hours, because we were specifically interested in the effects of ingestion of a meal in the early postprandial period. Although we cannot determine by our study design whether a postprandial augmentation occurs more than 2 hours after the meal, the other work in the enterically isolated colon showed augmented absorption to occur within the first 2 hours.

The morphometric measurements demonstrated an increase in crypt depth in the distal part of the colonic segment after selective extrinsic denervation of the jejunoileum but failed to show a similar change in the proximal colon. We had originally hypothesized that an increase in crypt depth or mucosal height would result in an increased capacity for absorption of water and electrolytes in the colon and thus serve as a mechanism to compensate for the transient decrease in absorption in the small intestine. The lack of similar changes in the proximal colon and the lack of an augmentation in colonic absorption by 12 weeks after denervation of the small bowel challenge this hypothesis. However, the suggested segmental increase in crypt depth in the colon after small bowel denervation demonstrates a structural adaptation in the colon to small bowel denervation that is, as yet, unexplained.

In summary, this study showed no change in the net absorption of water or electrolytes from an in situ proximal 35 cm of colon after selective extrinsic denervation of the jejunoileum. Moreover, we could not demonstrate postprandial augmentation of water or electrolyte absorption in the colon 2 hours after a meal either before or after jejunoileal denervation, questioning the importance or relevance of previous findings in enterically isolated colonic segments. These findings suggest that the intact colon does not contribute to the profuse early postoperative diarrhea after SBT during fasting or postprandially. From this study it seems that the colon is not involved primarily in causing the diarrhea.

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Inhibition of the Phosphatidylinositol 3′–Kinase Signaling Pathway Increases the Responsiveness of Pancreatic Carcinoma Cells to Sulindac

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Nonsteroidal anti-inflammatory drugs (NSAIDs) may be effective treatment for pancreatic cancer. We have previously demonstrated that NSAIDs suppress pancreatic cell growth in vitro by inhibiting cell cycle progression but have little effect on apoptosis. In fact, we have shown that NSAIDs, in some instances, increase Akt phosphorylation in human pancreatic carcinoma cells suggesting activation of the phosphatidylinositol 3′–kinase (PI3K)–Akt survival (antiapoptotic) pathway. We subsequently examined the effects of treating the human pancreatic cancer cell lines BxPC-3 and PaCa-2 with a specific inhibitor of the PI3K/Akt pathway, LY294002, in the presence or absence of the NSAID sulindac. The growth effects of sulindac (250 to 500 μmol/L) and/or LY294002 (1 to 100 μmol/L) were determined by a colorimetric proliferation assay and cell counts. The combination of low-dose LY294002 (10 μmol/L) and sulindac enhanced the growth inhibitory effects of sulindac in BxPC-3 and PaCa-2 cells. Treatment of both cell lines with the LY294002/sulindac combination altered the cell cycle distribution as determined by flow cytometry and also lowered the apoptotic threshold as measured with an enzyme-linked immunosorbent asssay to detect DNA fragmentation. These effects were associated with changes in the expression and/or phosphorylation level of proteins and kinases that regulate cell cycle progression and apoptosis. Taken together, our results suggest that inhibition of the PI3K/Akt signaling pathway may sensitize pancreatic tumor cells to therapy with NSAIDs such as sulindac. (J GASTROINTEST SURG 2003;7:354–363.)

KEY WORDS: Akt, PI3′-kinase, NSAIDs, pancreatic cancer

Nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit the enzyme cyclooxygenase (COX). COX catalyzes the conversion of arachidonic acid to prostaglandins and plays a central role in the inflammatory response. In addition to their anti-inflammatory properties, NSAIDs have also recently been shown to demonstrate antiproliferative and antineoplastic activities. For example, NSAIDs inhibit the growth of colon cancer cells in vitro, as well as suppress the growth of chemically induced colon carcinomas, in animal model systems, demonstrating the chemopreventative potential of NSAIDs.[1,2] Epidemiologic studies have also shown an association between prolonged NSAID use in humans and a reduction in the risk of colon cancer by 40% to 50%.[3] Furthermore, the NSAIDs sulindac and celecoxib can induce colonic polyp regression in patients with familial adenomatous polyposis, an inherited condition leading to colorectal cancer.[4,5] Whether the known ability of NSAIDs to inhibit COX and therefore prostaglandin production is required for their antineoplastic effects is not clear. Other cellular targets of NSAIDs have been identified including nuclear factor–kappa B and cGMP phosphodiesterase.[6] NSAIDs may therefore mediate their inhibitory effects by targeting multiple signaling pathways within the cell. Elu-

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cidation of these pathways will provide insight into the mechanism of NSAID action and how to modulate these pathways to increase the efficacy and further minimize the toxicity of NSAID therapy for the treatment of cancer.

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States with nearly identical incidence and mortality rates.[7] Other than surgical resection for early-stage disease, few viable options exist for patients with pancreatic cancer. Chemotherapy is the primary treatment option for most patients with pancreatic cancer, but even the most promising chemotherapeutic drugs, such as gemcitabine, have minimal positive impact on patient survival.[8] Clearly, novel targets and treatments must be identified. Previous work from our laboratory and others demonstrated that NSAIDs inhibit the growth of pancreatic adenocarcinoma cell lines in vitro, suggesting that NSAIDs may be effective treatment for pancreatic cancer.[9–11] In addition, we have recently shown that NSAIDs mediate their growth inhibitory effects in vitro primarily by altering cell cycle progression but have little effect on apoptosis in pancreatic cancer cells.[12] In the pancreatic cancer cell lines analyzed, NSAIDs appear to mediate their effects in a COX-2– and prostaglandin E_2–independent fashion[12] (and unpublished results). Thus other cellular signaling pathways may be utilized by NSAIDs to mediate their growth inhibitory effects.

The phosphatidylinositol 3–kinase (PI3K) signaling pathway is known to play a critical role in regulating cell survival. The serine/threonine kinase known as Akt or protein kinase B lies downstream of PI3K and has been shown to control the balance between cell survival and programmed cell death.[13] Akt can be activated by phosphorylation to inhibit apoptosis. One of the known targets of Akt is a proapoptotic member of the Bcl-2 family, BAD, which on phosphorylation by Akt is inactivated.[14] In the present study, we investigated the role of the PI3K/Akt pathway in mediating the inhibitory effects of NSAIDs in pancreatic cancer cells. We found that growth inhibitory concentrations of NSAIDs unexpectedly did not decrease Akt phosphorylation, and in some cases even increased phosphorylation. Increased Akt phosphorylation suggests activation of the PI3K/Akt pathway that may protect pancreatic tumor cells from undergoing NSAID-induced apoptosis. Conversely, inhibition of this survival pathway would be predicted to sensitize pancreatic cancer cells to drug treatment by promoting cell death. Pharmacologic inhibition of the PI3K/Akt pathway was achieved by treatment with the specific PI3K inhibitor LY294002. Effects on cell growth, cell cycle, apoptosis, and gene expression were determined after treatment with LY294002 and sulindac, either alone or in combination. Our results demonstrate that inhibition of the PI3K/AKt pathway enhances the inhibitory effects of the NSAID sulindac in pancreatic carcinoma cells.

MATERIAL AND METHODS

Cell Culture and Drug Treatment

Human pancreatic cancer cell lines BxPC-3 and PaCa-2 were obtained from American Type Culture Collection (Manassas, VA) and cultured as recommended. The NSAIDs sulindac (Sigma, St. Louis, MO), indomethacin (Sigma), and NS-398 (Biomol; Plymouth Meeting, PA) were dissolved in dimethyl sulfoxide (DMSO). The PI3K inhibitor LY294002 (Calbiochem, La Jolla, CA) was also dissolved in DMSO. For single-drug and combination treatment studies, compounds at the indicated concentrations or the solvent (DMSO) were added to cells plated the previous day.

Cell Growth

Cells (10^3/well) were plated in a 96-well plate and the following day, inhibitors were added. On day 3 after the addition of the drug, cell growth was assayed using a colorimetric proliferation assay, CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega Corp., Madison, WI). The percentage of cell growth was calculated relative to the growth of control-treated cells (100%). Each experimental point was set up in triplicate.

Alternatively, on day 3 after the addition of the drug, cells were harvested by trypsinization, stained with trypan blue, and counted manually with a hemacytometer. Cell growth was determined by averaging the cell counts and expressed as a percentage of the number of cells in the DMSO solvent control sample (set to 100%).

Western Blotting

Cells were lysed in RIPA buffer (phosphate-buffered saline solution, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium docecyl sulfate, 1 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 1 mmol/L Na_3VO_4), and the supernates were obtained. Cell lysates (10 μg total protein) were resolved by sodium docecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 10% or 4% to 20% gradient gels (Invitrogen, San Diego, CA) and transferred to Immobilon P membranes. The blots were probed with primary antibodies specific for the following proteins: phosphorylated extracellular signal–regulated kinase (ERK1/2), phosphorylated/total Akt, and phosphorylated/total p38 mitogen-activated protein (MAP) kinase (Cell Signaling, Beverly, MA);

total ERK1/2 (SC-94), p27, and actin (C-11) (Santa Cruz Biotechnology, Santa Cruz, CA); cyclin D1 and p21 (NeoMarkers, Inc., Fremont, CA); bcl-xl (Trevigen, Inc., Gaithersburg, MD); and bcl-2 (Transduction Laboratories, San Diego, CA) according to the manufacturer's protocol followed by ECL detection (Amersham, Piscataway, NJ).

Cell Cycle Analysis

Cells were plated in six-well plates, and the following day inhibitors were added for either 24 or 48 hours. Cells floating in the media were combined with the adherent cell layer, which was trypsinized. Cells (5×10^5) were washed, pelleted, and then incubated with 2 mg/ml RNase A in phosphate-buffered saline (200 μl) and 0.1 mg/ml propidium iodide in 0.6% Nonidet P-40 in phosphate-buffered saline (200 μl) on ice for 30 minutes. Samples were immediately analyzed by flow cytometry. Cell cycle phase distribution was determined using Modfit software to analyze DNA content histograms.

Apoptosis

Cells were plated in duplicate in 96-well plates (5×10^3/well). After drug treatment for 48 hours, apoptosis was measured using the Cell Death Detection Enzyme-Linked Immunosorbent Assay (ELISA) (Roche, Indianapolis, IN) to quantitatively determine the amount of cytoplasmic histone–associated DNA fragments induced by cells undergoing apoptosis. Cell lysates were placed into streptavidin-coated microtiter plates and incubated with antihistone-biotin and anti-DNA–peroxidase antibodies for 2 hours. After washing and incubation with substrate, the plates were read at 405 nm/L to quantitate the amount of nucleosomes bound to the plate. Relative apoptosis was determined by a ratio of the average absorbance of the treatment wells to the average absorbance of the control wells.

Statistical Analysis

Statistical significance between the combination treatments and either single agent alone was determined by a two-tailed Student's *t* test with a 95% confidence interval.

RESULTS

Effect of NSAIDs on Akt Phosphorylation in Pancreatic Cancer Cells

The effect of NSAID treatment on the PI3K/Akt survival pathway was determined in pancreatic tumor cells. Two pancreatic carcinoma cell lines, BxPC-3 (COX-positive) and PaCa-2 (COX-negative), were treated with the NSAIDs sulindac, indomethacin, and NS-398 for 4 hours at concentrations previously shown to be growth inhibitory.[10] Lysates were prepared and analyzed by Western blotting to detect phospho-Akt as a measure of PI3K/Akt pathway activation. NSAID treatment at early time points (1 and 4 hours) did not decrease Akt phosphorylation and, in fact, increased Akt phosphorylation in all cases at the 4-hour time point, with the exception of sulindac-treated BxPC-3 cells (Fig. 1 and unpublished results). After sulindac treatment for 24 hours, phospho-Akt was elevated in both cell lines, suggesting sustained rather than transient

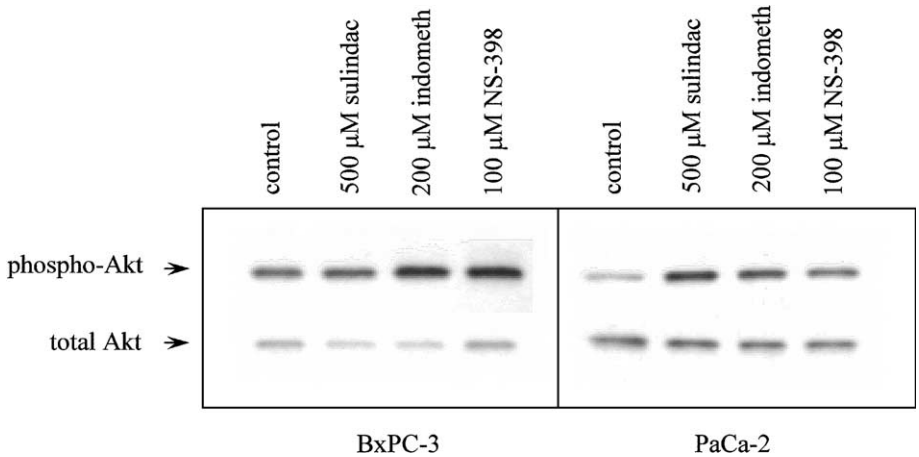

Fig. 1. Effect of NSAIDs on phosphorylation of Akt. BxPC-3 and PaCa-2 cells were treated with the NSAIDs as indicated for 4 hours. Cell lysates were prepared and analyzed by Western blotting with specific antibodies to detect phosphorylated Akt and total Akt levels. A representative blot is shown.

changes induced by sulindac in this signaling pathway. Taken together, our results show that rather than decreasing Akt phosphorylation, as expected from their growth inhibitory effects, NSAIDs may increase Akt phosphorylation, thus activating signaling pathways that protect pancreatic cancer cells from undergoing apoptosis. Because cell fate is determined by the balance between progrowth/survival (antiapoptotic) vs. proapoptotic pathways, inhibition of the PI3K/Akt survival pathway in pancreatic cancer cells may increase their sensitivity to NSAIDs by promoting cell death.

Growth Inhibition by the PI3′–Kinase Inhibitor LY294002

To inhibit the PI3K pathway in pancreatic cancer cells, BxPC-3 and PaCa-2 cells were treated with a specific pharmacologic inhibitor of this pathway, LY294002.[15] On day 3 after treatment, cell growth was monitored by means of a colorimetric proliferation assay (Fig. 2). LY294002 inhibited BxPC-3 and PaCa-2 pancreatic tumor cell growth in a dose-dependent manner (LY294002 IC_{50} = 20 μmol/L for PaCa-2 and 30 μmol/L for BxPC-3 cells). Inhibition of PI3K was confirmed by immunoblotting to detect phosphorylation of the downstream kinase target Akt (Fig. 2, *bottom panel*). Treatment with two concentrations of LY294002 for 24 hours decreased Akt phosphorylation dose dependently in both cell lines, confirming inhibition of the PI3K pathway.

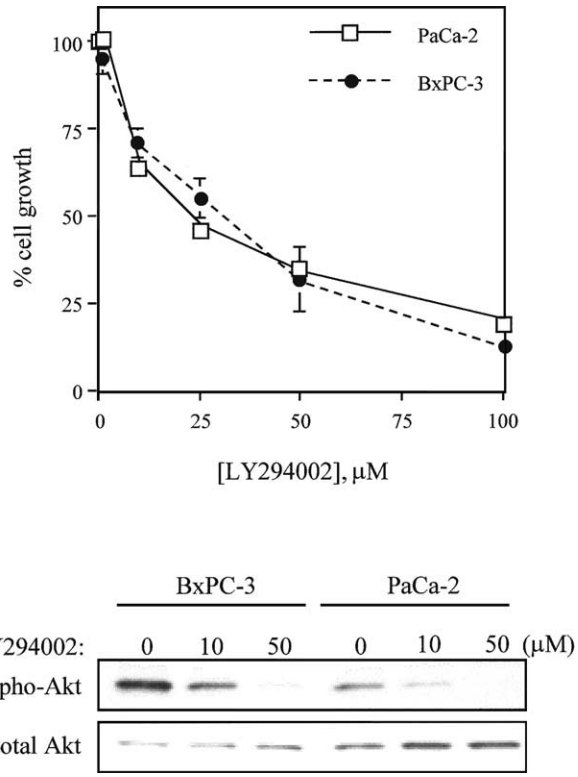

Fig. 2. Effect of the PI3K inhibitor LY294002 on pancreatic cell growth. PaCa-2 and BxPC-3 cells were treated with the indicated concentrations of LY294002. On day 3 following the addition of drugs, cell growth was determined by a colorimetric proliferation assay. Percentage of cell growth was determined relative to control-treated cells (100%). Means ± standard deviation from at least two independent experiments performed in triplicate are shown. Cell lysates were prepared after drug treatment for 24 hours and analyzed by Western blotting to detect phosphorylated and total Akt levels (*bottom panel*).

Growth Effects of LY294002 and Sulindac, Alone or in Combination

For combination inhibitor experiments, the NSAID sulindac was evaluated because sulindac, as well as celecoxib, is in clinical use for the prevention and treatment of colon cancer. To determine whether inhibition of the PI3K signaling pathway would increase the sensitivity of pancreatic tumor cells to sulindac, BxPC-3 and PaCa-2 cells were treated with LY294002 and sulindac, either alone or in combination. Doses of LY294002 (10 and 25 μmol/L) were chosen, which inhibited pancreatic cell growth between 25% and 50%, as shown previously. Sulindac concentrations were chosen on the basis of a dose response showing an IC_{50} of 250 μmol/L.[10] Cell counts were determined on day 3 after treatment, and the percentage of cell growth was calculated relative to control-treated cells (Fig. 3). Each compound alone inhibited the growth of both cell lines. The addition of LY294002 to sulindac increased the growth inhibitory effects over sulindac alone in BxPC-3 and PaCa-2 cells (Fig. 3, *A*). The inhibitory effects of the combination are additive. Akt phosphorylation after 24 hours of treatment with LY294002 in combination with sulindac was suppressed comparable to LY294002 alone in both cell lines, as confirmed by Western blot analysis (Fig. 3, *B*). Total levels of Akt were not significantly altered by inhibitor treatment for 24 hours.

Cell Cycle Effects of LY294002 and Sulindac, Alone or in Combination

To determine whether the growth inhibitory effects of the inhibitors are caused by changes in cell cycle progression, cell cycle analysis was performed on the cells after treatment. Specifically, BxPC-3 and PaCa-2 cells were treated with LY294002 and sulindac for 24 or 48 hours. Cells were harvested, stained

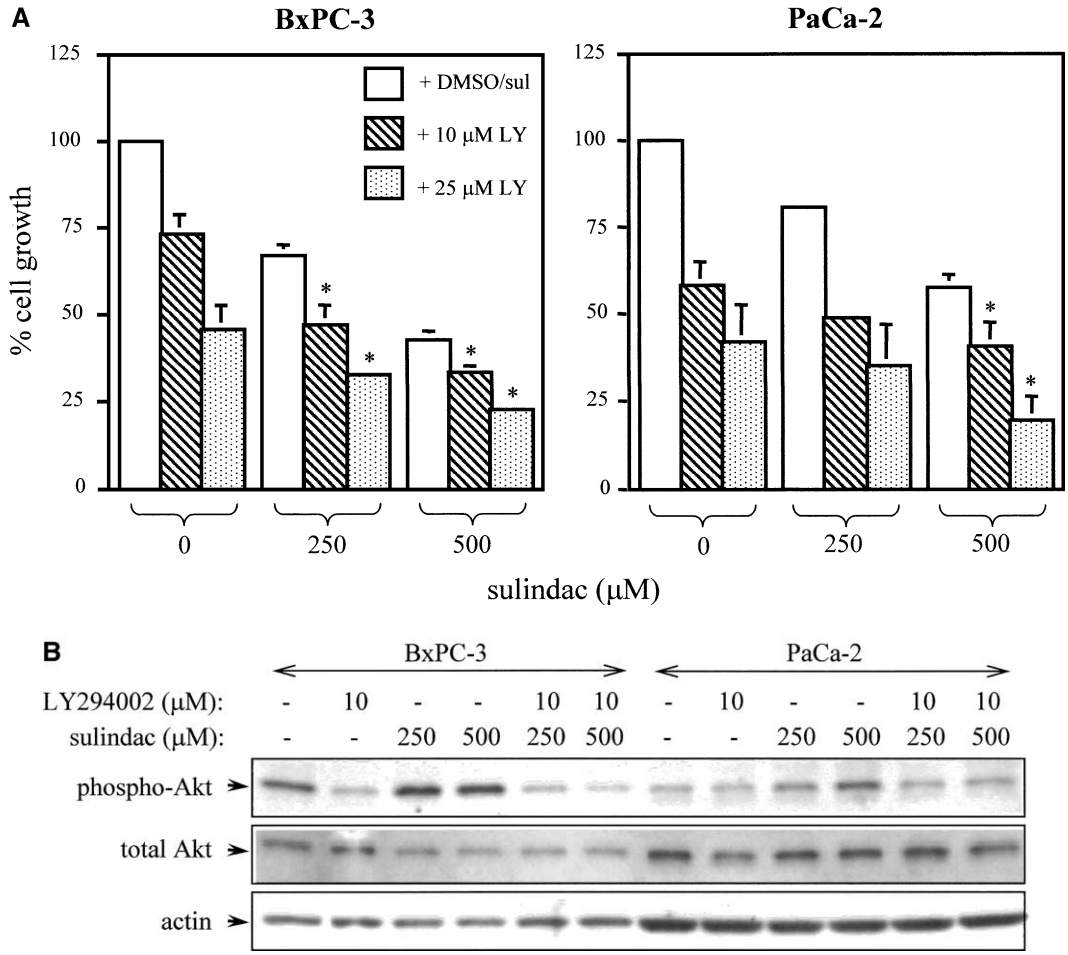

Fig. 3. Effect of LY294002 or sulindac, alone or in combination, on pancreatic cell growth. **A**, BxPC-3 and PaCa-2 cells were incubated with LY294002 (10 μmol/L = hatched bars; 25 μmol/L = stippled bars) and sulindac (250 μmol/L and 500 μmol/L), alone or in combination. After 3 days, cells were harvested and counted manually with a hemacytometer. Cell growth was calculated by averaging the cell counts and expressed as percentage of cell growth relative to the control value (set to 100%). The means ± SD from at least three separate experiments counted in duplicate are shown. *$P < 0.05$ vs. each agent alone. **B**, Cell lysates were prepared after treatment for 24 hours and analyzed by Western blotting to detect phosphorylated Akt and total levels of Akt. Actin levels were determined to confirm equal protein loading. A representative blot is shown.

with propidium iodide, and analyzed by flow cytometry (Table 1). Treatment of BxPC-3 cells with two different doses of either LY294002 or sulindac for 24 hours caused the cells to accumulate at the G_0/G_1 and/or G_2/M phase (Table 1, *A*). The combination of LY294002 and sulindac caused BxPC-3 cells to accumulate mainly at the G_2/M phase. After 48 hours, cell cycle changes in BxPC-3 cells treated with LY294002 and/or sulindac persisted with a relative shift in accumulation to the G_0/G_1 phase at the lower combinations (Table 1, *B*). At the highest combination, the BxPC-3 cells accumulated at G_2/M. PaCa-2 cells treated with LY294002 for 24 hours accumulated at the G_0/G_1 phase (Table 1, *C*). Treatment with the combination of LY294002 and sulindac resulted in G_0/G_1 accumulation of PaCa-2 cells at the lower combinations and G_2/M accumulation at the highest combination. By 48 hours, the cell cycle phase distributions of LY294002- and/or sulindac-treated PaCa-2 cells had returned to normal, suggesting that the cell cycle effects in PaCa-2 cells are more transient than those in BxPC-3 cells (data not shown). Taken together, our results demonstrate that the growth inhibitory effects of the inhibitors alone or in combination can be attributed, in part, to cell cycle alterations in both cell lines.

Apoptosis Induced by LY294002 and/or Sulindac

We have previously shown that pancreatic carcinoma cells are relatively resistant to undergoing apoptosis in response to NSAIDs including sulindac.[10]

Table 1. Cell cycle phase distribution of BxPC-3 and PaCa-2 cells treated with LY294002 and sulindac, alone or in combination*

Treatment	%G_0/G_1	%S	%G_2/M
A. BxPC-3 (24 hr)			
BxPC-3, control	47.1 ± 5.5	37.4 ± 3.9	15.5 ± 1.9
LY10	59.8 ± 3.1†	18.3 ± 1.1†	21.9 ± 2.6†
LY25	54 ± 6.8	8.0 ± 1.1†	38 ± 5.8†
sul250	64.6 ± 5†	17.0 ± 2.8†	17.4 ± 2.3
sul500	56.2 ± 6.5	13.7 ± 2.8†	29.4 ± 5.6†
LY10 + sul250	61.5 ± 4.8†	8.4 ± 1.3†	30.1 ± 4.3†
LY10 + sul500	41.6 ± 11.6	17.5 ± 7.4†	40.8 ± 16.3†
LY25 + sul250	42.6 ± 6.3	9.4 ± 0.8†	48 ± 7.2†
LY25 + sul500	33.5 ± 3.4	22.3 ± 1.9†	44.1 ± 1.5†
B. BxPC-3 (48 hr)			
BxPC-3, control	56.6 ± 5.5	29.1 ± 4.8	14.2 ± 1.2
LY10	52.4 ± 2.6	32.6 ± 1.7	14.9 ± 2
LY25	67.1 ± 1.8†	16.6 ± 2.7†	16.2 ± 3.1
sul250	65.9 ± 6.1	24.6 ± 4.3	9.4 ± 2.5
sul500	78.9 ± 2.2†	11.4 ± 0.8†	9.7 ± 1.7
LY10 + sul250	81.5 ± 1.7†	8.6 ± 1.4†	9.6 ± 2.1
LY10 + sul500	69.7 ± 4.1†	10.7 ± 2.3†	19.6 ± 2.3†
LY25 + sul250	74.2 ± 1.2†	9.6 ± 1.9†	16.2 ± 0.6†
LY25 + sul500	54 ± 12	18.3 ± 9.2	27.6 ± 2.8†
C. PaCa-2 (24 hr)			
PaCa-2, control	39.5 ± 1.5	16.2 ± 0.9	44.2 ± 3
LY10	45.9 ± 1.8	9.2 ± 0.8†	44.8 ± 2.6
LY25	49.5 ± 2.1†	4.9 ± 1.1†	45.5 ± 3.2
sul250	39.4 ± 0.8	16.5 ± 1.3	44.1 ± 2.1
sul500	42.6 ± 2.1	14 ± 1.1	43.4 ± 3.3
LY10 + sul250	47.2 ± 1.9†	8.4 ± 2.2†	44.3 ± 4.1
LY10 + sul500	39 ± 4.9	12.8 ± 2.6	48.1 ± 2.3
LY25 + sul250	47.9 ± 2.5†	6.9 ± 0.6†	45.1 ± 3.1
LY25 + sul500	30.8 ± 3.8	6.6 ± 0.7†	62.6 ± 3.1†

*BxPC-3 and PaCa-2 cells were treated with LY294002 (LY, 10 or 25 μmol/L) and sulindac (sul, 250 or 500 μmol/L) alone or in combination for 24 hours (**A** and **C**) or 48 hours (**B**). Cells were harvested, stained with propidium iodide, and analyzed by flow cytometry. Cell cycle phase distributions were calculated using Modfit software.
†$P < 0.05$ vs. control.

To determine whether treatment with the combination of LY294002 together with sulindac could promote programmed cell death in pancreatic carcinoma cells, apoptosis was measured in BxPC-3 and PaCa-2 cells treated with the inhibitors alone or in combination. Apoptosis was measured after 48 hours of treatment with the use of an ELISA to detect DNA fragmentation (Fig. 4). LY294002 alone did not induce apoptosis in either cell line. Sulindac alone at 500 μmol/L induced slightly more apoptosis relative to control-treated cells. Treatment with the combination of LY294002 and sulindac caused BxPC-3 and PaCa-2 cells to undergo more cell death than either compound alone, as shown by the increase in relative apoptosis. These effects are synergistic. These results suggest that inhibition of the PI3K pathway increases the growth inhibition of sulindac by lowering the apoptotic threshold.

Changes in the Expression of Regulatory Proteins and in Kinase Phosphorylation

We have shown that inhibition of the PI3K pathway increases the inhibitory effects of sulindac in both BxPC-3 and PaCa-2 cells by affecting cell cycle progression as well as apoptosis. To understand how the inhibitory effects are mediated at the protein level, the expression of cell cycle regulatory proteins was determined by Western blot analysis. Lysates were prepared after treatment with LY294002 and/or sulindac for 24 hours. Treatment of BxPC-3 cells with LY294002 or sulindac alone increased the expression of the cyclin-dependent kinase inhibitors p21 and p27 (Fig. 5, *A*). Treatment of BxPC-3 cells with the LY294002/sulindac combination increased p21 and p27 protein levels over treatment with either agent alone. Sulindac (500 μmol/L) treatment

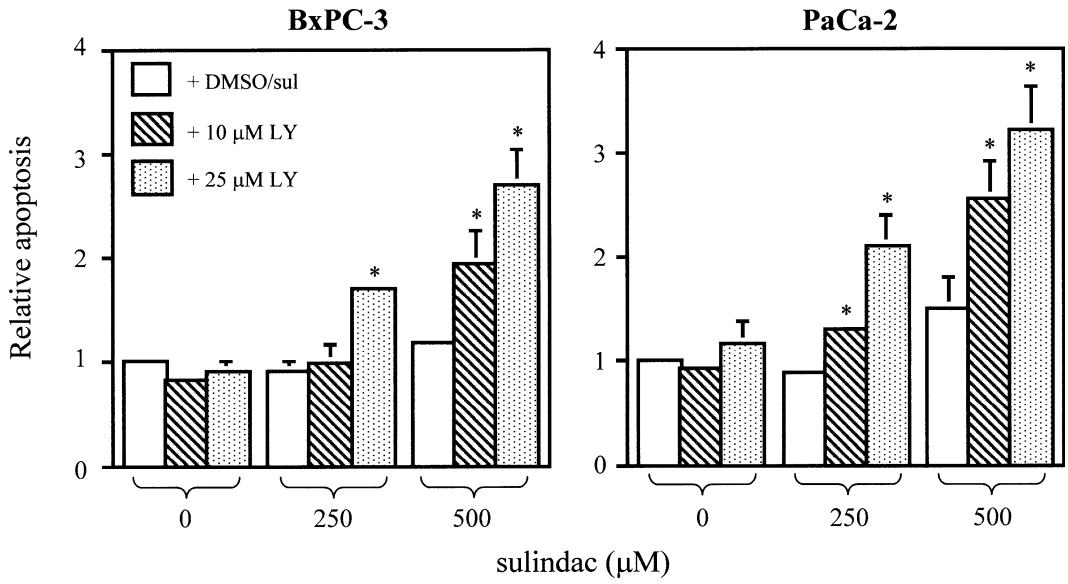

Fig. 4. Apoptosis induced by LY294002 or sulindac, alone or in combination. BxPC-3 and PaCa-2 cells were incubated with LY294002 (10 μmol/L = hatched bars; 25 μmol/L = stippled bars) and sulindac (250 μmol/L and 500 μmol/L), alone or in combination. After 48 hours, apoptosis was measured using an enzyme-linked immunosorbent assay to detect DNA fragmentation. Relative apoptosis was determined compared to the level of apoptosis detected in control-treated cells (set equal to 1). Means ± SD from two independent experiments performed in duplicate are shown. *$P < 0.05$ vs. each agent alone.

of BxPC-3 cells decreased the expression of another cell cycle regulatory protein, cyclin D1, which regulates progession through the G_1 phase. LY294002 treatment alone had no effect on cyclin D1 expression but did increase cyclin D1 suppression in combination with the 500 μmol/L dose of sulindac. In PaCa-2 cells, LY294002 in combination with sulindac markedly potentiated the decrease in cyclin D1 levels but did not increase p21 or p27 expression over the single-agent treatments. These results are consistent with the ability of LY294002 and sulindac to alter cell cycle progression in both cell lines but through distinct molecular circuitry.

To determine the effect of inhibitor treatment on pathways that influence cell fate, the activation of two MAP kinase family members was evaluated by monitoring the extent of phosphorylation. Phosphorylation, and therefore activation of the ERK1/2 by MAP kinase (MEK1/2), ultimately regulates the expression of genes involved in cell growth and survival.[16] In PaCa-2 cells, phospho-ERK1/2 levels increased after treatment with sulindac alone for 24 hours (Fig. 5, *B*). The addition of LY294002 potentiated this increase in phospho-ERK1/2 at the 500 μmol/L dose of sulindac, reflecting sustained activation of ERK1/2 in response to LY294002/sulindac inhibition. The level of phosphorylated ERK1/2 was also slightly elevated in BxPC-3 cells treated with sulindac (500 μmol/L) alone for 24 hours. Phosphorylation of p38 MAP kinase, a proapoptotic kinase,[16] was increased by sulindac alone as well as by the combination in both cell lines. Total levels of ERK and p38 MAP kinase did not change with any of the treatments. In addition, no change in the expression of the apoptotic regulatory protein Bcl-2 was detected in either cell line (data not shown).

To determine the persistence of the effects on the PI3K/Akt and MEK/ERK pathways, cell lysates were analyzed after inhibitor treatment for 48 hours. After 48 hours, the level of phospho-Akt in both cell lines was no longer elevated by sulindac alone as seen at 24 hours (Figs. 3, *B* and 6). Also, 48 hours' combination treatment decreased Akt phosphorylation below that seen with either LY294002 or sulindac alone. Furthermore, total Akt protein levels were decreased by sulindac alone and combination treatments in the BxPC-3 cell line at 48 hours, in contrast with unchanged levels at 24 hours. ERK1/2 phosphorylation was decreased in BxPC-3 cells after 48 hours treatment with LY294002 in combination with 500 μmol/L sulindac. In PaCa-2 cells, elevated phospho-ERK levels observed at 24 hours had returned to baseline by 48 hours (Figs. 5, *B* and 6). The relative decrease in phosphorylation of ERK and Akt by 48 hours suggests less activation of these antiapoptotic pathways that may therefore lower the apoptotic threshold in the combination-treated cells.

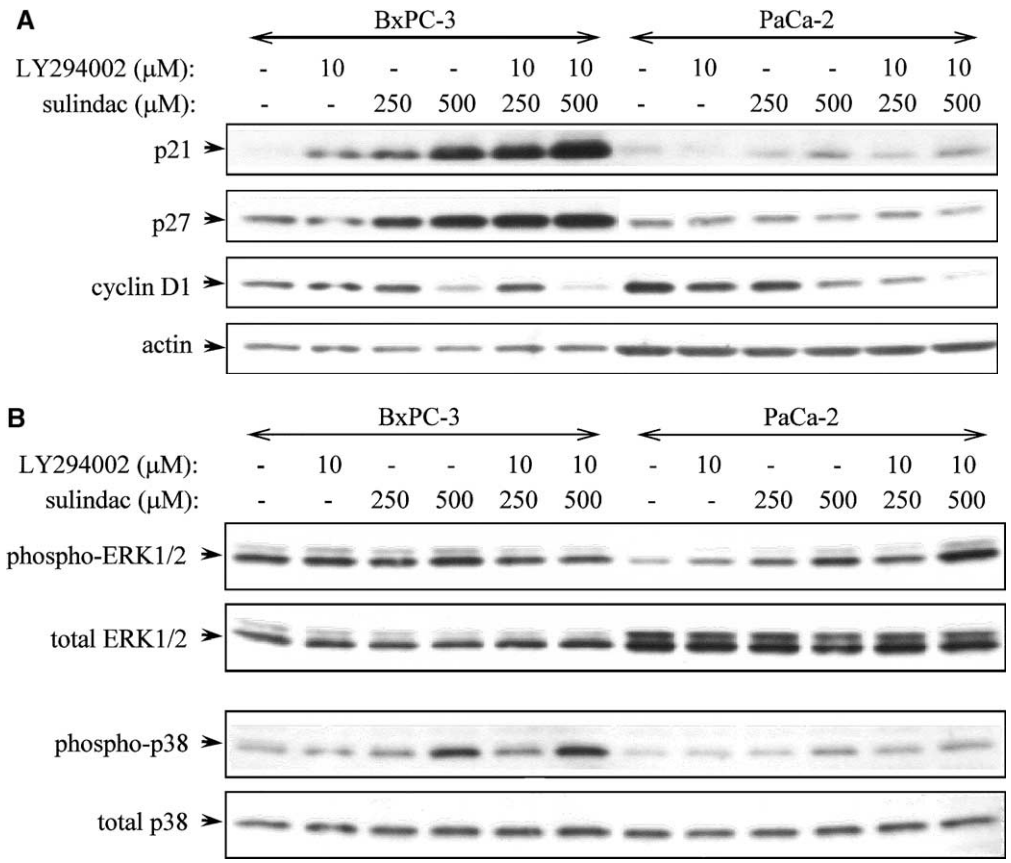

Fig. 5. Effect of LY294002 and sulindac, alone or in combination, on the expression of cell cycle regulatory proteins or kinase phosphorylation. BxPC-3 and PaCa-2 cells were treated with LY294002 and/or sulindac for 24 hours. Cell lysates were prepared and analyzed by Western blotting to detect the expression of the indicated cell cycle regulatory proteins (**A**) or kinase phosphorylation (**B**). Actin levels were determined to confirm equal protein loading. A representative blot is shown.

DISCUSSION

The antineoplastic properties of NSAIDs suggest their potential for use as chemotherapeutic and/or chemopreventative agents. NSAIDs have been shown to inhibit the growth of tumor cells in vitro by causing cell cycle arrest and inducing apoptosis.[17,18] Two different signaling pathways, ERK and Akt, have been reported to be involved in mediating NSAID-induced apoptosis. The NSAID sulidac and its metabolites were recently shown to induce apoptosis and inhibit ERK phosphorylation in colon cancer cells; treatment with an MEK inhibitor potentiated the apoptotic effect of the NSAIDs.[19] These findings demonstrate that inhibition of the MEK/ERK pathway by sulindac metabolites leads to programmed cell death. The cell survival pathway involving the antiapoptotic kinase Akt also plays a role in NSAID-induced apoptosis. Specifically, treatment of human prostate cancer cells with the COX-2 inhibitor celecoxib induced apoptosis and blocked phosphorylation of Akt, demonstrating that Akt inhibition may be involved in initiating programmed cell death.[20] Thus the activation level of the PI3K-Akt and MEK/ERK pathways may dictate whether cells undergo apoptosis in response to NSAIDs.

In the present study, we evaluated the role of the PI3K/Akt pathway in mediating the growth inhibitory effects of NSAIDs in pancreatic cancer cells. We report that rather than being inhibited, the level of phosphorylated Akt unexpectedly increased in response to NSAID treatment. The resistance of pancreatic tumor cells to undergoing NSAID-induced apoptosis may be associated with the inability of NSAIDs to decrease Akt phosphorylation and therefore inhibit this antiapoptotic pathway. Moreover, phosphorylation and activation of Akt by NSAIDs in pancreatic tumor cells may actually protect the cells from undergoing apoptosis. Therefore inhibition of the PI3K/Akt pathway would be expected to augment NSAID-induced apoptosis and increase sensi-

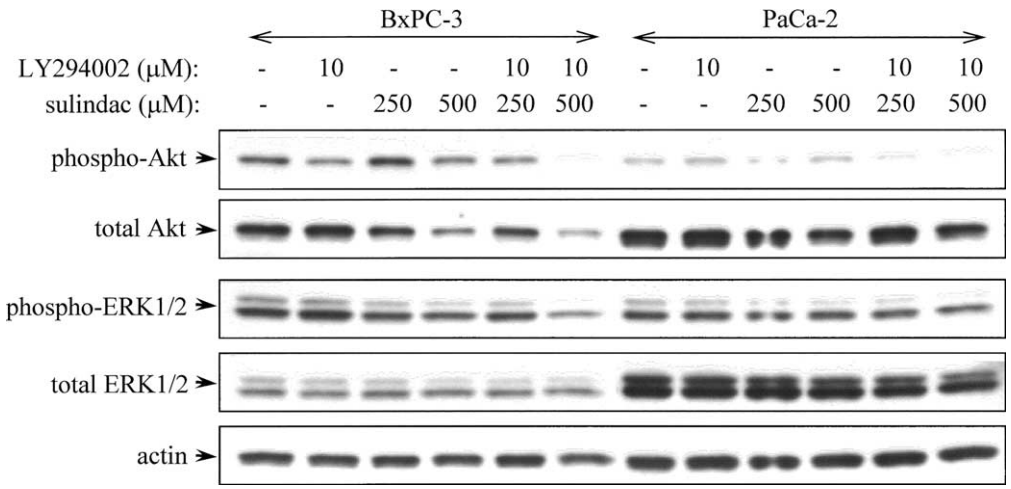

Fig. 6. Kinase phosphorylation after treatment with LY294002 and/or sulindac for 48 hours. Cell lysates were prepared after drug treatment for 48 hours and analyzed by Western blotting to detect levels of phosphorylated Akt and ERK1/2. Actin expression and total Akt and ERK1/2 levels confirmed equivalent protein loading. A representative blot is shown.

tivity to these agents. This hypothesis was tested in the present study by treating two different human pancreatic carcinoma cell lines with the NSAID sulindac and the PI3K inhibitor LY294002.

Treatment with LY294002 augmented the growth inhibitory effects of sulindac alone in both BxPC-3 and PaCa-2 cells. We also showed that the growth inhibitory effects of the LY294002/sulindac combination were mediated by altering cell cycle progression as well as lowering the apoptotic threshold. Changes in the expression of the cyclin-dependent kinase inhibitors p21 and p27 as well as cyclin D1 were enhanced by treatment with the combination compared with the individual agents in BxPC-3 cells; in PaCa-2 cells, the expression of cyclin D1 was further decreased by the combination. Modulation of the expression of these cell cycle regulatory proteins correlated with the observed cell cycle and growth inhibitory effects.

Increased ERK phosphorylation was observed after treatment of both pancreatic cell lines with sulindac for 24 hours. This may be an attempt by the cells to overcome the sulindac-induced growth inhibition. Activation of the ERK pathway in pancreatic cells regulates cell growth since we have found that treatment with a specific MEK inhibitor suppresses ERK phosphorylation and cell growth (unpublished results). Decreased phosphorylation of ERK1/2 and Akt by 48 hours combined with increased phosphorylation of p38 MAP kinase by 24 hours may contribute to lowering the apoptotic threshold by inhibiting survival pathways and activating proapoptotic pathways. In addition, total Akt levels were decreased by sulindac alone in BxPC-3 cells and further suppressed in the presence of LY294002. Lower Akt protein levels may also turn off survival signals and therefore lower the apoptotic threshold. Akt and other signaling molecules have previously been shown to be degraded during apoptosis in a caspase-dependent manner.[21] Inhibition of the proapoptotic p38 MAP kinase stimulates proliferation of the pancreatic cancer cell line PANC-1, identifying p38 MAP kinase as a negative regulator in pancreatic cells.[22] Combination inhibitor treatment clearly influences multiple signaling pathways in pancreatic tumor cells, and the ultimate outcome of treatment depends on the additive effects of the various pathways.

As shown in this study, LY294002 and sulindac increase sulindac's growth inhibitory effects in two different human pancreatic carcinoma cell lines. Interestingly, however, inhibition is accomplished by separate but overlapping pathways in the pancreatic cancer cell lineages examined. For instance, common mediators identified here include cyclin D1 and Akt. The other signals appear to be unique to each cell line. Although the molecular circuitry in these two pancreatic cancer cell lines is distinct, they both greatly depend on PI3K/Akt signaling for their survival.

The PI3K pathway has been shown to influence the response of pancreatic cancer cells to chemotherapy. Specifically, inhibition of the PI3K/Akt antiapoptotic pathway enhances gemcitabine-induced apoptosis in pancreatic tumor cells.[23] The potential importance of the Akt pathway in pancreatic cancer is also implicated by the finding that one of the Akt family members, Akt2, is overexpressed in approxi-

mately 20% of human pancreatic ductal adenocarcinomas.[24] Accordingly, NSAID activation of the PI3K/Akt pathway may contribute to the drug resistance of pancreatic cancer cells. The design of NSAIDs or COX inhibitors that inhibit the PI3K/Akt pathway in pancreatic tumor cells would be predicted to induce apoptosis more effectively and thus have greater therapeutic potential in the clinic. Alternatively, a combined chemotherapeutic approach with NSAIDs and PI3K/Akt inhibitors in pancreatic adenocarcinoma may result in more effective tumor kill and allow the use of lower drug doses to minimize toxicity.

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Development of a Therapeutic Adenoviral Vector for Cholangiocarcinoma Combining Tumor-Restricted Gene Expression and Infectivity Enhancement

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Cholangiocarcinoma is an invasive malignancy that is most often unresectable upon diagnosis and unresponsive to chemotherapy and radiation. While adenoviral gene therapy has shown promise in treating many tumors, systemic toxicity and low tumor transduction efficiency have hampered its application in many gastrointestinal cancers. To overcome these difficulties, we have constructed an adenoviral vector utilizing a tumor-specific promoter (TSP) for selective transgene expression and a vector with an RGD-motif in the fiber-knob region for infectivity enhancement. In seeking a TSP for cholangiocarcinoma, Secretory Leukoprotease Inhibitor, Midkine, Gastrin Releasing Peptide, VEGF, Cox-2M, and Cox-2L promoters were configures in adenoviral vectors, and evaluated in cholangiocarcinoma cells lines (Oz and SkChA-1). Luciferase assays demonstrated that Cox-2 promoters (M and L) showed the highest promoter activity, with Cox-2M appearing slightly stronger than Cox-2L. Infectivity enhanced vectors with RGD-motif in the fiber-knob region were also constructed with the luciferase transgene driven by a CMV control and the Cox-2M and Cox-2L promoters. Subsequent luciferase assays comparing the unmodified vectors to the RGD-modified versions demonstrated higher levels of luciferase activity than the RGD-infected cells. This paradigm was then applied to a therapeutic HSV-TK/GCV model by constructing RGD-enhanced HSV-TK vectors driven by Cox-2M and Cox-2L promoters. *In vitro* cytocidal effect analysis confirmed that the RGD-modified, cox-2 (M and L) driven vectors showed a stronger cytocidal effect upon gancyclovir administration than the vectors with wild-type fiber. The Cox-2 promoter demonstrates a favorable selectivity profile for cholangiocarcinoma, and RGD-modification further enhances transduction efficiency. This combination has potential to overcome the obstacles to clinical application of adenoviral gene therapy in cholangiocarcinoma. (J GASTROINTEST SURG 2003;7:364–371.)

KEY WORDS: Cholangiocarcinoma, adenoviral gene therapy, tumor-specific promoter

Cholangiocarcinomas are slow growing, invasive tumors of the intrahepatic and extrahepatic biliary ducts. They constitute approximately 2% of all reported cancers in the United States, with an incidence of 1 to 2 per 100,000 people.[1–3] Although the spread of this cancer is generally very slow and distant metastasis are extremely rare, the prognosis for patients with this disease is dismal. The average 5-year survival is only 10%, with a median survival of 1.5 years.[4] Complete surgical resection provides the only chance for cure, however, only 10% of patients present with early stage disease and are candidates for curative resection.[5] The vast majority of patients have unresectable tumors and succumb to the disease within a year of diagnosis, usually from liver failure or infectious complications of biliary obstruction.[1,2,6,7] Various combinations of brachytherapy, external beam radiation, and/or chemotherapy have resulted in limited gains in medial survival.[5] Thus, a novel approach is needed to improve therapeutic outcome. Gene therapy for this tumor represents such a novel approach.

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The adenovirus has been preferred for cancer gene therapy for a variety of reasons. Adenoviruses can infect a broad range of human cells, both dividing and nondividing, with high gene transfer efficiency.[8,9] Also, adenoviruses have a low pathogenicity, typically causing only mild "cold-type" symptoms. Finally, the widely used E1-deleted adenovirus vector can accommodate large insertions of DNA (up to 7.5 kb) and are stable enough to maintain transgene expression through successive rounds of viral propagation.[10] These benefits, as well as the relative ease of genetic modification and production, provide the adenovirus with a distinct advantage in the field of cancer gene therapy.

Despite the promise, previous studies and Phase I clinical trials with adenoviral vectors have revealed obstacles to clinical application. First, when injected systemically, the adenovirus shows hepatotropism, which can lead to significant hepatic dysfunction and even death when it involves a toxin gene strategy.[11] Thus, systemic treatment of metastatic disease or systemic leakage after locoregional treatment incurs significant risks. Secondly, while adenoviral vectors can infect many human cancer cells, adenoviral transduction efficiency in many gastrointestinal cancers is very low.[12] This is because infectivity largely depends on the presence of the coxsackie adenovirus receptor (CAR) on cellular surface and the expression of CAR has been shown to be extremely low in those tumor cells.[13–15]

To circumvent the issue of adenoviral hepatotoxicity, we applied transcriptional targeting with tumor-specific promoters (TSP). Using this strategy, we can limit the expression of a toxic transgene to only the target cells by selecting proper TSP that is specifically active in the target cells. These TSP sequences do not affect the vector transduction efficiency, however they can regulate the transgene expression of infected cells and eliminate the potential toxicity of ubiquitously expressed toxin genes. To mitigate hepatotoxicity, many different tumor specific promoters with the "liver-off" profile have been developed and utilized in targeting cancer. Some of the most successful promoters include: the midkine (MK) promoter, which has been used to target pediatric solid tumors;[16] secretory leukoprotease inhibitor (SLPI) for cervical and ovarian cancer;[17,18] cyclooxygenase-2 (Cox-2) for pancreatic, colon, and gastric cancer;[19] and gastrin releasing peptide (GRP) and vascular endothelial growth factor (VEGF) for lung cancer.[20–23] However, none of these TSPs have been evaluated in cholangiocarcinoma cells. In this study, we evaluated all of these tumor specific promoters for utility in cholangiocarcinoma, and then incorporated the most suitable promoter into the genomic backbone of our candidate vector.

Secondly, to overcome the problem of low transduction efficiency, we applied our infectivity enhanced vector with RGD-4C motif in HI-loop that allows CAR-independent vector transduction.[26,27] This modification allows the vectors to bind an alternate group of cell surface receptors, the integrins, in addition to the primary CAR receptor. By incorporating an Arg-Gly-Asp (RGD) motif into the HI loop of the viral capsid fiber knob, the vector binds to integrin including: $\alpha 5\beta 1$, $\alpha 8\beta 1$, $\alpha v\beta 1$, $\alpha v\beta 3$, $\alpha v\beta 5$, $\alpha v\beta 6$, and $\alpha v\beta 8$.[24–27] Additionally, these integrins are expressed at increased levels in many tumor cells.[28,29] This modification does not otherwise alter the high level of transgene expression or recombinant genomic stability exhibited by the adenovirus.[27,30] This infectivity enhancement strategy, combined with the transcriptional targeting of our tumor specific promoter, was the basis for our development of a therapeutic vector for use in cholangiocarcinoma.

The challenges to the successful application of adenoviral gene therapy have led to the development of a new generation of adenoviral vectors, with higher infectivity in tumor cells and lower toxicity in the liver. We present the data of one such vector, an infectivity-enhanced, tumor-specific vector developed for therapeutic application in cholangiocarcinoma.

MATERIALS AND METHODS

Cell Lines

The human cholangiocarcinoma cell lines Sk-ChA-1 and Oz were gifts of Dr. A. Knuth, Ludwig Institute for Cancer Research, London, United Kingdom. These cells were maintained in RPMI-1640 medium (Mediatech, Herdon, VA) supplemented with L-glutamine (2 mM), penicillin (1000 IU/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal bovine serum (FBS) (Summit Biotechnology, Ft. Collins, CO) at 37C in a humidified 5% CO_2 atmosphere. MKN-45 and Kato-3 cells (Japanese Collection of Research Bioresources [JCRB] JCRB0254 and JCRB0611, respectively; Tokyo, Japan) were used as controls and were also maintained in RPMI 1640 (Mediatech) supplemented with L-glutamine (2 mM), penicillin (1000 IU/ml), streptomycin (100 μg/ml), and 10% heat-inactivated FBS at 37C in a humidified 5% CO_2 atmosphere. The transformed human embryonic kidney cell line 293 (Microbix, Toronto, Ontario, Canada) was used for viral propagation and titering and maintained in Dulbecco's modified Eagle medium (DMEM; Mediatech) supplemented with L-glutamine (2 mM), penicillin (1000 IU/ml), streptomycin (100 μg/ml), and 5%

heat-inactivated FBS at 37C in a humidified 5% CO_2 atmosphere.

Recombinant Adenoviral Vectors

To analyze gene promoter activity and gene transfer efficiency, recombinant adenoviral vectors encoding the firefly luciferase reporter gene were employed. The control vector, AdCMV Luc, is an E1-deleted, replication-incompetent recombinant adenovirus expressing the transgene luciferase under the ubiquitous cytomegalovirus (CMV) promoter.[19] It was constructed using homologous recombination in *Escherichia coli* using the AdEasy system as previously described.[31] AdMK Luc containing the midkine (MK) promoter,[16] AdCox-2M Luc and AdCox-2L Luc containing the medium and long promoter sequences of cycloxygenase-2 (Cox-2M and Cox-2L) respectively,[19,32,33] AdSLPI Luc with secretory leukoprotease inhibitor (SLPI) promoter,[18] AdGRP Luc and AdVEGF Luc driven by gastrin releasing peptide (GRP), and vascular endothelial growth factor (VEGF) promoters respectively,[20–23] were constructed as previously described. RGD AdCMV Luc is an infectivity-enhanced vector containing the RGD-4C motif in HI-loop of fiber-knob region and the luciferase transgene driven by the CMV promoter.[30] RGD Ad Cox-2M Luc and RGD AdCox-2L Luc, containing the RGD-4C motif and the luciferase transgene driven by Cox-2 promoters, were constructed by recombination as described previously.[19,34] AdCMV TK, AdCox-2M TK, AdCox-2L TK, RGD AdCox-2M TK, and RGD AdCox-2L TK were constructed to express the herpes simplex virus thymidine kinase transgene using the adenoviral vector backbone with wild-type or RGD-modified fiber-knob region under control of the respective promoter regions (Fig. 1).[19,31] The viruses were propagated in the E1-*trans*complementing cell line 293, purified by double cesium chloride density gradient ultracentrifugation and dialysis in phosphate-buffered saline (PBS) with 10% glycerol. The viral preparations were stored at −80C. Titration was performed by plaque forming assay with 293 cells and optical density-based physical titration.[35] The vp/pfu ratios of the RGD modified vectors were 20-30, and those of unmodified vectors were 5-10.

Analysis of Reporter Gene Expression

The firefly luciferase transgene was used for analysis of the relative efficiency of adenoviral vector expression. Twenty-four hours after plating 50,000 cells/well in 24 well plates, 10, 50, 100, 250, or 1000 viral particles/cell were added in 250 μl DMEM with 5% FBS and incubated for 2 hours. This medium was then removed and replaced with cell appropriate complete medium. After 48 hr of incubation, the medium was removed and cells were lysed with cell culture buffer (CCLB) (Promega, Madison, WI) after washing with PBS. The cell lysate was spun (13,000 rpm for 1 min) and the cell extract (1 μl) was added to Luciferase assay reagent (20 μl) (Promega) for analysis of emitted light in a Lumat LB9501 luminometer (1.0 sec) (Berthold Systems, Aliquippa, PA). The results were standardized by protein concentration as determined by the DC protein assay (Bio-rad, Hercules, CA). All assays were repeated in triplicate.

Analysis of Toxin Gene Killing

The cytotoxicity with gancyclovir was analyzed after transduction with each adenoviral vector expressing herpes simplex virus-thymidine kinase. Ad CMV Luc was used as a negative control. Twenty-four hours after plating 3000 cells/well in 96 well plates, the medium was removed and 500 viral particles/cell in 100 μl DMEM with 5% FBS was added. After 5 hr of incubation, this media was replaced with cell appropriate medium supplemented with 0, 10, 100, or 1000 μM concentrations of gancyclovir (GCV; Merck). The cells were incubated for 5 days and the number of surviving cells was analyzed by the MTS method using the Cell Titer 96 Aqueous Non-Radioactive Cell Proliferation Assay as described by the manufacturer (Promega, Madison, WI). Results were measured at a wavelength of 490 nm in an automated E-max spectrophotometer (Molecular Device Corporation, Sunnyville, CA) and standard curves were generated by plating counted cells and calculating for experimental groups using the SOFTmax computer software (Molecular Device Corporation). All experiments were repeated in triplicate and results were displayed as percentages against the number at 0 μM GCV.

RESULTS

Tissue Specific Promoter Activity in Cholangiocarcinoma Cell Lines

To determine a suitable promoter for the gene therapy of cholangiocarcinoma, we evaluated the relative strength of 6 promoters in cholangiocarcinoma cells. Promoter-driven, luciferase-expressing adenoviral vectors were utilized to evaluate each promoter in an adenoviral vector construct, using the cholangiocarcinoma cells (Oz and SkChA-1) (Fig. 2). In both cell lines, the Cox-2 (M and L) promoter demonstrated very high activity,

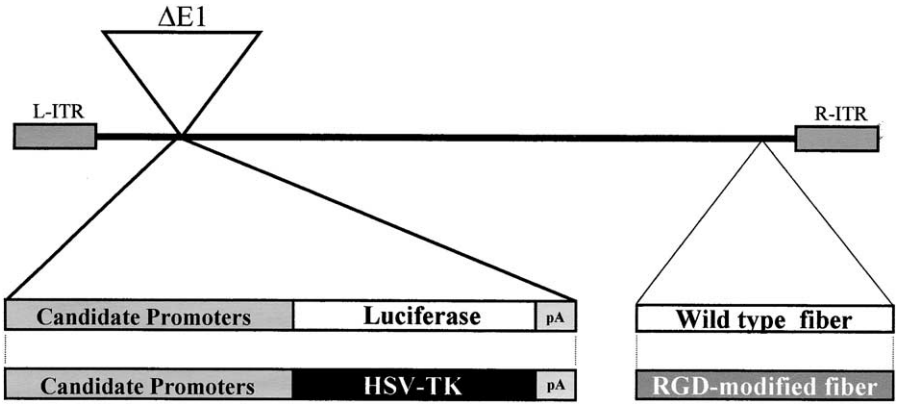

Fig. 1. Structure of the recombinant adenoviral vectors. Adenoviral vectors were constructed by inserting the expression cassettes into the E1-deleted region of the adenovirus-type 5 vector backbone. The following promoters were placed 5′ of the transgene to drive expression: CMV, Cox-2M, Cox-2L, GRP, SLPI, MK, VEGF. Also, RGD-4C motifs including arg-gly-asp (RGD) sequences were configured into the HI-loop of the fiber-knob region of the vector backbone.

in the same order of magnitude as the CMV promoter, which is known to be very strong (Fig. 2). The midkine promoter was active but weaker than Cox-2 promoter. Thus, Cox-2 promoter was the most promising for use in cholangiocarcinoma among the tested promoters. These results are in accordance with the Cox-2 RNA level analyzed by RT-PCR (data not shown).

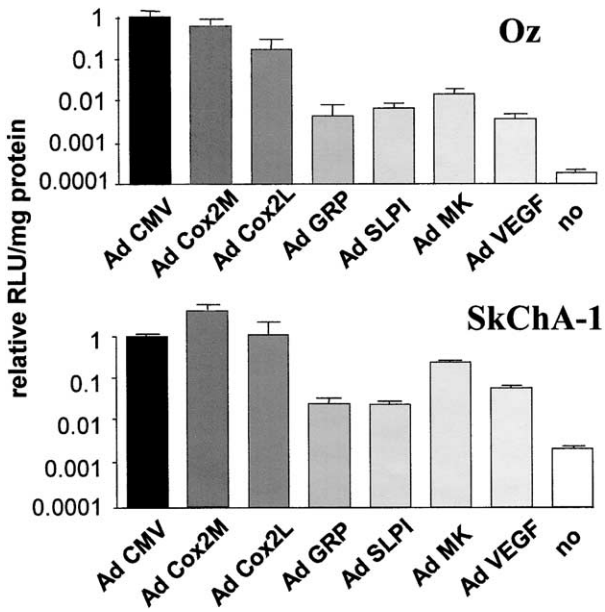

Fig. 2. Candidate promoter activity in cholangiocarcinoma cell lines. Each cholangiocarcinoma cell line (Oz and SkChA-1) was infected with respective candidate promoter-driven luciferase expression vectors (m.o.i. 50), and promoter activity was analyzed by luciferase assay after 48 hours. The results were standardized by protein concentration and displayed relative to the nonspecific CMV promoter. (*Upper panel*, Oz; *lower panel*, SkChA-1.)

Integrin Targeted Enhancement of Transgene Expression

To determine whether incorporation of the RGD-motif in the HI loop of the fiber-knob region would enhance the infectivity of adenoviral vectors in the cholangiocarcinoma cells, we evaluated the transduction efficiency between the vectors with wild-type and RGD-modified fibers using adenoviral vectors with the CMV promoter-driven luciferase expression cassette (Fig. 3). In both cell lines and at all three multiplicities of infection (10, 100, and 1000 viral particles per cell), the RGD modified vectors outperformed the unmodified versions by almost 2 orders of magnitude. Clearly, the RGD modification of the vectors improved transgene expression in these cell lines. These results are compatible with the rich $\alpha v\beta 5$ integrin expression observed by flow cytometric analysis (data not shown).

Combining Infectivity Enhancement and Transcriptional Targeting in an Adenoviral Vector Construct

To determine the functionality of infectivity enhancement in a tumor-specific promoter context, we constructed RGD-modified luciferase-encoding vectors driven by the promoters Cox-2M and Cox-2L.

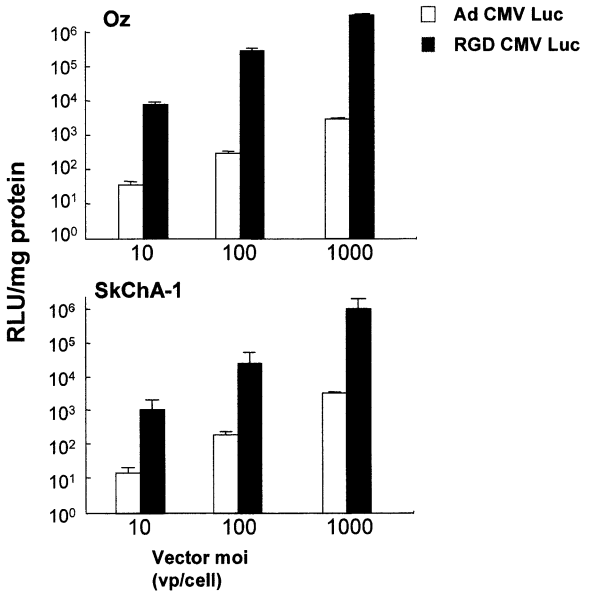

Fig. 3. Infectivity Enhancement with RGD-modified vectors in cholangiocarcinoma cell lines. Each cholangiocarcinoma cell line (Oz and SkChA-1) was infected with CMV promoter-driven luciferase expression vectors with RGD-modified or unmodified fiber (m.o.i. 10, 100, 1000). Luciferase activity was analyzed after 48 hr and results were standardized by protein concentration. (*Upper panel*, Oz; *lower panel*, SkChA-1.)

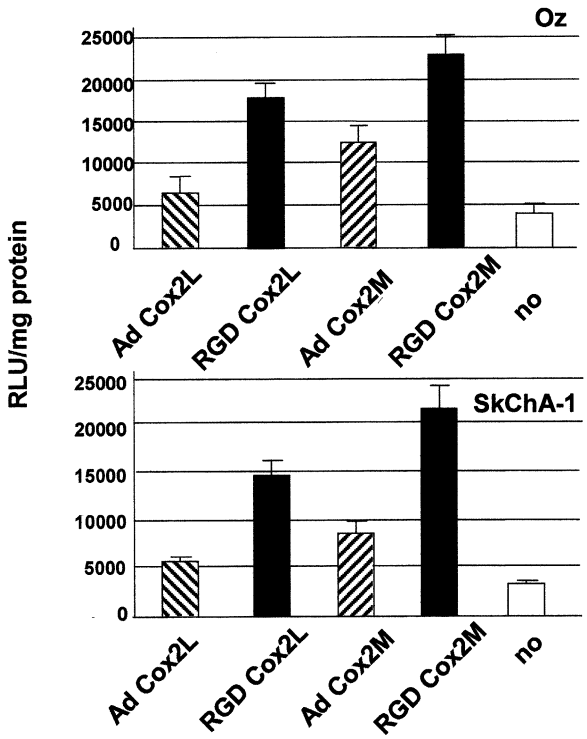

Fig. 4. Efficacy of RGD-modified, Cox-2 promoter-driven vector activity in cholangiocarcinoma cell lines. The cholangiocarcinoma cell lines (Oz and SkChA-1) were infected with Cox-2M or Cox-2L promoter-driven luciferase expression vectors (m.o.i. 50). Luciferase activity was measured at 48 hr and results were standardized by protein concentration. (*Upper panel*, Oz; *lower panel*, SkChA-1.)

At a multiplicity of infection of 50 viral particles per cell, we compared the relative luciferase expression in the cholangiocarcinoma cells infected by either RGD modified or unmodified vectors, driven by Cox-2. The results indicated that the RGD modified vectors were superior to the unmodified versions in both cell lines (Fig. 4). This confirmed the functionality of the combination of infectivity enhancement and promoter-based transcriptional targeting.

Infectivity Enhancement and Transcriptional Targeting in a Therapeutic Adenoviral Vector Construct

To determine whether our results could be translated to therapeutic gains in tumor cell killing, we evaluated infectivity-enhanced, Cox-2 promoter-driven vectors in the toxin gene/prodrug paradigm of herpes simplex virus thymidine kinase/gancyclovir. We compared the unmodified and infectivity-enhanced versions of Cox-2 (M and L) adenoviral vectors encoding the transgene thymidine kinase (Fig. 5). While Cox-2 promoter-driven TK vectors with unmodified fiber (AdCox-2M TK and AdCox-2L TK) showed weak cytocidal effect, those with RGD-modified fiber (RGDCox-2M TK and RGDCox-2L TK) showed significantly stronger cytocidal effect. Of note, in the SkChA-1, more than 70% of the cells were killed with RGD-modified, Cox-2-driven TK-vectors (Fig. 4).

DISCUSSION

Cholangiocarcinoma is a devastating disease that affects approximately 2500 people in the United States each year.[5] Current treatment options are limited and offer little improvement in the overall poor prognosis. Cancer gene therapy offers an alternative approach to this disease. The vast majority of these tumors can be reached endoscopically or *via* the percutaneous-transhepatic route; they are particularly well suited for a locoregional gene therapy approach. Local injection of adenoviral vectors has shown promising results in recent clinical studies.[36] Furthermore, the compartmental nature of the biliary duct system would allow minimal systemic dispersion of the adenoviral vector, which decreases the potential toxicity. We have reported that adenoviral vectors can be delivered to the biliary epithelium in a human liver with

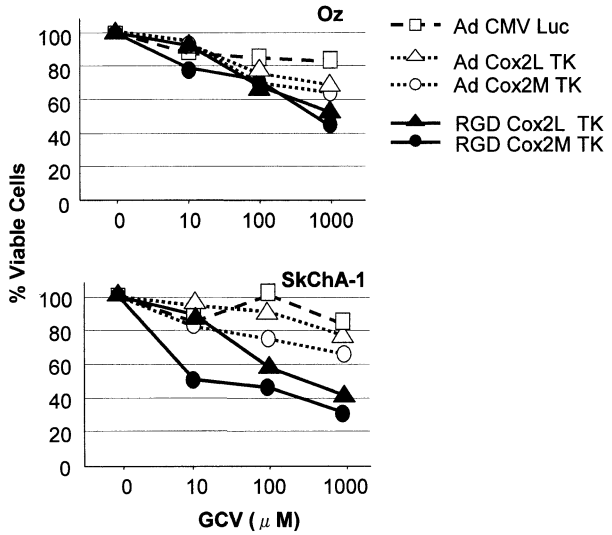

Fig. 5. The cytocidal effect of RGD-modified Cox-2 promoter-driven HSV-TK expression vectors. Each cholangiocarcinoma cell line (Oz and SkChA-1) was infected with Cox-2M or Cox-2L promoter-driven HSV-TK expressing vectors with RGD-modified or unmodified fiber (m.o.i. 500), and exposed to various concentrations of gancyclovir (GCV). After 5 days of incubation, viable cell numbers were analyzed using the MTS method. Results are displayed as percentages versus the number at 0 μM GCV. AdCMV Luc was used as a negative control vector. (*Upper panel*, Oz; *lower panel*, SkChA-1.)

successful transgene expression.[37] If we can develop optimal vectors for this disease, a gene therapy approach can be a very promising option.

Few groups have attempted to study cholangiocarcinoma from a gene therapy perspective, so information on vector targeting in this disease is extremely limited. Furthermore, practically applicable tumor specific promoter for cholangiocarcinoma has not been identified.

In this study, we focused on promoter-based transcriptional targeting and identified the Cox-2 promoter as the most promising promoter for this entity of disease. Several groups have demonstrated cholangiocarcinoma cells have an increased expression of Cox-2.[28,29,38–41] Hayashi et al. performed immunohistochemistry staining and PCR on cholangiocarcinoma cells to demonstrate the relationship between Cox-2 expression and malignant transformation of biliary duct epithelium,[38] in the same manner reported in other gastrointestinal cancers as well.[42,43]

In our work, Cox-2 promoters were the strongest promoters, and Cox-2 M appeared stronger than Cox-2L promoter in most instances (Fig. 2). Thus, Cox-2 M promoter is the leading promoter in the context of the promoter strength in cholangiocarcinoma cells. Our prior work showed that the Cox-2 promoter activity *in vivo* in liver is minimal (1/100 and 1/30000 of CMV promoter for Cox-2M and Cox-2L promoter, respectively).[19] In the context of therapeutic index, Cox-2L promoter indicated the highest promise for its therapeutic utility. Additionally, Cox-2L promoter has enough strength in cholangiocarcinoma cells since Cox-2L promoter-driven TK expression vector showed strong cytocidal effect in suicide gene therapy context (Fig. 5). Thus, Cox-2 L promoter is most promising among "liver-OFF" promoters evaluated in this study.

From the viewpoint of transduction efficiency, we have shown that the RGD-4C motif in HI-loop of fiber knob region greatly augmented the gene delivery with adenoviral vector.[27] Integrin expression in cholangiocarcinoma is reported to be relatively high and the flow cytometry analysis indicated $\alpha v\beta 5$ expression is very high in the cells we used.[44–46] Thus, it is reasonable to have augmented transduction with RGD-modified vectors mediated by CAR-independent, integrin-mediated binding to the target cells. Also, previous work by Reynolds et al. has demonstrated that RGD-fiber modification augments tumor transduction without increasing liver transduction *in vivo*.[34] This data strongly suggests that the infectivity-enhanced vectors are promising for this entity of disease.

CONCLUSION

We have presented the data showing that the major obstacles to targeting gene therapy for cholangiocarcinoma can be overcome. With a combination of currently available vector innovations, we can selectively express transgenes in cholangiocarcinoma cells, at a higher level of infectivity than has been previously possible. Recent trends in adenoviral vector research and design are shifting toward the use of conditionally replicative viruses for targeting cancer. This next generation of adenoviral vectors can provide cell killing as well as lateral spread through conditional viral replication based on the presence of promoter sequences.[8,9] Our work provides strong support for the use of a Cox-2 promoter in future cholangiocarcinoma targeting. Also, the infectivity enhancement afforded by RGD modification will be similarly applicable in vector design, and we hope that these results will translate into a future clinical therapy for cholangiocarcinoma.

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Discussion

Dr. Nagi: Before any questions are asked, let me say, a lot of the current research that we are doing in our lab is not only based on the RGD and the COX-2 promoter, but it is also based on a replicative model of these adenoviruses. We will actually have a poster on display this afternoon regarding that in GI cancers with the replicative model, which adds not only the cell killing, but also that there can be lateral dispersion when the cells are killed and more viruses are released to infect the surrounding cells.

Dr. H.J. Sugerman (Richmond, VA): I presume that, since you had to write this abstract a while ago, that you have been continuing to work. So, between then and now, have you been able to evaluate your animal model at all with your vector and your drug?

Dr. Nagi: A lot of our current work is actually based on these two modifications but in a replicative model, and that is what we have been trying to apply to other GI cancers as well as cholangiocarcinoma. So we have not advanced this specific vector to a mouse model. We wanted to do some primary cells, but they are hard to come by, too.

Incidence and Management of Biliary Pancreatitis in Cholecystectomized Patients: Results of a 7-Year Study

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Data are lacking concerning the frequency of biliary acute pancreatitis in the postcholecystectomy patient. The aim of this study was to identify patients at risk for biliary pancreatitis after cholecystectomy and to describe the therapeutic management of these patients, based on an analysis of 278 unselected patients with acute pancreatitis during a 7-year period. A biliary etiology was presumed in the presence of laboratory findings of cholestasis that could not be explained by another disease, together with the absence of any other known etiology of acute pancreatitis. A biliary cause of disease was found in 132 (47%) of 278 patients. Seventeen (13%) of 132 patients had a history of cholecystectomy. Endoscopic retrograde cholangiopancreatography was performed in all patients with a suspected biliary cause of acute pancreatitis. It showed bile duct stones, microlithiasis, or sludge in 14 patients, and was consistent with typical findings at the papilla of Vater after stone passage in another three patients. No surgical bile duct exploration was necessary. One patient with severe disease and infected pancreatic necrosis died of septic multiorgan failure. (J GASTROINTEST SURG 2003;7:372–377.)

KEY WORDS: acute pancreatitis, etiology, cholecystectomy, endoscopic retrograde cholangiopancreatography, prognosis

Gallstones represent one of the main causes of acute pancreatitis, accounting for 35% to 65% of all cases, depending on the population studied. The clinical relevance of biliary acute pancreatitis is further emphasized by evidence from recent studies demonstrating that a biliary cause of acute pancreatitis is more prevalent than was previously acknowledged. These conclusions are based on the detection of biliary sludge and occult microlithiasis in 67% to 74% of all patients who had what was formerly termed "idiopathic acute pancreatitis."[1–3]

Patients with a biliary etiology should undergo cholecystectomy during their initial hospitalization in order to reduce the risk of recurrent acute pancreatitis or other biliary complications.[4–6] Patients who are left with a gallbladder in situ are at higher risk of developing recurrent biliary symptoms, as compared to patients undergoing cholecystectomy.[7] Although the management of the biliary tract in patients with acute pancreatitis and an in situ gallbladder is well established,[6] data are lacking on the frequency, severity, and therapeutic strategy for management of biliary acute pancreatitis in the postcholecystectomy patient. The aim of this study, therefore, was to identify patients at risk for biliary pancreatitis after cholecystectomy, to assess the causes of biliary acute pancreatitis in these patients, and to describe the therapeutic management of their disease.

PATIENTS AND METHODS

Patients

All patients with acute pancreatitis who were admitted to the Department of Visceral and Transplantation Surgery at the University of Bern, Switzerland, during a 7-year period (from January 1994 to December

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2000) were prospectively entered into a database in a consecutive and unselected manner. The diagnosis of acute pancreatitis was determined by clinical, laboratory, and radiographic parameters. Clinical and laboratory severity staging of acute pancreatitis was carried out using Ranson's prognostic signs,[8] the Acute Physiology and Chronic Health Evaluation (APACHE) II scoring system,[9] and determination of serum C-reactive protein levels. Contrast-enhanced computed tomography was performed within 48 to 96 hours of admission and was repeated weekly in patients whose clinical conditions did not improve. Mild and severe acute pancreatitis was defined in accordance with the Atlanta classification.[10]

Etiology of Pancreatitis

The etiology of acute pancreatitis was determined by a careful history, physical examination, and radiographic and laboratory findings. A *biliary* cause of acute pancreatitis was assumed with laboratory findings of cholestasis, including elevated serum levels of bilirubin (>26 μmol/L), alkaline phosphatase (>120 U/L), γ-glutamyl transferase (>45 U/L), and elevation of serum alanine aminotransferase (ALT) to three times the upper limit of normal (>120 U/L) in the absence of any other disease causing cholestasis (e.g., pancreatic tumor, drug-induced acute pancreatitis, or primary liver disease). Furthermore, the presence of cholelithiasis was assessed by transabdominal ultrasound imaging, and endoscopic retrograde cholangiopancreatography (ERCP) was performed in all patients with a suspected biliary etiology of acute pancreatitis. *Alcohol-related* acute pancreatitis was determined by the patient's history, physical examination results, and laboratory findings, such as elevated γ-glutaryl transpeptidase. *Idiopathic* pancreatitis was diagnosed only after the exclusion of biliary microlithiasis by ERCP and after other rare causes of acute pancreatitis were ruled out, such as hypercalcemia, hyperlipidemia, pancreatic tumors, or other etiological factors (e.g., ischemia, trauma, infection).[11]

Management of Patients With Acute Pancreatitis

All patients with acute pancreatitis were observed in an intermediate care or intensive care unit and treated according to the standards of the Department of Visceral and Transplantation Surgery at the University Hospital of Bern. A treatment strategy based on the severity of the disease and infection of the necrotic pancreatic tissue has been published previously.[12]

RESULTS

Between January 1994 and December 2000, a total of 278 patients (111 women and 167 men; median age 52 years [range 17 to 96 years]) with acute pancreatitis were seen. Patient characteristics and disease severity according to the Atlanta classification are summarized in Table 1. A biliary etiology was found in 132 patients (47%). Ninety-seven patients (35%) suffered from ethanol-induced acute pancreatitis, and in 49 patients (18%) the etiology was classified as "other" or "unknown." All cases of biliary acute pancreatitis were confirmed by ERCP. Of the 132 patients with biliary acute pancreatitis, 17 had a history of prior cholecystectomy (6% of all patients with acute pancreatitis; 13% of all patients with biliary acute pancreatitis) (Fig. 1). In four patients with biliary acute pancreatitis after cholecystectomy, the interval between removal of the gallbladder and recurrent biliary acute pancreatitis was as short as 1 to 4 months, suggesting that bile duct stones overlooked at the time of cholecystectomy were most likely responsible for the recurrent biliary acute pancreatitis. Thus these four patients were not included in the "postcholecystectomy biliary acute pancreatitis" group (see Fig. 1). In the remaining 13 patients (5 men and 8 women; median age 69 years [range 20 to 75 years]), the interval between cholecystectomy and recurrent biliary acute pancreatitis was a median 4 years (range 1 to 25 years).

ERCP findings and laboratory results in 13 patients with "true" biliary pancreatitis are presented in Table 2. In 10 patients bile duct stones and/or sludge was detected, whereas in three other patients the ERCP findings were consistent with typical findings at the papilla of Vater after stone passage, as implied by detection of a torn or swollen papilla. A dilated ductus choledochus (1.5 cm) was found in three cases in the absence of a periampullary tumor. One stone was incarcerated at the level of the papilla.

Therapeutically, eight patients required a papillotomy and concomitant extraction of gallstones or sludge, whereas in one patient (No. 5) with an edematous papilla of Vater, only a papillotomy was performed. In one patient (No. 2) with sludge in the common bile duct, clearance was achieved by passage of a balloon catheter. In the remaining three patients, ERCP may be termed diagnostic because the bile drainage into the duodenum was considered sufficient and no papillotomy was necessary. No surgical bile duct exploration was necessary in any of these patients. Laboratory findings of cholestasis correlated with the ERCP findings. The length of the cystic stump was detectable in only seven patients and varied in length between 3 and 21 mm. With regard to severity of disease, eight patients had severe acute pancreatitis and five had mild disease.

Table 1. Characteristics of 278 patients with acute pancreatitis treated between January 1994 and December 2000

	Biliary etiology (n = 132)	Etiology other than biliary (n = 146)	*P* value
Female	81 (61%)	30 (21%)	$P < 0.01$
Male	51 (39%)	116 (79%)	
Median age (yr)	69 (range 20–96)	41 (range 17–63)	$P < 0.005$
Severity of disease			
Mild	80	84	
Severe	52	62	NS
Mean hospital stay (days)	28 (range 6–231)	24 (2–191)	NS
No. of in-hospital deaths	3	7	NS

NS = not significant.

Three of four patients with pancreatic necrosis of more than 50% of the gland had infected necrosis, as determined by fine-needle aspiration, and thus required surgical intervention by laparotomy, necrosectomy, and continuous retroperitoneal lavage. One patient (No. 6) suffered from sterile pancreatic necrosis and was treated conservatively in the intensive care unit.

Microorganisms isolated from the infected pancreatic tissue included gram-negative germs (No. 11), coagulase-negative staphylococci (No. 9), and both gram-negative and gram-positive organisms and *Candida* (No. 10). Cultures of bile taken preoperatively at the time of ERCP were sterile in all of these three patients.

Clinical conditions such as prolonged fasting and/or rapid weight loss, pregnancy, liver cirrhosis, sickle cell anemia, or solid organ transplantation/cyclosporin medication, which are known to be associated with the formation of sludge and microlithiasis, were not present in any of the 13 patients. With regard to the outcome, one patient (No. 11) with infected pancreatic necrosis died of septic multiorgan failure 131 days after admission. Thus the mortality rate for the postcholecystectomy group was 8% (1 of 13). In the remaining 12 patients, the median length of hospital stay was 15 days (range 9 to 112 days).

Information regarding the index cholecystectomy in 13 patients with biliary pancreatitis after removal of the gallbladder is summarized in Table 3. ERCP and/or intraoperative findings revealed choledocholithiasis or biliary sludge in five patients (5 of 13; 38%).

During a mean follow-up period of 50 months, none of the surviving 12 patients was readmitted to a hospital for recurrent biliary symptoms. In the group of 146 patients with an etiology other than biliary, 14 (9.6%) had a history of cholecystectomy. These patients were excluded from our study because of a clearly established etiology, the lack of laboratory findings consistent with cholestasis, and normal sonographic findings in the biliary tree. Nevertheless, six of these patients underwent ERCP, which confirmed the absence of a biliary cause.

DISCUSSION

Only a limited amount of data are available regarding the frequency and severity of biliary pancreatitis in

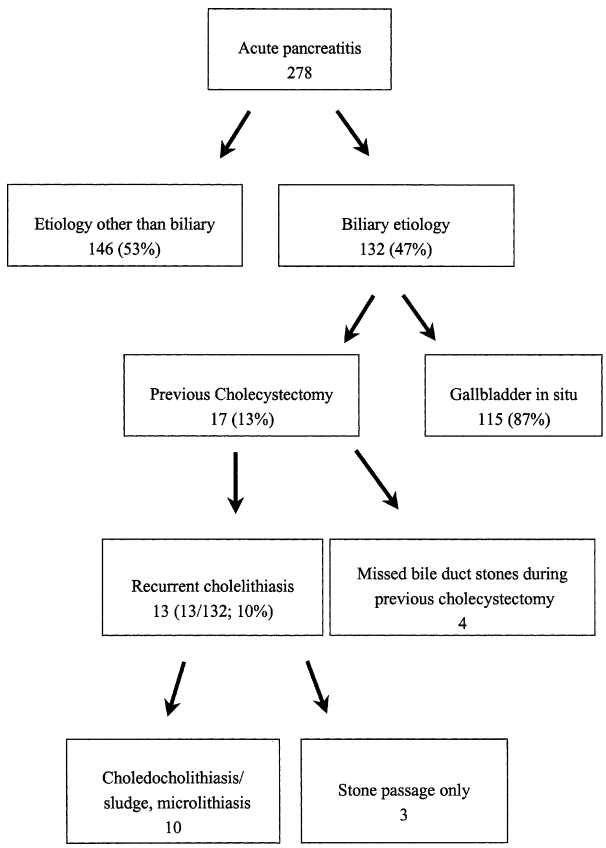

Fig. 1. Patients with acute pancreatitis grouped according to etiology of pancreatitis and treatment of cholecystectomy.

Table 2. ERCP and laboratory findings in 13 patients with biliary pancreatitis after cholecystectomy

Patient	Sex/age (yr)	Disease severity*	Time after cholecystectomy (y)	ERCP findings	Laboratory findings				Therapy	Length of hospital stay (days)
					Bilirubin (<26 μmol/L)	γ-GT (<45 U/L)	Alkaline phosphastase (<120 U/L)	ALT (<41 U/L)		
1	M/32	Mild	3	Choledocholithiasis, cholangitis, dilated biliary ducts	36	197	189	58	ERCP; papillotomy, stone extraction	19
2	M/66	Mild	1	Normal biliary and pancreatic ducts, sludge	41	425	137	378	Diagnostic ERCP only, balloon passage	9
3	F/74	Severe	4	Choledocholithiasis, duodenal diverticulum	11	500	149	134	ERCP; papillotomy, stone extraction	9
4	M/65	Severe	8	Dilated ductus choledochus, choledocholithiasis/ microlithiasis	20	33	57	19	ERCP; papillotomy, stone extraction (microlithiasis)	11
5	F/62	Severe	3	Edematous papilla of Vater normal biliary and pancreatic ducts, no concrements	43	N/A	113	405	ERCP; papillotomy	9
6	F/75	Severe	25	Choledocholithiasis	16	43	65	510	ERCP; papillotomy, stone extraction	29
7	F/60	Mild	8	Bile ducts not dilated, signs of stone passage; torn papilla of Vater	63	390	216	96	Diagnostic ERCP only	10
8	M/20	Mild	9	Dilated biliary ducts, sludge, pneumocholangiography	45	16	127	222	Diagnostic ERCP only	15
9	F/71	Severe/Infected	3	Choledocholithiasis, incarcerated stone	281	412	238	187	(1) ERCP; papillotomy, stone extraction (2) Laparotomy, necrosectomy	71
10	F/70	Severe/Infected	4	Sludge, no choledocholithiasis	39	346	120	652	(1) ERCP; papillotomy, drainage of sludge (2) Laparotomy, necrosectomy	112
11	F/73	Severe/Infected	10	Signs of stone passage; bloody fringed papilla of Vater; dilated common bile duct	28	228	172	220	(1) Diagnostic ERCP only (2) Laparotomy, necrosectomy	131
12	M/69	Severe	2	Choledocholithiasis	56	271	561	311	ERCP; papillotomy and stone extraction	21
13	F/73	Mild	1	Small common bile duct stones	48	210	180	281	ERCP; papillotomy, stone extraction	14

ERCP = endoscopic retrograde cholangiopancreatography; GT = glutamyl transferase; ALT = alanine aminotransferase; N/A = not available.
*Mild vs. severe acute pancreatitis was determined according to the Atlanta criteria. Infected necrosis was detected in cultures obtained by fine-needle aspiration and by intraoperatively collected tissue samples.

Table 3. Procedures and findings during index cholecystectomy performed either at a community hospital or at the university hospital

Patient	Preoperative ERCP/ papillotomy	Choledocholithiasis or sludge at ERCP	Intraoperative cholangiography	Common bile duct exploration	Choledocholithiasis
1	Yes/Yes	Yes	Yes	No	No
2	No	No	No	No	No
3	No	No	No	No	No
4	No	No	No	No	No
5	No	No	No	No	No
6	No	No	Yes	No	No
7	No	No	Yes	Yes	Yes
8	N/A		Yes	Yes	Yes
9	Yes/Yes	Yes	Yes	No	No
10	No	No	Yes	Yes	No
11	N/A		Yes	No	No
12	Yes/Yes	Yes	No	No	No
13	No	No	No	No	No

ERCP = endoscopic retrograde cholangiopancreatography; N/A = not available.
Management of cholelithiasis and indications for preoperative ERCP, intraoperative cholangiography, and common bile duct exploration have changed considerably over time.

patients with a history of a cholecystectomy. The present study, which reviewed data from a prospectively documented series of 278 patients with acute pancreatitis, demonstrates that more than 10% of all cases of biliary acute pancreatitis occur in patients with a history of cholecystectomy. It must be kept in mind, however, that patients with biliary pancreatitis after cholecystectomy, especially those with severe pancreatitis, are more likely to be referred to a tertiary referral center, as represented by this institution. Consequently the prevalence reported in this series may be overestimated. Sungler et al.,[13] in a series of 155 patients with biliary acute pancreatitis, found 22 patients (14%) with a history of prior cholecystectomy. However, no information is given in that report regarding the interval between cholecystectomy and recurrent biliary acute pancreatitis, and some cases in which bile duct clearance was not achieved at the time of cholecystectomy may have been included. In our study, four patients were excluded from the "postcholecystectomy" group because pancreatitis had developed within a few weeks or months after cholecystectomy, suggesting that bile duct stones overlooked at the time of cholecystectomy may have been the cause of the recurrent episode of pancreatitis in these cases.

Sand et al.[14] reported recurrent biliary colics and stones in 9% of 296 patients, and common bile duct stones at the time of cholecystectomy was a risk factor for recurrent biliary tract disease. Four percent (12 of 296) underwent reoperation. In this series no surgical bile duct exploration was necessary because ERCP was successful in all 17 patients. Patients underwent ERCP to assess the biliary tree, to remove calculi and sludge from the common bile duct, and to provide free biliary drainage, usually by means of a papillotomy. In line with this data is a report from Sweden in which endoscopic sphincterotomy for retained or recurrent common bile duct stones in 147 patients after cholecystectomy was found to have a failure rate of only 5.4% (8/147).[15]

Duodenal diverticula were found to be associated with an increased risk of recurrent bile duct stones after cholecystectomy.[16] However, of 157 patients with duodenal diverticula and a history of cholecystectomy, only 13 (8%) had an episode of biliary pancreatitis or recurrent choledocholithiasis[17] and, similarly, in this study such a finding was present in only one patient. Mackenzie et al.[17] calculated the risk for recurrent biliary tract disease after cholecystectomy in the presence of duodenal diverticula to be 5.5% 10 years after and 10.2% 15 years after cholecystectomy.

A recent study reporting on 13 cases of recurrent bile duct stones after cholecystectomy found an increased frequency of recurrent bile duct stones when the choledochus diameter was greater than 10 mm.[18] In the present study, a diameter of 15 mm or more was considered dilated, and this finding was present in only three patients. Thus, on the basis of our data, we cannot conclude that bile duct diameter is a risk factor for recurrent bile duct stones.

CONCLUSION

In the experience of this institution, more than 10% of all patients with a biliary etiology for acute

pancreatitis had undergone prior cholecystectomy. ERCP, papillotomy, and stone or sludge extraction are the treatments of choice for these patients. Surgical bile duct exploration is hardly ever necessary.

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Arginase Acts as an Alternative Pathway of L-Arginine Metabolism in Experimental Colon Anastomosis

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L-Arginine is the substrate for the nitric oxide synthase (NOS) pathway that is essential for gastrointestinal wound healing. L-Arginine is also the substrate for the enzyme arginase which metabolizes L-arginine to ornithine and subsequently to proline and polyamines both known to interact in cell proliferation and collagen synthesis. Two distinct isoforms of arginase exist. The temporal expression of the L-arginine metabolism in experimental colon anastomosis was investigated. Male Lewis rats underwent laparotomy. A left-sided colotomy was performed and the colon reanastomosed using 6-0 prolene. Sham operation was performed in controls. On days 2, 5, 10, 14, and 28 after the surgery the anastomosis was excised. The tissue at the anastomosis (ANAST) as well as above and below the anastomosis (PDC) and from sham colon was harvested and analyzed for distinct arginase isoform I (AI) and arginase isoform II (AII) activity, protein and mRNA expression as well as immunohistochemistry. iNOS protein and mRNA expression were investigated in parallel. A mean of 3 to 4 separate rats were analyzed per time point. Statistical analysis was performed by student's *t*-test, significance was reached when $P < 0.05$. AI activity, protein, and mRNA expression were significantly upregulated at the anastomosis compared to sham controls and PDC colons at all time points. The maximum was achieved at days 10 to 14 after wounding, and decreased to baseline levels thereafter. Inflammatory cells stained positive for AI. AII protein was not detectable. However RT-PCR showed low baseline expression. iNOS expression was upregulated early but for a shorter time period after wounding and reverted quickly to undetectable levels. In anastomotic healing, AI upregulation suggests a prolonged metabolism of arginine via arginase to polyamines and proline to provide substrate for collagen synthesis and cell proliferation. The functional implication of this arginase pathway further needs to be elucidated. (J GASTROINTEST SURG 2003;7:378–385.)

KEY WORDS: Colon anastomosis, arginase, wound healing, nitric oxide

Nitric oxide (NO) is a small but highly reactive molecule acting as a mediator in physiological settings such as sepsis, arthritis, vasodilatation, platelet aggregation, and many more. In the gastrointestinal tract, NO also acts as a mediator of colitis,[1] electrolyte transport,[2] motility including postoperative ileus,[3,4] mucosal defense,[5] and probably tumor development.[6,7]

Wounding is an injury to a distinct site of the body electing a specific cascade of events leading to successful healing.[8] The arginine pathway plays a vital role in wound healing since L-arginine becomes an essential amino acid after wounding with almost undetectable levels in the wound milieu.[9] Studies have shown that arginine itself has advantageous effects on cutaneous healing by enhancing collagen deposition and breaking strength.[10] This effect seems to be at least partially mediated via NO since iNOS knockout mice have delayed healing.[11] The reason why arginine becomes depleted in the wound milieu or after burn injury is speculative, but could be due to a fast metabolism of arginine via arginase to ornithine and urea.[12,13] This alternative pathway provides polyamines and proline from ornithine via ornithine decarboxylase (ODC) and ornithine aminotransferase (OAT) respectively, both key elements for cell proliferation and collagen synthesis.[14] Polyamines serve as regulators of epithelial regeneration after wounding.[15]

Arginase is the first enzyme in the urea cycle, which is predominant in the liver and which serves as "waste cycle" of ammonia. However, with the ongoing dis-

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covery of the regulation of nitric oxide, arginase has been discovered as one distinct regulatory element of NO synthesis. Arginase exists in two isoforms, AI and AII respectively. They differ in cellular sublocalization and in tissue distribution. AI, or liver type, is the cytosolic isoform whereas AII is the mitochondrial isoform, which is located in the kidney, prostate, small intestine, and breast.[16] Several cells can express both isoforms such as murine macrophages[17] and rat aortic endothelial cells.[18] AI is increased in experimental glomerulonephritis[19] or LPS-induced inflammation.[20] AII however is constitutively expressed in the epithelium of the small bowel where it metabolizes arginine to ornithine which is then released into the portal circulation or serves as precursor for polyamine synthesis.[21–23]

Arginase isoforms can be regulated by cytokines such as TGF-β and IL-4, cAMP, and oxygen tension.[17,24,25] After trauma the Th1/Th2 imbalance with Th2 predominance is reflected by a predominance of the arginase inducing (AI) cytokines such as Il-4, Il-10, and TGFβ. The extent of arginase induction after trauma reflects clinical outcome.[26]

In gastrointestinal healing, the NOS inhibitor MITU reduces colon anastomotic breaking strength.[27] In gastric ulcer healing, NO donors accelerate healing[28] probably through enhanced vasodilatation.[29] Furthermore, NO-releasing derivatives of 5-aminosalicylic acid have marked anti-inflammatory capacity.[30] On the other hand, L-arginine has dose dependent beneficial effects on TNBS-induced colitis[31] and arginine supplemented immunonutrition reduces anastomotic leakage.[32]

In view of the importance of NO as a mediator of gastrointestinal functions and anastomotic healing, the alternative pathway of arginine metabolism through arginase was investigated in healing rat colon anastomosis.

MATERIALS AND METHODS

Male Lewis rats (Charles River, Germany) weighing between 250–300 g were allowed to acclimatize for 1 week prior to the experiment.

Under intraperitoneal Ketamine/Rompun anesthesia, rats underwent median laparotomy. The left-sided colon was dissected in an avascular plane, divided and subsequently reanastomosed using 6-0 prolene by full layer sutures. The laparotomy was closed by running suture. Sham laparotomy operation was performed in controls. On days 2, 5, 10, 14, and 28 after surgery the anastomosis was excised. The edge 5 mm above and below the suture line was considered "anastomosis" (ANAST). The 5 mm above and below these regions were harvested and considered proximal/distal colon (PDC). A piece of the ascending colon was also harvested as control. Animal experiments were performed under approval of the animal welfare committee of the University of Tuebingen.

The tissue was homogenized in lysis buffer (50 mM tris-buffer, pH 7.5 containing 300 mM NaCl, 1% triton [v/v] and 1 mM PMSF) for arginase activity which was measured by newly formed urea (nmol urea/min/mg protein) as described previously.[33] Briefly, 12.5 μl tissue extract was mixed with equal volumes activation buffer (10 mM MnCl in 50 mM Tris, pH 7.5) and incubated for 10 minutes at 55C. An equal volume of 0.5 M L-arginine, pH 9.7, was added and the mixture incubated at 37C for 1 hour. The reaction was stopped by adding 400 μl of a phosphoric/sulfuric acid mixture. Urea formation was detected by colorimetric reaction at 570 nm using IPSF (isonitrosopropiophenone) as color reagent and boiling for 45 min. Urea alone was run as standard. Blanks were run in parallel where the acid mixture was added immediately to stop the reaction. Arginase activity was expressed as urea formed per min and mg protein (nmol/min/mg protein) in tissue extract. Protein concentrations were measured by colorimetric reaction using Biorad procedure (Biorad, Hercules, CA).

Specificity of the arginase activity was tested by using L-valine as inhibitor which was added directly to the reaction mix (20 mM). L-glycine (20 mM) was used as control for L-valine.

For western blotting, equal amounts of proteins from the ANAST and PDC were separated by 8% (iNOS) or 15% (AI and AII) SDS-PAGE gels under reducing conditions. After transfer onto nitrocellulose membranes, the membranes were blocked with 1% caseine (pH 7.5) and probed with Arginase I (1:2000), AII (1:500), or iNOS (1:500) antibodies in PBS-Tween 0.5%. Secondary antibodies were conjugated with HRP. Detection was performed by chemiluminescence. Liver and kidney were positive controls for AI and II respectively. Stimulated mouse macrophages (RAW 267.4 cells) served as positive control for iNOS. Monoclonal AI and iNOS specific antibodies were from Transduction (Transduction Laboratories, San Diego, CA). Rat specific AII antibody was kindly provided by M. Mori.[23]

The tissue was minced and further squeezed in TRIzol reagent (Life Technologies, Grand Island, NY) for RNA extraction following the manufacturers instruction. RNA was subjected to DNAase digestion and reprecipitated. Equal concentrations of RNA were used for RT-PCR using a Perkin-Elmer RT-PCR kit (Perkin-Elmer, Branchburg, NJ). GAPDH was used as housekeeping gene.

The primers were obtained from the gene bank using PC-gene program and were specific for rat AI, AII, and iNOS. The primers were as follows (DNA was separated onto 2% agarose gel):

AI (532 bp): 3′-5′: GTG GAC AAG CTG GGA ATT GGC
5′-3′: TCC CAC ACC CTG AAT TTC ATT CC
AII (280 bp): 3′-5′: CAC TTC TGA GGA AGA GGC CAA GG
5′-3′: AGA GTC CAC AGA TGT TCT CAT TAA GGC
iNOS (262 bp): 3′-5′: TTG GGT CTT GTT AGC CTA GTC
5′-3′: TGT GCA GTC CCA GTG AGG AAC
GAPDH (400 bp): 3′-5′: GTG GAG TCT ACT GGC GTC TTC
5′-3′: CAT GCC AGT GAG CTT CCC GTT

After paraffin embedding, 1 μm tissue slices were obtained and prepared for immunohistochemistry as follows. The tissue sections were incubated in 0.5% SDS in blocking buffer (TBS, pH 7.4) for 5 minutes. After blocking in blocking buffer containing 10% mouse serum and 5% albumin for 1 hour at 37C, the sections were incubated overnight at 4C with the primary antibody (1:100 in blocking buffer). Sections without the primary antibody were used as negative controls. After washing in TBS, the slides were incubated one hour with a secondary horseradish peroxidase coupled rabbit anti-mouse antibody (1:35, DAKO, Glostrup, DK). Detection was performed using DAB reagent in the dark. Slides were counterstained and fixed. Liver and kidney were used as positive controls for AI and II staining respectively.

The data was analyzed by Statview program comparing the anastomosis (ANAST) and the proximal/distal colon (PDC) using student's unpaired *t*-test. Statistical significance was reached when $P < 0.05$. Data is the mean of 5 separate time course experiments. At each time point one rat was analyzed yielding in total 3 to 4 separate data points per day. The results are expressed as mean ± SEM.

RESULTS

Surgery was clinically well tolerated by all animals, no infection or dehiscence occurred at the anastomosis.

Arginase activity at the anastomosis was significantly elevated compared to sham operated animals and the colon above and below the anastomosis (Fig. 1). There was no difference in arginase activity between colon tissue from the descending and ascending part (data not shown). Arginase activity raised rapidly after surgery but remained elevated until day 10 before reverting to background levels. The elevated enzymatic activity was located strictly at the anastomosis since the tissue above and below did not demonstrate an increase in arginase activity. The specificity of the anastomotic arginase activity was demonstrated by the fact that it was almost completely inhibited by L-valine, an arginase inhibitor (Fig. 2).

The elevated arginase activity was solely due to an increase in AI isoform since AII was not detected by western blotting (data not shown). In accordance

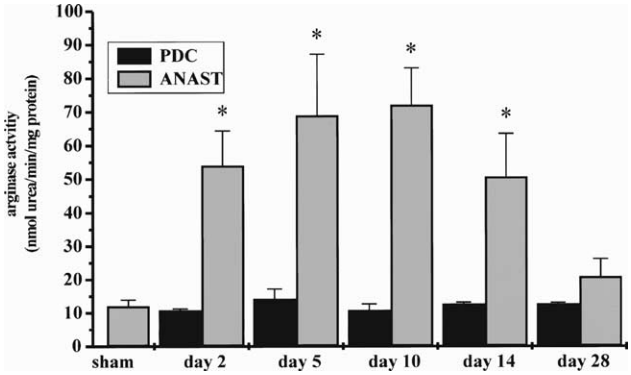

Fig. 1. Arginase activity measured as newly formed urea (nmol/min/mg protein) at the anastomosis and the colon proximal/distal thereof. The arginase activity at the anastomosis (ANAST) is significantly elevated between day 2 and 14 compared to the PDC. Mean of 3 to 4 different sets of experiments (mean ± SEM). $P < 0.05$ when comparing PCD versus ANAST at one time point.

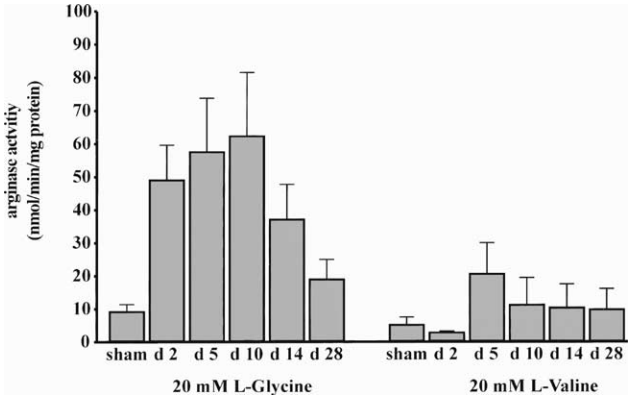

Fig. 2. Arginase activity in the presence of L-valine (20 mM) as inhibitor. The left side shows the arginase activity in the presence of 20 mM L-glycine as control for L-valine. In the presence of L-valine, arginase activity was significantly lower compared to the control demonstrating specificity of arginase activity. Mean of 3 to 4 sets of experiments (mean ± SEM).

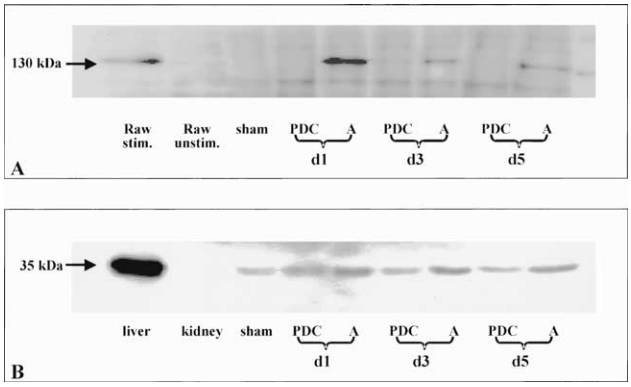

Fig. 3. Representative western blot for AI (*lower panel*) and iNOS (*upper panel*) for days 1, 3, and 5 after colon anastomosis. Equal amounts of protein were separated by SDS-page gel. IFN/LPS stimulated RAW cells were used as control for iNOS and liver extract was used as control for AI.

with the enzyme activity data, AI protein expression raised early after wounding at the ANAST but not in proximal/distal colon. Figs. 3 and 4 show one representative western blot for the early phase after anastomosis (day 1, 3, and 5) and one representative blot for the later phase of healing (day 2, 7, and 14). iNOS expression was only detectable at day 1 and 2 in the anastomosis but neither in the proximal/distal colon nor at later time points (Figs. 2 and 3, *upper panel*) indicating a shorter expression time.

Immunohistochemistry using AI and AII specific antibodies did not show any expression of AII neither in control nor in anastomotic tissue (data not shown). In a timely manner, AI upregulation occurred after day 2. At day 7 and 14 there was strong positive staining for AI where expression is strictly

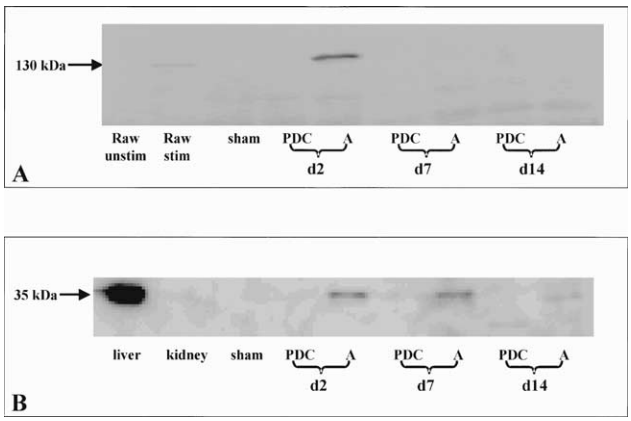

Fig. 4. Representative western blot for AI (*lower panel*) and iNOS (*upper panel*) for days 2, 7, and 14 after colon anastomosis. Equal amounts of protein were separated by SDS-page gel. IFN/LPS stimulated RAW cells were used as control for iNOS and liver extract was used as control for AI.

located in the granulation tissue at the anastomosis (Fig. 5). Staining appeared to occur at the base of the granulation tissue formation and to move to the top of the granulation wall. Inflammatory cells mainly stained positive (Fig. 6). Sometimes endothelial cells of blood vessels in the granulation tissue also stained positive for AI. Controls sparing out the primary antibodies stained continuously negative for AI.

RT-PCR using specific primers showed an upregulation of iNOS mRNA during the early phase after operation (day 1 until day 5) at the anastomosis and the surrounding tissue with the anastomosis having a higher upregulation of expression. AI mRNA expression was also upregulated during the early phase after wounding however specifically at the anastomosis but not in the surrounding colon tissue. AII showed a basal expression that was not affected by wounding. At later time points (day 7, 14 or 21) after operation neither iNOS nor AI was detectable at the transcriptional level (not shown). GAPDH was used as housekeeping gene and showed equal expression demonstrating equal loading of RNA for RT-PCR (Fig. 7).

DISCUSSION

The results of this study show for the first time that arginase acts as an alternative pathway of arginine metabolism in healing colon anastomosis. The mRNA expression of the isoform AI peaks early after wounding whereas the protein expression is detectable until day 14. The activity is highest around day 10 to 14 after wounding and reverts to baseline levels thereafter. In contrast, the iNOS expression that also peaks early after wounding at the anastomosis rapidly reverts to undetectable after day 2.

Arginine is essential for dermal wound healing through the involvement of iNOS pathway as shown by wound healing studies in iNOS knock-out animals and inhibitor studies.[10] In colon healing iNOS is upregulated at the anastomosis and inhibition of this iNOS activity using specific inhibitors (MITU) lowers colon bursting pressure.[27] The main source of iNOS in colon healing is supposed to be macrophages.[27] In our experiments inflammatory cells detectable around day 7 stain for AI isoform suggesting that macrophages and lymphocytes might be the primary source of arginase since these invade the wound later than neutrophils.[34] However, immunostaining for AI demonstrates a faint staining in the granulation tissue at the serosal part of the colon suggesting that newly ingrown fibroblasts also express AI. Dermal wound derived fibroblasts have been shown to express AI, which suggests a role in cell proliferation and matrix synthesis.[35] Interestingly, AI

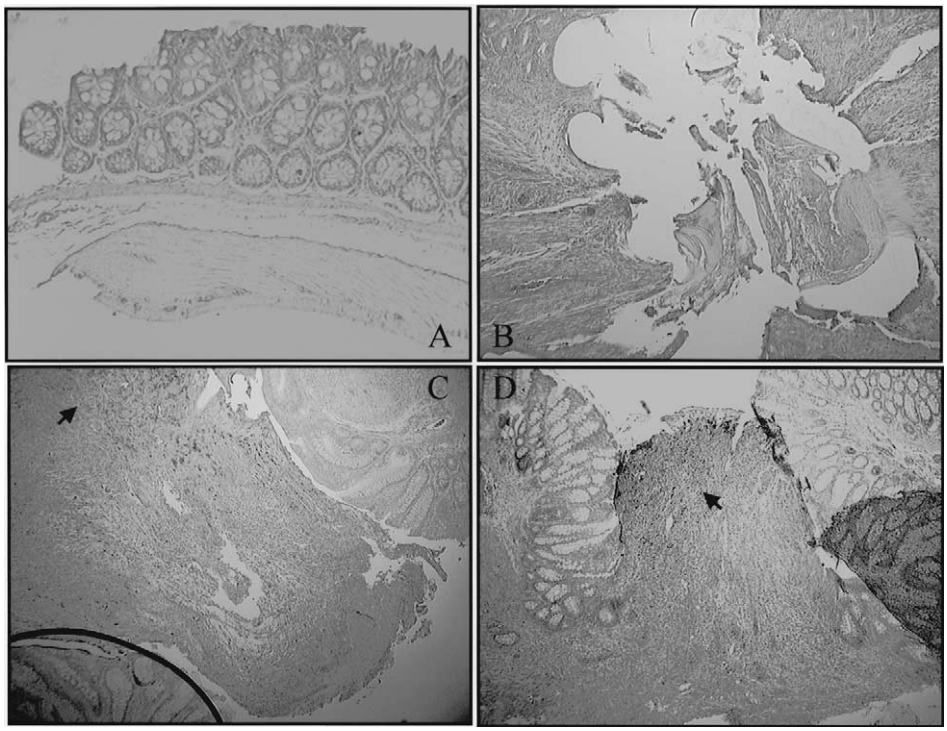

Fig. 5. Representative tissue sections of the anastomosis stained for AI showing control (**A**), day 2 (**B**), day 7 (**C**), and day 14 (**D**). Staining without primary antibody (negative control) was continuously negative (data not shown). Results show strong staining of inflammatory cells at day 7 and 14 occurring at the base of the granulation tissue and moving to the top of the granulation tissue (*arrow*). (Original magnification ×40.)

is strictly expressed in the newly formed granulation tissue suggesting a specific role in colonic wound healing. The surrounding tissue does neither stain for AI nor AII demonstrating that the upregulation is not due to a systemic but a local effect. This is underlined by the RT-PCR data showing that the proximal and distal colon tissue does not express AI. AII which is constitutively expressed in small bowel epithelial cells[36] is neither detectable by western blotting nor by immunostaining and is not regulated during healing.

Arginine is provided either by nutrition or by internal new synthesis. In the small intestine arginine can be metabolized from internal glutamine and glutamate.[22] Arginine becomes essential after wounding and supplementation of arginine improves dermal repair[37] and lowers the rate of anastomotic dehiscence.[32] Trauma is accompanied by a systemic upregulation of AI in

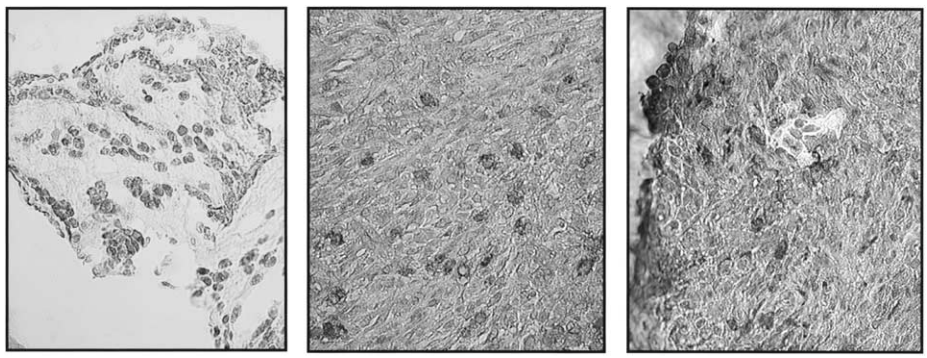

Fig. 6. Enlargement of sections from anastomosis at day 2 (*left panel*), day 7 (*middle panel*), and day 14 (*right panel*). There is no staining at day 2 at the anastomosis. At day 7 and 14 mainly inflammatory cells stain positive. (Original magnification ×100.)

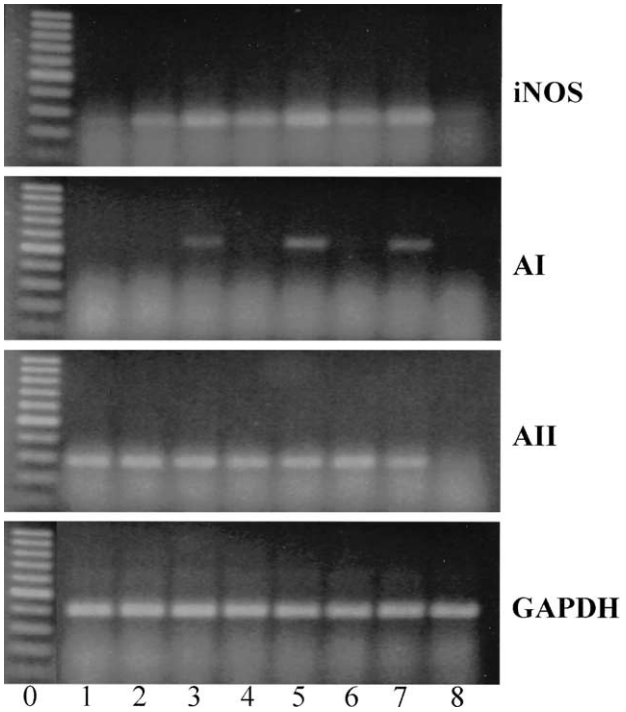

Fig. 7. One representative 2% agarose DNA gel of the RT-PCR products for iNOS (280 bp), AI (532 bp), AII (280 bp), and GAPDH (400 bp). There is a distinct upregulation of AI mRNA at the anastomosis at day 1, 3, and 5 but not in the PDC. AII shows low baseline expression. iNOS mRNA upregulation is not limited to the ANAST but also detectable in the PDC. 0 = marker; 1 = sham; 2 = PDC day 1; 3 = ANAST dl; 4 = PDC day 3; 5 = ANAST day 3; 6 = PDC day 5; 7 = ANAST day 5; 8 = blank master mix.

splenic tissue and mononuclear cells and has been suggested to be a marker for outcome.[38] In case of colon healing, however, the effect of arginase is strictly local, suggesting that this upregulation has a distinct role.

Arginase is the first enzyme in the cascade of enzymes for polyamine synthesis which occurs via ornithine synthesis through the enzyme ornithine decarboxylase (ODC). Ornithine can alternatively be metabolized to proline via ornithineaminotransferase (OAT) which serves as substrate for collagen synthesis. So far no study has shown the functional implications of arginase upregulation in wound healing. Arginase is upregulated in colon cancer tissue[39] and in mesenteric epithelial cells during the weaning phase[22] suggesting a possible marker for high proliferation or dedifferentiation. Furthermore, arginase activity mediates endothelial cell proliferation,[40] whereas inhibitors of ornithine decarboxylase (ODC) serve as antineoplastic agents[41] and nitric oxide inhibits cell proliferation through inhibition of ODC.[42] Together, this suggests arginase and the other enzymes in the polyamine pathway play a significant role in cell proliferation.

The duality of two pathways metabolizing the same amino acid suggests a distinct function of both pathways for successful healing. Colon healing appears from the serosal side of the bowel wall with new granulation tissue formation which is then recovered by newly formed epithelial layer.[34] The fact that both pathways appear in the same granulation tissue is not occlusive since they appear in a timely manner with iNOS peaking early and arginase peaking later. iNOS is found in the epithelium of colon from ulcerative colitis and Crohn's disease where it is a marker for inflammation.[43] However, arginase is also increased in biopsies from inflammatory colitis,[39] suggesting arginase could also serve as a mediator in inflammatory disease. In vitro data has shown arginase can limit arginine substrate for NO synthesis.[44] The present data could support the hypothesis that arginase upregulation limits NO synthesis, since it peaks at later time points after wounding. The coordinated appearance of arginase in the granulation tissue however suggests a more distinct function of arginase for wound healing. This is supported by the appearance of TGF-β1 expression in colon anastomosis which also peaks around day 7[45] and is known to be one of the most potent inducers of arginase.[46]

The data regarding the influence of arginine on colon healing is controversial. Arginine-free diet deteriorated colon bursting pressure, but in the same study arginine feeding (3% supplementation) failed to show any beneficial effect.[47] This, however, might have been due to the experimental design of the study since arginine was only administered for 3 days postoperatively. In this study no effect of treatment was noted on the composition of the cellular infiltrate at the anastomosis. Colonic lavage with the iNOS inhibitor L-NAME before performing the anastomosis resulted in a lesser anastomotic bursting pressure at day 3 and 6 which was unrelated to the collagen content of the anastomosis.[48] In gastric ulcer healing arginine accelerated the healing rate probably by enhancing blood flow. This effect, however, could be abolished through addition of difluoromethylornithine (DMFO), an ODC inhibitor, suggesting that the arginine mediated effect is on one hand due to NO-mediated vasodilatation and on the other hand to ODC-mediated polyamine formation.[49]

CONCLUSION

The present work shows that the arginine pathway during wound healing is more complicated than expected. The sequential upregulation of initially iNOS and subsequently arginase points toward a specific role of both pathways in colon healing. iNOS has been

shown to increase bursting pressure and collagen deposition,[27] however the exact mechanism of action remains unclear so far. Arginase could act in a similar manner through a different mechanism of action. Function studies regarding the role of arginase are currently underway.

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Combined Endoscopic/Laparoscopic Intragastric Resection of Gastric Stromal Tumors

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Myogenic neoplasms of the stomach are the most common submucosal mass. Their natural history is indeterminate, and surgical resection is advised regardless of size. These lesions have typically required open resection, but a variety of laparoscopic techniques have been described. We report results of endoscopically guided, laparoscopic intragastric resection. Fourteen lesions have been excised in 13 patients in the last 3.5 years. There were eight women and five men with a mean age of 57 years (range 34–72). All patients were asymptomatic, and no lesions had mucosal ulceration. Eight lesions were located at the gastroesophageal junction, two each at the incisura and posterior body, and one each in the fundus and anterior wall of the corpus. All lesions were predominantly intraluminal, and three were transmural. The diagnosis of a myogenic lesion was confirmed by endoscopic ultrasound in eight patients. The laparoscopic/endoscopic technique included two or three, 2 or 5 mm intragastric trocars; endoscopic suture passage and specimen removal; and laparoscopic intragastric suture repair of the gastric defect. The mean operative time was 186 minutes. The mean size of the resected specimens was 3.8 cm (range 1.5–7.0). There was no mitotic activity on histopathology, and all were considered pathologically benign. The median length of stay was 3.8 days (range 3–8). There was no mortality or operative morbidity. At a mean follow-up of 16.2 months (range 1–32) there has been no local recurrences. A combined laparoscopic/endoscopic intragastric resection is most appropriate for intraluminal, benign-appearing submucosal lesions of the proximal stomach. (J GASTROINTEST SURG 2003;7:386–392.)

KEY WORDS: Endoluminal laparoscopy, laparoscopic gastrectomy, gastric stromal

The intra-abdominal application of laparoscopic surgery has been widely adapted for numerous traditional open operations, from cholecystectomy to pancreaticoduodenectomy.[1–3] These laparoscopic adaptations are typically a nuanced variation of the same operation performed at laparotomy. An additional level of operative innovation is exemplified by intraluminal laparoscopic surgery. Broadly interpreted, this includes specialized approaches to vesicular organs, such as the stomach, bladder and uterus, that previously required transabdominal access. The distensibility and relatively large intraluminal space of the stomach fosters consideration of intragastric approaches to benign gastric pathology. Retrogastric pancreatic pseudocysts represent a contiguous disease process accessible by intraluminal laparoscopic techniques as it has beckoned the interventional endoscopists.[4,5]

Gastric stromal tumors represent a heterogeneous group of mesenchymal neoplasms of the gastric wall. While they include truly malignant and benign forms, they embody a spectrum of malignant potential predicted primarily by size and mitotic activity. The natural history is indeterminate for an individual lesion, although all are presumed to grow and increase their metastatic potential. The expanded use of diagnostic endoscopy has exposed a number of asymptomatic submucosal masses that can currently be accurately characterized by endoscopic ultrasound.[6–8] The failure to predict the natural history favors excision in acceptable risk patients, even when asymptomatic. We reviewed our experience with intragastric resection of presumed benign gastric stromal tumors to assess feasibility and outcome.

MATERIALS AND METHODS

In March of 1998 an initial attempt at intraluminal resection of gastric stromal tumors was per-

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formed.[9] Since that time a registry has been maintained of patients who have undergone this technique. Patients were considered candidates for the operation if they were found to have a suspected benign submucosal, intraluminal mass at the gastroesophageal junction or posterior wall. Gastroscopy was the prime diagnostic study which was supplemented by endoscopic ultrasound. Endoscopic ultrasound was used to determine the gastric layer of origin, size, and transmural involvement but was only available for the Cleveland Clinic patients. Patient characteristics included age, indication for the initial endoscopy, and comorbid medical conditions. Endoscopic information regarding location and local complications such as ulceration were noted. Operative data included findings at diagnostic laparoscopy, if closure of the gastric defect was required, and operative time as determined by time of induction to closure of trocar sites. Pathologic interpretation of the specimen included size measurements and assessment of mitotic activity leading to a final determination of a benign, indeterminate, or malignant stromal tumor. Postoperative data collected included an evaluation of morbidity including but not exclusive to wound and intra-abdominal infection, gastric leak, and pulmonary complications. Length of stay was recorded. A systematic follow-up schedule is performed with surveillance endoscopy and EUS if available at 6 and 18 months postoperatively.

OPERATIVE TECHNIQUE

The following approach was typically used in all patients, although some degree of variation occurred.

Table 1. Equipment required for laparoendoscopic intragastric surgery

Endoscopic equipment
Diagnostic video-gastroscope
Overtube
Endoscopic injector needle
Dilute epinephrine
2-0 Vicryl sutures on SH-needles
Biopsy forceps to grasp and pass sutures
Endoscopic snare/basket for specimen retrieval
Laparoscopic equipment
Dual input, video monitors
Split-leg operating table
2–5 mm trocars with internal stabilization capability
2–5 mm laparoscope
2–5 mm insulated laparoscopic instruments
Hook
Graspers
Scissors
Needle driver
Endoloop
2-0-silk sutures on SH-needles

Successful performance of the operation requires adequate preparation of specialized equipment which is summarized in Table 1. Advanced laparoscopic and endoscopic skills are required with a flexibility to favor one modality in specific circumstances, e.g., retroflexed endoscopic visualization may be equivalent to the laparoscopic view and eliminate one intragastric port site.

Following induction of general anesthesia, the endotracheal tube is carefully secured. The patient is positioned on a split-leg operating table, and the operating surgeon stands between the legs. One assistant is required for the laparoscopic procedure, and a surgical endoscopist is positioned at the head of the operating table. The resection can be performed with either 2 or 5 mm laparoscopic instruments depending on availability. The advantage of using 2 mm instruments is the elimination of closure of the gastric wall port sites.

An initial diagnostic laparoscopy and endoscopy are performed. The peritoneal cavity is accessed at the umbilicus by an open or closed technique. If 5 mm instruments are used for the intragastric portion, a 10 mm trocar is used at the umbilicus to allow passage of sutures to close the gastric trocar sites at the completion of the operation. A laparoscope is inserted to exclude metastatic disease and unsuspected transmural extension of the stromal tumor. Only the anterior gastric surface need be inspected for transmural involvement, which generally converts the procedure to a standard laparoscopic excision. Transmural extension of posterior lesions are completely and readily resectable with our intragastric approach and obviates the need for visualization of the posterior gastric surface.

Diagnostic endoscopy is also performed to visualize the lesion and plan trocar placement. Patients selected for this technique had previously visualized, predominantly intraluminal masses making intraoperative ultrasound unnecessary. Placement of the operating trocars is an important aspect of the operation with the goal of triangulating the three trocars for maximal intertrocar distance and distance from the lesion, while avoiding traversing the lesser or greater omentum. This can be achieved by the combination of digital palpation of the abdominal wall, endoscopic view, and laparoscopic view with minimal pneumoperitoneum. Occasionally a spinal needle is used to simulate trocar position and direction prior to placement. Maximal gastric distension and release of the pneumoperitoneum will then allow trocar placement into the stomach with endoscopic guidance (Fig. 1). Intragastric stabilization of the trocars is required by a balloon (5 mm trocars, Entec Corp., Madison, CT) or flanges (2 mm trocars, Imagyn Surgical, Newport Beach, CA). Place-

ment of two trocars are needed if endoscopic visualization alone is used, and three trocars for laparoscopic visualization. Use of the intragastric laparoscope helps eliminate the potential for left-right image inversion which is common if the endoscope provides visualization.

Submucosal and intramuscular injection of dilute epinephrine is performed with an endoscopic sclerotherapy needle or transabdominal spinal needle, facilitating a hemostatic dissection and demarcation of the mass from the submucosa and normal muscle fibers (Fig. 2). The dissection is accomplished with hook cautery after circumferential incision of the mucosa at the base of the lesion. Care is taken to not disrupt the lesion which is characteristically well demarcated. Retraction of the mass can be done by grasping the overlying mucosa or endolooping the lesion. Complete resection may result in a transmural defect. Once excised, the lesion is delivered through the mouth after placing it in a plastic bag (Catch purse, Hakko Trading Co., Japan), or with use of an endoscopic snare. The gastric wall defect is closed with intragastric suturing and knot-tying. Suture needles are passed and removed endoscopically with esophageal injury avoided with use of an overtube (Fig. 3). Adequate closure is verified with gastric distension under laparoscopic inspection. Closure of gastric port sites is done with the same trocars after removal from the stomach and resecuring at the abdominal wall (Fig. 4). A nasogastric tube is placed for decompression until a normal gastrografin study is performed on the first postoperative day. The diet is advanced as tolerated, and patients are discharged on acid-suppressive medication. Follow-up endoscopy and if available, ultrasonography is performed at 6 and 18 months postoperatively.

RESULTS

A total of 14 gastric stromal tumors have been excised in 13 patients over a 40 month period ending in July, 2001. No patient had symptoms referable to the stromal lesions, and patients were initially endoscoped for a diverse array of symptoms including gastroesophageal reflux disease, anemia, dyspepsia and globus. There were eight women and five men with a mean age of 57 years (range 32–72) (Table 2). The mean maximum diameter of the lesions based on specimen measurement was 3.8 cm (range 1.5–7.0). Most lesions were located in the proximal stomach, eight at the gastroesophageal junction, two each on the posterior body and incisura, one each in the fundus and anterior body. During excision, three lesions were transmural requiring intragastric repair of a full thickness gastric wall defect. Histopathology revealed lesions of low malignant potential, and one Schwannoma. The mean operative time was 186 minutes (range 120–320) with the majority of the operative time required for suturing the gastric defect, which varied based on the experience of the operating surgeon. There was no operative mortality,

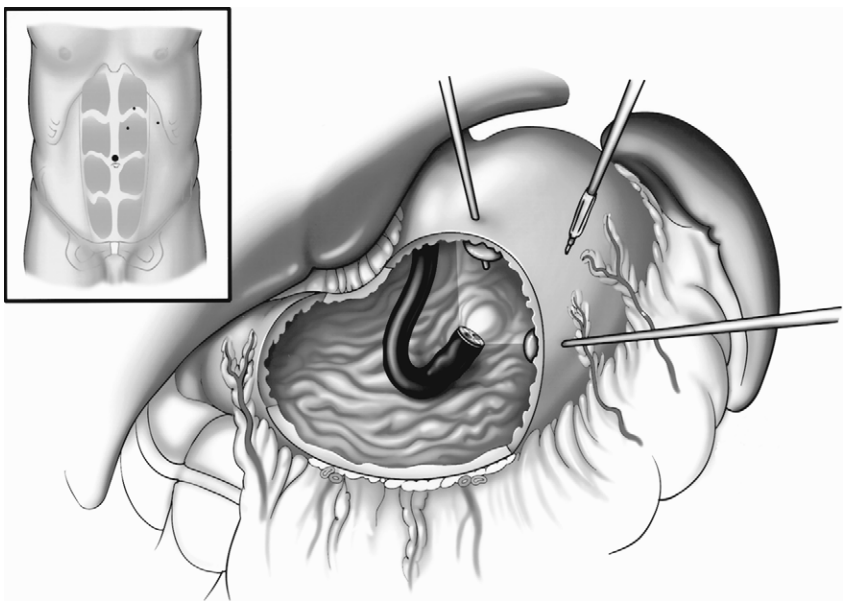

Fig. 1. Endoscopic placement of intragastric, balloon stabilized trocars. Maximal distance between trocars increases technical use of laparoscopic instruments. *Inset* shows typical placement of all trocars including the initial umbilical site for general laparoscopic exploration.

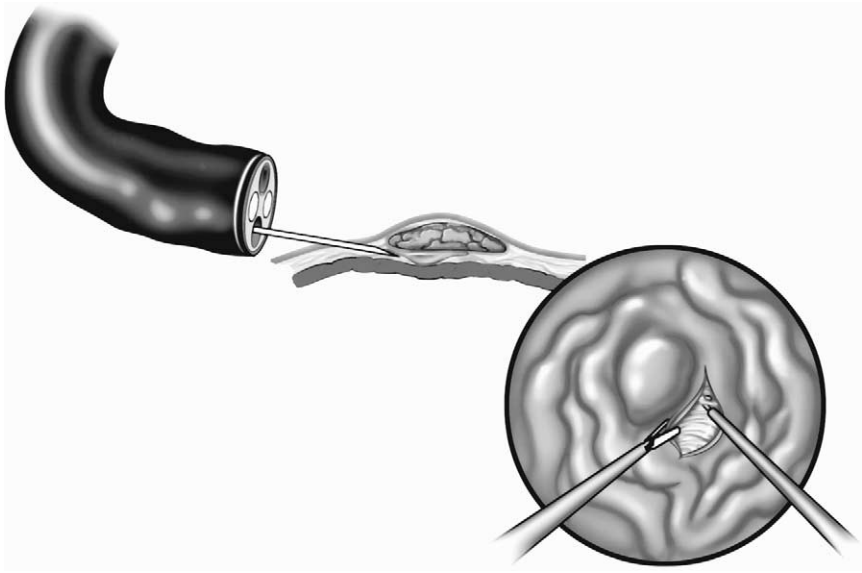

Fig. 2. Endoscopic submucosal injection of saline with epinephrine to facilitate bloodless enucleation. *Inset* shows use of hook cautery for excision.

nor procedure-related morbidity including leak or gastrointestinal bleeding. The mean length of stay was 3.8 days (range 2–8). One patient required a protracted stay as a consequence of multiple comorbid medical diseases including morbid obesity, congestive heart failure, and chronic renal insufficiency requiring hemodialysis. At a mean follow-up of 16.2 months (range 1–40) there have been no clinical or endoscopic recurrences.

DISCUSSION

Gastric stromal tumors are common submucosal neoplasms whose natural history is indeterminate and require excision when diagnosed. Our data indicates that minimally invasive, intragastric surgical excision is a safe and effective method of definitive treatment. This operative approach is straightforward with proper preparation and a plastic application of both endoscopic and

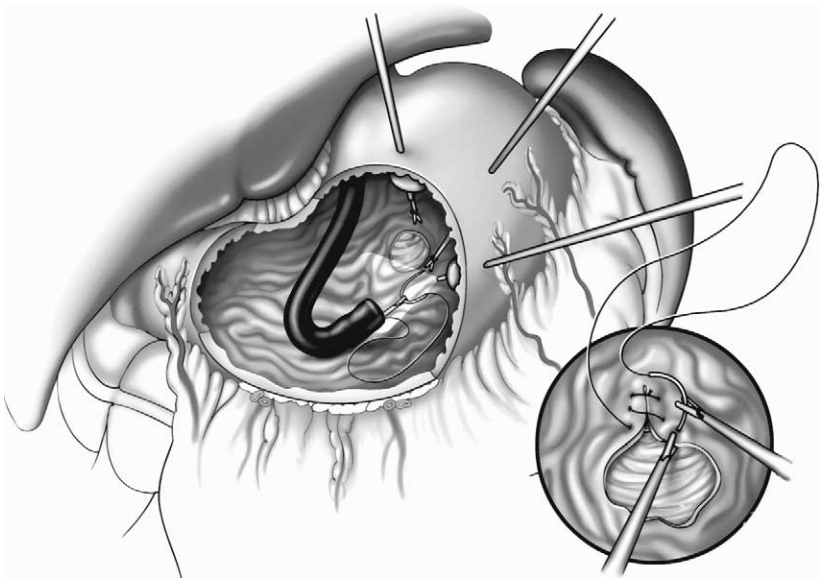

Fig. 3. Endoscopic passage of suture into stomach for laparoscopic repair of mucosal-mural defect (*inset*). Laparoscopic intragastric visualization improves suturing ability.

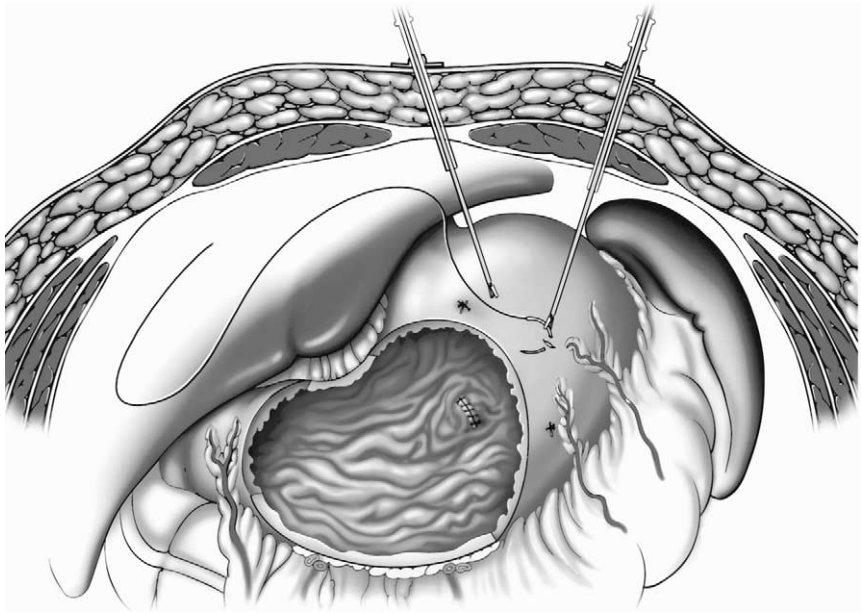

Fig. 4. Closure of gastric trocar sites with repositioned placement of the operating trocars.

laparoscopic techniques. This is the largest series of intragastric surgery for gastric stromal tumors, and it supports the low morbidity and early recovery inferred to by earlier case-reports.[9,10,11] Unlike most advanced laparoscopic procedures, no conversions to laparotomy were required, highlighting a high likelihood of success, and steep learning curve when applied by surgeons with extensive laparoscopic and endoscopic experience. The low morbidity and flexibility of this technique may challenge the application of aggressive endoscopic procedures such as endoscopic mucosal resection (EMR) that are less well-controlled surgical procedures.[12]

Submucosal masses of the stomach are common incidentally found abnormalities at the time of endoscopy.[13] Although stromal neoplasms are the most frequent cause of a submucosal mass, other possibilities include a gastric varix, carcinoids, ectopic pancreatic rest, lipoma, and an extragastric mass.[14] The endoscopist is faced with the challenge of managing a lesion whose diagnosis may be suspected, but unconfirmed. Mucosal biopsies may be nondiagnostic, and surveillance endoscopies might be advised with their attendant costs and patient anxiety. The scope of this clinical dilemma is compounded by the number

Table 2. Operative and pathologic results

Patient no.	Age (yr)	Tumor size (cm)	Location	Pathology	Op time (min)	Length of stay (day)	Follow-up (mo)
1	67	2.5	GE Junction	Benign	200	4	40
2	62	5.8	GE Junction	Benign	160	3	37
3	67	5.0	GE Junction	Benign	160	5	25
		4.5	Fundus	Benign	180		
4	34	2.8	GE Junction	Benign	120	3	16
5	72	2.4	Posterior Body	Benign	180	4	13
6	47	4.4	GE Junction	Benign	140	3	15
7	54	3.2	GE Junction	Benign	155	3	20
8	42	5.3	GE Junction	Benign	130	3	9
9	66	7.0	Incisura	Benign	320	4	12
10	68	2.0	Incisura	Benign	300	8	10
11	56	1.5	Proximal Body	Schwannoma	190	5	7
12	39	3.4	GE Junction	Benign	180	2	5
13	66	4.0	Posterior Body	Benign	185	3	1
Mean	57	3.8	—	—	186	3.8	16.2

of endoscopic procedures performed for the myriad of dyspeptic complaints.[15] The advent of endoscopic ultrasound has become a useful tool to characterize these submucosal lesions. Endoscopic ultrasound can accurately distinguish solid from liquid, extragastric origin, mural layer of origin, and when necessary, a route for aspiration cytology.[16] The combination of these attributes has resulted in the ability to distinguish solid neoplasms arising from the muscular layer consistent with stromal tumors, and benign versus malignant.[17–19] The diagnosis of a myogenic tumor should be followed by surgical excision. This recommendation is based on the fact that these neoplasms represent a spectrum of malignant potential, the exact nature of which is indeterminate for a given lesion. Their natural history is predicted by size and nuclear grade.[20–22] Small lesions with infrequent mitotic activity are considered benign, but growth over time will increase the malignant potential and lead to local symptoms such as bleeding and pain.[23,24] Endoscopic ultrasound has improved our ability to diagnose these lesions and brought to our attention smaller lesions for surgical consideration instead of endoscopic surveillance. Timing of surveillance is also difficult to determine when the doubling time for malignant lesions is suggested to be approximately 16 months.[25] While possibly increasing the number of patients considered for operation, this should only be advantageous in definitively treating younger, asymptomatic patients with the highest curative potential.[26]

Numerous operative approaches have been utilized for these neoplasms and include a variety of laparoscopic as well as traditional open procedures.[27–32] Complete excision of the neoplasm is required without disruption,[33] and should include a margin of normal gastric wall for malignant lesions. Our technique of enuclation has already been successfully applied (no recurrences) at open surgery for benign or low grade gastric sarcomas.[13] A lymphadenectomy is not necessary, nor an anatomic resection unless dictated by the location of the lesion. There is a higher anatomic distribution of these neoplasms in the proximal stomach,[14] including the gastroesophageal junction, which can limit exposure and obviate an extended resection with a traditional operation. The majority of our patients had lesions at the gastroesophageal junction and the intragastric route affords excellent access, and should be considered the preferred method for small lesions. The growth pattern for stromal tumors is unpredictable, and range from intraluminal to exophytic serosal lesions. This technique is particularly ideal for predominantly intraluminal lesions and transmural involvement does not preclude its use. Full thickness defects of the gastric wall can be closed with intra- or extra-gastric suturing depending on the location. Extragastric approaches to intraluminal masses, especially laparoscopic techniques, are loathsome, as the lesions can be difficult to locate and invariably lead to an extended gastric wedge resection, if laparoscopic staples are used. Linear resection of inherently spherical masses with endoscopic staplers results in unintended extended resection of normal stomach. Limited, direct excision of these masses, analogous to open surgery, is preferred and is readily achieved with our intragastric approach. Enucleation is achieved in patients without transmural involvement with our technique and preliminary endoscopic follow-up indicates this is adequate. We have broadened the success of conservative local excision,[34] to include enucleation of small lesions. The risk of local recurrence is likely to be the greatest concern of this technique, although in our operative experience, it should be rare given the encapsulated nature and clear demarcation of these lesions.

Our initial experience indicates that combined laparoscopic/endoscopic excision of selected gastric stromal tumors is safe and effective. It is advised for predominantly intragastric, likely benign lesions of the proximal stomach and gastroesophageal junction.

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Modulation of Growth Factor and Cytokine Expression by Nitric Oxide During Rat Colon Anastomotic Healing

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We have previously shown that inhibition of nitric oxide generated by inducible nitric oxide synthase (iNOS) results in impaired colon anastomotic healing. Therefore, we proceeded to assess whether disruption of iNOS activity alters the normal pattern of growth factor expression during anastomotic healing. Two groups of male Sprague-Dawley rats underwent distal colonic division and anastomosis, jugular venous catheterization and subcutaneous placement of polyvinyl alcohol sponges. The first group (n = 10) received q8 hour intravenous injections of 10 mg/kg L-N-iminoethyl-lysine (L-NIL, a selective inhibitor of iNOS), while the second group (n = 12) received equal volumes of saline. On postoperative day 5, animals were sacrificed and anastomotic bursting pressure was determined. Histologic sections of the anastomosis were subjected to *in situ* hybridization versus mRNA of the proteins listed below. Positive controls were reacted with a poly-thymidine (poly-T) probe versus ubiquitous mRNA poly-adenine tails. Positively stained cells were quantified using a calibrated optical grid encompassing 0.5 mm^2 area centered over the anastomosis. Results are reported as the number of positive cells per 1000 cells positive for poly-T. L-NIL treated animals demonstrated an 18% decrease in wound fluid NO_X compared to controls (29.2 ± 1.2 vs. 34.6 ± 2.0 μM, mean ± SEM; $P = 0.035$). This corresponded to a 17% decrease in anastomotic bursting pressure (153 ± 4 vs. 182 ± 8 mm Hg, mean ± SEM; $P < 0.05$). L-NIL also markedly increased the number of cells expressing transforming growth factor-β, tumor necrosis factor-α, vascular endothelial growth factor, and both inducible and endothelial forms of nitric oxide synthase. L-NIL had no effect on the expression of basic fibroblast growth factor. The data demonstrate that iNOS inhibition markedly disrupts the profile of cytokine and growth factor mRNA normally expressed during anastomotic healing. This provides in vivo evidence that NO modulates gene expression during anastomotic healing. (J GASTROINTEST SURG 2003;7:393–399.)

KEY WORDS: Nitric oxide, colon anastomosis, wound healing, growth factors

Wound healing is characterized by a precise series of events that involve a cascade of cellular and biological activities resulting in the restoration of tissue integrity. Most wounds, including gastrointestinal anastomoses, follow the same series of events characterized at first by inflammation, then by proliferation of collagen producing cells with concomitant collagen accumulation, and finally by reorganization of the scar tissue. Though the populations of cells present in the wound throughout the progression of healing have been well described, little is known about the cellular and sub-cellular interactions that regulate normal healing.

Nitric oxide (NO) has been shown to play an important role in wound healing. Inhibitors of NO production impair cutaneous healing[1] while transfer of inducible nitric oxide synthase (iNOS) cDNA by injection into subcutaneously implanted sponges enhances collagen deposition.[2] Additionally, we have shown that NO production from iNOS is vital for

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the optimal healing of colonic anastomoses.[3] iNOS gene and protein activities, normally not present in uninjured colon, have been quantified in anastomotic tissues over the course of healing.

Nitric oxide (NO) is a highly reactive nitrogen-oxygen radical which is best known to act as a biochemical signaling molecule via activation by guanylate cyclase.[4] Recent data suggest that NO may function to selectively regulate gene expression by modulating the binding of transcription factors.[5] Because of the differential levels of iNOS expression and activity over the course of the healing wound we hypothesized that NO production from iNOS influences the pattern of growth factor and cytokine gene expression during colon anastomotic healing.

MATERIALS AND METHODS

Animals

All animal experiments were performed in compliance with the guidelines set forth by the National Institutes of Health for the care and use of laboratory animals under protocols approved by the Sinai Hospital of Baltimore Institutional Animal Care and Use Committee. Animals were allowed one week to acclimatize to our facility prior to experimentation. Rats were housed at 21C with a regular 12 hour light-dark cycle, and allowed free access to tap water and standard chow food throughout the experiment (Teklad LM-485, Harlan Teklad, Madison, WI).

Jugular Access and Colonic Anastomosis

Male Sprague-Dawley rats (270–320 g) were anesthetized with IP injection of 50 mg/kg sodium pentobarbital and their necks, backs, and abdomens were shaved and painted with betadine. A 1 cm incision was created over the right neck. The right jugular vein was exposed and the distal end of a short length of sterile Silastic catheter (0.03 inch I.D. $\times$ 0.065 inch O.D.; Dow Corning, Midland, MI) was placed in the vein with its tip in central venous position under direct visualization. The proximal catheter was subcutaneously tunneled over the right shoulder and exteriorized through a small inter-scapular incision. The proximal catheter was attached to an 18 gauge tubing adapter (Beckton-Dickinson, Franklin Lakes, NJ). The catheters were aspirated to remove air and flushed with 0.5 cc normal saline. The wounds were closed with running 4-0 silk suture. Two sterile polyvinyl alcohol sponges were then inserted subcutaneously on the back of the rat in a position caudal to the catheter hub. Next, the abdomen was opened through a small midline incision. The colon was divided 1.5 cm proximal to the pelvic reflection with attention to preserving the marginal artery. A single layer, inverting anastomosis was created with interrupted 6-0 polypropylene sutures spaced at approximately 3 mm apart. The abdominal wall incision was closed with a two-layer, running 3-0 silk suture.

iNOS Inhibition and Sacrifice

At the time of operation, animals were randomized to receive q8 hour injections (300 μl each) of either saline (n = 12) or L-N-iminoethyl-lysine (L-NIL, n = 10; Alexis Biochemical, San Diego, CA) resuspended in saline at a dose of 30 mg/kg/day. This dose of L-NIL was found in preliminary experiments to consistently and significantly decrease wound NO synthesis. All catheters were flushed with an additional 300 μl of normal saline following each injection. On postoperative day 5 animals were reanesthetized. Anastomotic segments were isolated with ligatures placed 1 cm proximal and distal to anastomosis. Using a 16 gauge angio-catheter inserted into the lumen of isolated bowel, the segments were infused with normal saline using a Medfusion intravenous infusion pump (Medex Inc., Duluth, GA). Continuous pressure was monitored by connecting the pump and anastomotic segment to a pressure transducer (American Edwards Laboratories, Irvine, CA) via a 3-way stopcock. Bursting pressure was measured as peak pressure attained prior to anastomotic disruption.

Nitrite/Nitrate Determination

Cardiac blood obtained by cardiac puncture was centrifuged to extract the plasma fraction. Wound fluid squeezed from the PVA sponges was centrifuged to remove cellular debris. Plasma and wound fluid samples were filtered through 10,000 MW cut off Ultrafree-MC (Millipore Corporation, Bedford, MA) to remove proteins. Total nitrite and nitrate content of each sample was determined (as a measure of NO production) using a commercially available colorimetric assay kit based on a nitrate reductase reaction (Oxford Biomedical Research, Oxford, MI).

In Situ Hybridization

Immediately following bursting pressure determination, anastomotic segments were excised, opened longitudinally and rinsed in cold, sterile saline. Sections of tissue spanning the anastomotic scar were embedded in O.C.T. compound, immediately snap frozen in liquid nitrogen and stored at −70C until sectioning.

Probe sequences for the mRNA of the proteins listed in Table 1[6–12] were obtained from GenBank using an Internet Entrez nucleotide sequence and the probe sequences were verified for uniqueness to each specific cytokine via a BLAST search of the NR (Non-Redundant GenBank CDS translations + PDB + SwissProt + PIR + PRF databases) resource search (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov). The oligomer probes were then generated via a DNA synthesizer at the Johns Hopkins Medical Institutions Core Laboratory Facility (Baltimore, MD). The individual probes were poly-end labeled with digoxigenin-11-dUTP using a terminal deoxyribonucleotidyl transferase (TdT) kit (Boehringer Mannheim Corporation, Indianapolis, IN), and stored at 4C until use.

Sections of each specimen (7 μ thick) were cut using a −20C cryostat and placed onto RNAse free slides. Serial sections from each animal were used for each of the probes. The sections were then fixed for 15 minutes in 10% paraformaldehyde in PBS with 20 mM $MgSO_4$, and subsequently dehydrated in a series of graded ethanol solutions. The slides were air dried at room temperature for 30 minutes followed by a 1 hour incubation period in prehybridization solution (2× hybridization solution mixed with 60% deionized formamide and 1% saponin, Sigma Corporation, St. Louis, MO). Sections were hybridized with 10 nanomolar pre-labelled probe in prehybridization solution overnight (18 hours) in a 100% humidity chamber at 37C. Slides were then serially washed for 30 minutes each in 5x, 5x, 2x, 1x, 0.5x, and 0.1x saline-sodium citrate (SSC) solution.

Non-specific binding from tissue antigens were then blocked by incubation of slides in 0.1 M Tris-HCl, pH 8.0, with 200 mg/10 ml of normal goat serum (Transduction Labs, San Diego, CA), and 10 millimolar sodium dodecyl sulfate (SDS). Tissue sections were subsequently incubated in blocking solution containing alkaline phosphatase-conjugated FAB fragments versus digoxigenin (1:100 dilution; Boehringer Mannheim Corporation, Indianapolis, IN).

Slides were washed twice for 10 minutes in PBS plus 2% saponin, and developed with a modified McGadey's reagent (20 millimolar nitro-blue-tetrazolium plus 40 millimolar bromo-chloro-indoyl-phosphate in 0.1 molar Tris-HCL, pH 9.5; Sigma Corporation, St. Louis, MO). Reactions were terminated by exposure to 2% paraformaldehyde in water prior to application of coverslips.

Positive cells, defined as having a dark-blue cytoplasm, were quantified in a blinded fashion using a Leica microgrid and micrometer (Fisher Scientific, Pittsburgh, PA) centered over the anastomosis (100 × magnification). All positive cells within a one square millimeter area of tissue were counted. Grids were positioned in the same tissue area in serial sections. The total number of cells positive for each cytokine in each animal was divided by the total number of poly-T positive cells in serial sections from the same animal. The results were reported as cytokine-positive cells per 1000 cells at the anastomosis. Verification of appropriate staining was achieved by hybridization with the IL-6 sense sequence as a negative control.

Statistics

Comparison between treatment groups was accomplished using the Student's *t* test.

RESULTS

All animals survived until sacrifice and there was no evidence of sepsis from the jugular catheters. There were no apparent anastomotic complications (i.e., disruption or abcess). Postoperative weight gains are shown in Fig. 1. After postoperative day 1, L-NIL treated animals gained minimally, but significantly more weight postoperatively than saline-treated controls.

L-NIL treatment successful, inhibited NO production as measured by total nitrite and nitrate con-

Table 1. Probe sequences for in situ hybridization

Abbreviation	Protein	Probe Sequence
iNOS[6]	Inducible nitric oxide synthase	TCTCTCTCCTCTCCCCCGTCCCCTATTCTC
eNOS[7]	Endothelial nitric oxide synthase	CCTGCTGACTGGGCCTGGATCGTGCCCCCC
bFGF[8]	Basic fibroblast growth factor	AGCCAGGTACCGGTTCGCACACACTCC
TGF-β1[9]	Transforming growth factor beta	CCCCCTCCCCGCAGGGCTGAAGAGACCCCC
TNF-α[10]	Tumor necrosis factor alpha	GCTGAGCCAGCGTGCCAACGCCCTCCTGGC
VEGF[11]	Vascular endothelial growth factor	GCACCCATGGCAGAAGGAGGAGGGCAGAAT
IL-6 sense orientation[12]	Negative control	CGGAGAGGAGACTTCACAGAGGATACC
Poly-T	Positive control	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTT

centrations of both plasma (24.3 ± 2.5 vs. 31.9 ± 2.5 μM, mean ± SEM; $P = 0.044$) and wound fluid (29.2 ± 1.2 vs. 34.6 ± 2.0 μM, mean ± SEM; $P = 0.035$) (Fig. 2). Inhibition of iNOS by L-NIL resulted in a 17% decrease in anastomotic bursting pressure compared to saline controls (153 ± 4 vs. 182 ± 8 mm Hg, mean ± SEM: $P < 0.05$) (Fig. 3).

Hybridization and staining of the poly-thymidine probe, the positive control for the in situ hybridization technique, demonstrated similar numbers of cells in the anastomoses of L-NIL and saline-treated animals (positive control: 4387 ± 335 in L-NIL treated animals, 4053 ± 201 in saline controls; negative control: 3 ± 3 with L-NIL, 3 ± 2 with saline; mean ± SD). Inhibition of NO production from iNOS by L-NIL resulted in a significant alteration in the anastomotic expression of 5 of 6 cytokines and growth factors studied on postoperative day 5. L-NIL treated animals demonstrated a 2.5-fold increase and a 10-fold increase in cells expressing iNOS and eNOS respectively. L-NIL treatment also resulted in an increase in the number of cells expression mRNA of TNF-α (8-fold increase), TGF-β1 (3-fold increase), and VEGF (2-fold increase). Inhibition of iNOS activity had no effect on bFGF expression in 5-day anastomotic tissue (Fig. 4).

DISCUSSION

The present experiment demonstrates that the inhibition of NO production from iNOS impairs anastomotic healing. Additionally, disrupted iNOS activity results in a marked alteration in the expression of a number of wound-active proteins within anastomotic tissue. We have previously shown that inhibition of iNOS activity impairs anastomotic healing.[3] However interpretation of the results of this study was limited by the toxicity of S-methylisothiourea (MITU), the substance used for selective iNOS inhibition. At a dose that would consistently block iNOS activity (as assessed by nitrate and nitrate concentration in plasma and cutaneous wound fluid), the animals treated with MITU lost significantly more weight postoperatively than saline-treated control animals. L-NIL allows a reduction in dosage without accompanying weight loss in the animals. L-NIL treatment, not only decreased NO production but also diminished anastomic bursting pressure confirming our previous findings. The dose of L-NIL utilized in the present studies was chosen as having inhibitory effects on anastomotic NO synthesis without impairing animal well-being. On the contrary the L-NIL-treated group gained weight when compared to saline-treated controls, an effect for which we have no ready explanation.

There are a number of characteristics unique to gastrointestinal wounds in comparison to cutaneous wounds.[13] The wound environment of the GI tract is more varied than that seen in skin. The luminal contents of the tract progress from largely aseptic in the stomach to colonization by massive bacterial loads in the distal colon. The character of the luminal contents, such as pH and fluidity, also are markedly different in the proximal and distal bowel. Oxygen supply to healing tissue in the GI tract is entirely dependent upon blood flow and is recognized to be of paramount importance to successful anastomotic healing. Additionally, intestinal wounding results in activation of collagenase along the entire GI tract

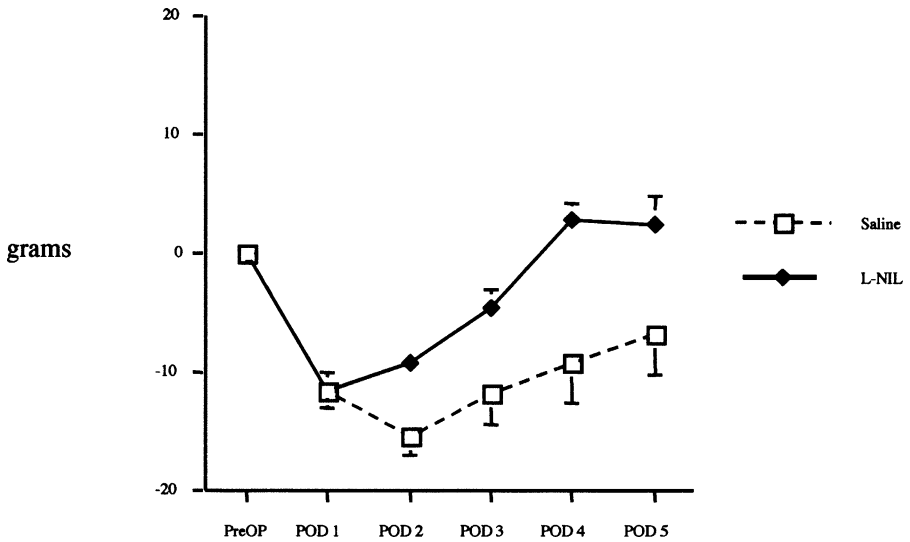

Fig. 1. Postoperative weight gain of saline and L-NIL treated animals (mean ± SEM).

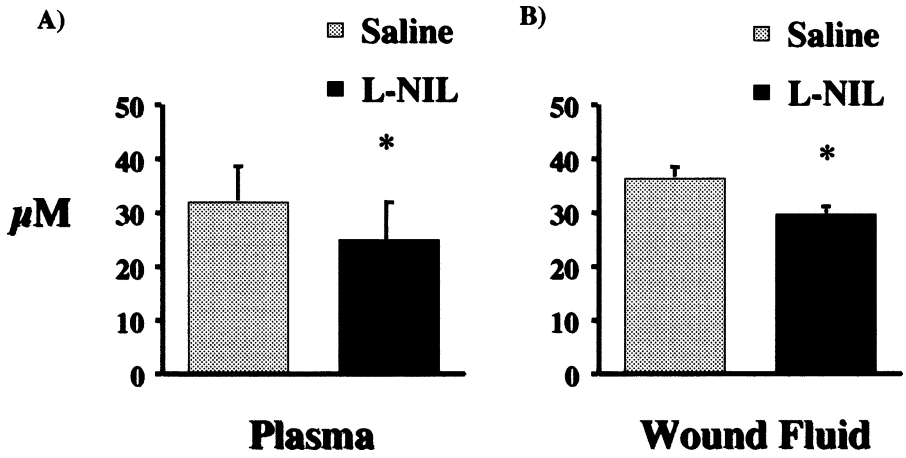

Fig. 2. Total nitrite/nitrate concentration in plasma (**A**) and wound fluid (**B**) (mean ± SEM; $^*P < 0.05$).

and this is a proportionally greater activation than that seen in skin after wounding.

NO plays a number of roles in gastrointestinal tissues. Fischer et al. demonstrated the constitutive isoforms of nitric oxide synthase to be abundantly expressed throughout the uninjured gastrointestinal tract. Immunohistochemically, eNOS activity was localized to GI vascular endothelium, and nNOS activity was localized to intramural ganglia.[14] NO production from eNOS has been implicated in regulating blood flow to gastrointestinal mucosa (presumably via activation of guanylate cyclase), and this has been linked to healing of gastric ulceration.[15,16] Additionally, NO production from iNOS has been identified as a potential cause of the inflammatory damage noted in ulcerative colitis.[17]

Recently NO has been shown to potentially regulate gene expression. delaTorre et al. noted that nitrosylation of the thiol binding site of NF-kappa beta, a transcription factor important for facilitating

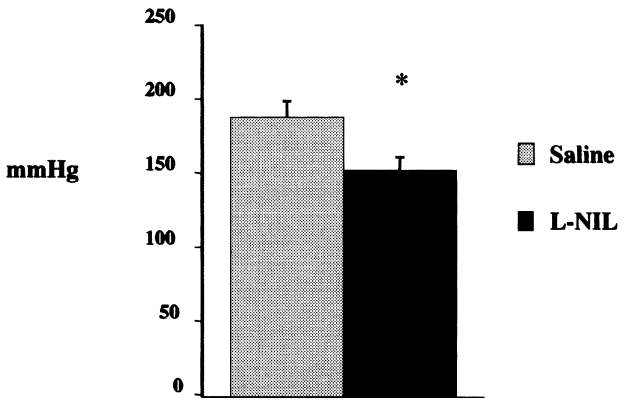

Fig. 3. Mean anastomotic bursting pressure of saline and L-NIL treated animals (mean ± SEM; $^*P < 0.05$).

iNOS gene activity, significantly impaired its capacity for DNA binding.[18] They subsequently showed this was selective for NF-kappa beta and not for all transcription factors (e.g. c-jun).[19] Interestingly, this phenomenon could explain the upregulation of both iNOS and TNF-α gene activities in response to L-NIL treatment in the present study. Both of these proteins are known to be regulated by NF-kappa beta activation. Walley et al. reported a similar phenomenon in a mouse model of acute lung injury.[20] TNF-α protein and mRNA, induced after tracheal LPS exposure, was markedly upregulated by pretreatment of the mice with L-NAME, another inhibitor of NO production. This was also shown to be via a NF-kappa beta dependent pathway.

In this study, the number of anastomotic cells expressing TGF-β mRNA was increased by L-NIL treatment. L-NAME produced a similar increase in TGF-β expression in the renal cortex of hypertensive rats as compared to controls.[21] Additionally, in this study VEGF expression was also increased by iNOS inhibition. This was in contrast to the effect of iNOS inhibition in cutaneous excisional wound as reported by Frank et al. where mice treated with 2.5 mgs of L-NIL twice daily demonstrated a marked decrease in both VEGF mRNA and protein in the wound.[22] However direct comparisons are difficult in that these studies represent two different types of wounds and the dose of L-NIL in the study by Frank et al. was at least 7 times that of ours.

Clearly disruption of iNOS activity significantly affects the expression of a number of genes in healing colonic tissue. However, from this study it is difficult to identify which genes are primarily responsible for the impaired healing. Both TGF-β1 and VEGF have been shown to improve wound heal-

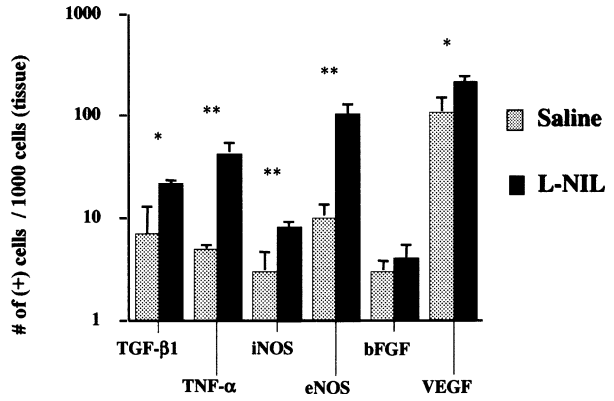

Fig. 4. Inhibition of NO production from iNOS by L-NIL resulted in a significant alteration in the anastomotic expression of 5 of 6 cytokines studied on postoperative day 5 (mean ± SD; L-NIL, n = 5; saline, n = 9; $*P < 0.05$; $**P < 0.001$).

ing,[23,24] yet upregulation of these genes following L-NIL treatment does not strengthen colonic anastomoses. One possible explanation may be found in the concurrent upregulation of TNF-α expression, which has been shown to have an inhibitory effect on collagen deposition in wounds.[25–27]

A major limitation of this study is that only a single time point was assessed and thus it is difficult to conclude whether the impaired healing was due to disruption or delay of the inflammatory phase of healing or a true interruption of cell function by altering gene expression. If the former were the case, then the decreased bursting pressure would reflect delayed healing which ultimately may achieve normal strength. Further study is required to investigate these issues.

CONCLUSION

Nitric oxide production from iNOS is required for optimal healing of colonic anastomoses. The aim of this study was to determine whether disruption of iNOS activity markedly altered the pattern of expression of a number of cytokines, the expression and activity of which felt to be integral to normal wound healing. Although it is difficult to ascertain exact mechanisms of how these changes impact their effects, the net expression pattern is correlated with impaired healing. This may represent one mechanism by which NO modulates the wound healing process.

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Introduction to Papers from the 2001 AHPBA/AASLD Surgical Forum

Steven M. Strasberg, M.D., President, American Hepato-Pancreato-Biliary Association

Each year since 1994, the American Hepato-Pancreato-Biliary Association (AHPBA) has met in conjunction with the American Association for the Study of Liver Diseases (AASLD) at the annual meeting of the AASLD in November. The first meeting took place even before the formal founding of the AHPBA. Initially the concept was to focus on all of the surgical papers, including those on transplantation, during 1 day of the 3-day AASLD meeting. However, in 1994, the plan broadened to include a postgraduate course called the AHPBA/AASLD Surgical Forum. The first Forum was held on November 5, 1994, in Chicago, Illinois, and the subject was laparoscopic hepatopancreatobiliary surgery. When the AHPBA was formally established in that year, it was decided that the presidency would be 2 years in duration and that the president would be responsible for organizing the Forum. The first president, Dr. Michael Henderson, established a format that divided the day into thirds covering liver, biliary, and pancreatic topics, respectively, and alternating benign and malignant subjects in successive years. This pattern was continued through the subsequent presidencies of Drs. William Meyers and Henry Pitt. However, each president brought his own style to the meeting. Dr. Meyers used a short debate format that was lively and occasionally provocative, whereas Dr. Pitt employed a case presentation mode that included audience voting.

Recently, the AHPBA and The Society for Surgery of the Alimentary Tract (SSAT) reached an agreement that the JOURNAL OF GASTROINTESTINAL SURGERY would be the official journal of the AHPBA, as it already was for the SSAT. One of the aspects of the agreement was that the AHPBA would use its meetings as a source of papers for the JOURNAL. This included the biannual "Americas" meeting and the annual AHPBA/AASLD Surgical Forum. Papers presented at the Americas meeting are oral presentations or posters and are in suitable style for conversion into publications. Many papers from the 2001 Americas meeting have appeared in the JOURNAL in an effort organized by Dr. Henry Pitt. The question was how to organize the Forum so it would also contribute.

The format adopted for the Forum held on November 1, 2001 was the mini-symposium, because it was thought that such an approach would readily translate into informative publications. An expert was asked to organize a summary symposium lasting about 2 hours in a developing area of surgery using four or five speakers to present the relevant areas of the topic. The papers that follow are the product. The topics chosen for mini-symposia were "Intraductal Papillary Mucinous Tumors" (Pancreas), "Biliary Injury" (Biliary), and "Hepatocellular Carcinoma in Cirrhosis" (Liver). The mini-symposium leaders—Dr. Michael Sarr of the Mayo Clinic, Dr. William Chapman, then of Vanderbilt University, and Dr. Scott Helton of the University of Illinois, are acknowledged leaders in their fields and selected outstanding speakers. The day was highly successful. After the meeting, the three symposium leaders and the speakers wrote the three papers that follow. They are current detailed presentations of the subjects and will be of interest to all gastrointestinal surgeons. It is hoped that this will be the first of many such contributions to the JOURNAL OF GASTROINTESTINAL SURGERY from the Forum.

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Treatment Strategies for Hepatocellular Carcinoma in Cirrhosis

W. Scott Helton, M.D., Adrian Di Bisceglie, M.D., Ravi Chari, M.D., Ph.D., Myron Schwartz, M.D., Jordi Bruix, M.D.

The diagnostic and therapeutic approach to treating patients with hepatocellular carcinoma (HCC) has become a complex process that is rapidly evolving and changing as the biology and natural history of this disease becomes better understood.[1,2] The presence of cirrhosis, chronic active hepatitis, and multicentric tumors significantly limits treatment options. For patients who are known to be at high risk for developing HCC, early detection and accurate tumor staging are poor and costly. It was widely accepted for many years that partial or total hepatectomy with liver transplantation offered the only chance for long-term, disease-free survival for patients with HCC.[3] Unfortunately, few patients with HCC are candidates for surgical resection or transplantation at the time of diagnosis because of advanced tumor stage, multicentric disease, or comorbid medical conditions.[4] For those patients with HCC who are candidates for either partial or total hepatectomy, major obstacles remain and include the following: universal recurrence of HCC after partial hepatectomy; inadequate supply of cadaveric organs, which leads to increased wait times and substantial numbers of patients removed from the transplant list because of tumor progression; and limited ability to completely ablate tumors with current technologies.

The preceding issues have led to significant changes in the management of patients with cirrhosis who are either at risk for or develop HCC. Unfortunately, there is little evidence from randomized clinical trials to support current practices, many of which are considered standards of care. For this reason, a symposium to address several controversial practices in the management of HCC was convened by the American Hepato-Pancreato-Biliary Association at their annual meeting in Dallas, Texas, in November of 2001. The specific topics to be discussed and invited speakers included the following: (1) "The Ethics, Economic Implications, and Results of Screening for HCC in High-Risk Patients" (Adrian Di Bisceglie, Professor of Medicine, St. Louis University); (2) "The Results and Technical Limitations of Radiofrequency Ablation for HCC" (Ravi Chari, Associate Professor of Surgery, Vanderbilt University); (3) "Adult Living-Donor Liver Transplantation for HCC" (Myron Schwartz, Associate Professor of Surgery, Mount Sinai Hospital, New York); (4) "The Need for Adjuvant Therapy Following Resection, Ablation, or Transplantation for Patients With Cirrhosis, HCC, and Hepatitis B or Hepatitis C" (W. Scott Helton, Professor of Surgery, University of Illinois at Chicago); and (5) "Optimizing Treatment Strategies in HCC: The Barcelona Clinic Liver Cancer Group Experience" (Jordi Bruix, M.D., Barcelona, Spain).

THE ETHICS, ECONOMIC IMPLICATIONS, AND RESULTS OF SCREENING FOR HEPATOCELLULAR CARCINOMA IN HIGH-RISK PATIENTS

The risk for developing HCC is well documented and includes the presence of cirrhosis, chronic active viral hepatitis associated with elevated alpha-fetoprotein (AFP), age over 50 years, male sex; family history of HCC, and previously resected or ablated HCC. Once cirrhosis has developed, the risk of developing HCC is estimated to be 1% to 4% per year.[5] This well-documented risk for developing HCC has led to the commonly accepted practice of screening and surveillance of patients for HCC. In this regard, the objective of screening and surveillance for the development of HCC is to identify tumors at an early stage so that treatment can be initiated early with the hope of reducing disease-specific deaths. There are no randomized

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controlled trials comparing surveillance and no surveillance. Screening and surveillance should be restricted to patients with cirrhosis who would benefit from treatment if diagnosed with HCC. Patients who are not candidates for screening include the following: normal healthy subjects with no liver disease; healthy carriers of hepatitis B and hepatitis C; hepatitis C without cirrhosis; age over 40 years without a family history; and possibly patients who are not candidates for invasive therapy such as surgery and transplantation.

The candidate screening tests for HCC include serial AFP measurement, HCC-specific AFP variants, Des-gamma carboxyprothrombin, and hepatic ultrasound imaging. Other imaging tests such as contrast-enhanced CT or MRI, although more sensitive for detecting small tumors, have not typically been used in screening because of their increased costs. Most of the screening programs for detecting HCC have used AFP in combination with hepatic ultrasound. Serum markers other than AFP have no proved efficacy for early detection of HCC. Ultrasound has reasonable sensitivity (60% to 78%) and specificity (80% to 90%) but is both operator and equipment dependent.

There are significant ethical implications with screening for HCC. First, the overall ability to prolong survival in a group of patients at risk for developing HCC is limited. This is related to the fact that all forms of therapy for HCC in patients with cirrhosis other than transplantation are unlikely to result in 5-year disease-free survival. Hence the detection of a cancer in an asymptomatic patient for which there is no cure raises ethical concerns. Any effort by the clinician to detect HCC through screening must be counter-balanced by the patient's expectations and the need to preserve his or her quality of life, as well as by the use of current technologies and therapies with unproved benefits. For these reasons, patients should be informed as to the rationale, limitations (e.g., false positive and/or false negative rates), and consequences of screening prior to its initiation.

There are four potential outcomes of screening: true positive, false positive, true negative, and false negative. The hope and ideal consequence of obtaining a true positive outcome is that early treatment can be initiated and lead to an improvement in survival. Unfortunately, many patients are found to have advanced incurable disease at first detection. In fact, the results of several screening programs demonstrate that the majority of detected tumors are not curable and that survival for patients undergoing resection is limited.[6–8] Patients with false positive screening results undergo the risks, costs, and inconvenience of additional evaluation, and patients whose screenings are false negative receive inappropriate reassurance until their tumors are eventually discovered. The benefit of true negative screening is that patients receive appropriate reassurance that they do not have HCC.[9] Data on tumor doubling volume provide the rationale for the optimal surveillance interval for patients whose initial screenings are negative, which is every 6 months for AFP and hepatic ultrasound.[10]

Numerous studies have investigated the effectiveness of screening/surveillance for HCC. Farinati and Gianni[11] reported that using ultrasound and AFP every 6 months increased the percentage of patients who were eligible for transplantation (39% vs. 26%) and increased the 4-year survival (50% vs. 22%) and median survival (24 months vs. 8 months) compared to patients in whom HCC was discovered by chance. Bolondi et al.[6] reported that screening did not increase the percentage of patients who underwent surgical resection or transplantation but that 3-year survival and median survival both increased in patients whose tumors were found at screening compared to those in whom HCC was found by chance. Tong et al.[8] reported that of 173 patients with cirrhosis resulting from chronic viral hepatitis, 31 cases of HCC were detected by screening and surveillance. Only 4 of the 31 patients underwent resection; eight underwent transplantation and nine received palliative therapy. McMahon et al.[12] have demonstrated that screening for HCC in native Alaskans with chronic hepatitis B virus infection leads to improved resectability and 5- and 10-year survival. Sherman et al.,[7] on the other hand, found that screening chronic carriers of hepatitis B virus for HCC resulted in a resection rate of only 50%, and only 40% of these patients survived for more than 2 years. The cost of screening for HCC in patients at risk is substantial. The cost per tumor detected is estimated between $11,800 and $25,000 in 1996 U.S. dollars. The cost per additional year of life gained has been reported to vary from $26,000 to $113,000.[6,13]

To evaluate screening practices for HCC in the United States, Chalasani et al.[14] polled 473 members of the American Association for the Study of Liver Disease: 17% responded that they do not routinely screen for HCC; 57% reported that they use AFP and ultrasound for screening; and 12% use AFP, ultrasound and CT. Only one third of respondents believed that screening was cost-effective in nontransplant candidates, whereas 80% believed that screening was cost-effective for transplant candidates; 44% of respondents believed that screening prolongs survival and 64% believed that failure to screen for HCC in high-risk patients posed a malpractice liability.

In summary, patients at high risk for developing HCC can be identified by screening. Ultrasound in combination with AFP every 6 months is the most commonly employed method of screening for HCC. The identification of HCC by screening has marginal cost-effectiveness. Despite this, screening for HCC in

high-risk patients has become standard practice in both the United States and abroad. When viewed from an individual patient's perspective, screening is probably worthwhile for those who can be successfully resected or transplanted.

THE RESULTS AND TECHNICAL LIMITATIONS OF RADIOFREQUENCY ABLATION FOR HEPATOCELLULAR CARCINOMA

Few patients with HCC are candidates for surgical resection or transplantation at the time of diagnosis because of advanced tumor stage, multicentric disease, or comorbid medical conditions, as the presence of cirrhosis, portal hypertension, chronic active hepatitis, and/or multicentric tumors significantly limit treatment options.[15] Many patients with cirrhosis and HCC are never listed for transplantation because of complicating socioeconomic or medical problems, whereas others do not meet minimal listing criteria. Moreover, many patients listed for transplantation are subsequently removed from the waiting list because of tumor progression. For all of the preceding reasons, there has been a priority placed on the development and implementation of nonresection modalities for tumor ablation.[16,17] Ablative techniques used to treat HCC include transarterial embolization alone or with chemotherapy, percutaneous ethanol injection, radiofrequency ablation (RFA), microwave coagulation, laser coagulation, radionuclide injection, and combinations thereof. Percutaneous ethanol injection, although quite effective and inexpensive, should be used to treat only encapsulated HCC. Of the other techniques, RFA has gained tremendous recent popularity and is rapidly being disseminated throughout the medical community as a means of treating HCC.[18–20]

Radiofrequency ablation of HCC involves placing an electrocautery needle into the tumor under ultrasound or CT guidance and then applying radiofrequency alternating current (450 to 500 kHz) for variable periods of time (usually from 10 to 15 minutes) until the temperature of the tumor is raised consistently to above 60° C. Radiofrequency ablation is most commonly performed under conscious sedation in the radiology suite but can also be performed under general anesthesia using percutaneous, open, or laparoscopic techniques. The laparoscopic approach is preferable for patients who are unable to cooperate under conscious sedation, for patients with tumors on the surface of the liver, and for patients who have ascites or coagulopathy. The procedure is well tolerated, reasonably safe, and can be performed on an outpatient basis in most cases. Complication rates are low (0% to 12%) and include intraperitoneal bleeding, abscess, hemobilia, biliary strictures, pneumothorax, visceral injury, liver failure, and even death. There are four different types of RFA devices currently being marketed; all four differ in design and in how they deliver and sense energy delivery to tissue. To date, there have been no published trials comparing the efficacy of one device vs. another in the treatment of HCC.

Most studies demonstrate that RFA adequately ablates HCC up to 3 cm in size.[19,21] In fact, the survival rate following RFA in patients with HCC less than 3 cm is reported to be similar to that reported for patients with equivalent-stage disease undergoing surgical resection.[22] Recent reports suggest that tumors up to 5 cm may be adequately treated by the newest generation of RF devices.[23–27] However, reports of patients treated with these devices have less than 2 years' follow-up and local tumor recurrence approaches 40%.[28] Many transplant centers around the world are currently treating patients with HCC up to 5 cm with RFA prior to transplantation. To date, there are no published data on whether this approach adequately prevents local tumor progression or extrahepatic spread while the patient waits for a transplant or whether the incidence of post-transplant tumor recurrence decreases after transplantation.

Local tumor recurrence as a result of incomplete tumor ablation is common and underestimated by most clinicians reporting their experience with RFA.[29] Radiology imaging modalities (ultrasound, CT, and MRI) currently being used to monitor the completeness of RFA underestimate the thoroughness of ablation. Solbiati et al.[30] reported that all four patients treated successfully with RFA who subsequently underwent surgical resection (15 to 60 days later) had viable residual tumor. Dodd et al.[31] reported similar results in three patients with HCC smaller than 3 cm treated by RFA while awaiting liver transplantation. All of the tumors were labeled as "completely ablated" by lack of enhancement during the arterial phase of CT scanning. However, on histologic review of the explanted livers after transplantation, there was microscopic evidence of viable cancer within every single ablated tumor. These results demonstrate that currently used imaging modalities do not reliably detect residual cancer in ablated lesions. It is currently unknown, however, whether residual microscopic disease after RFA adversely affects long-term survival with or without liver transplantation. The answer to this important question can only be provided in the context of a prospective randomized clinical trial.

Several recently reported prospective randomized and nonrandomized clinical trials have compared RFA with percutaneous ethanol injection in patients with cir-

rhosis and small HCCs (<5cm)[21,32,33]; two of these are only in abstract form.[32,33] These studies consistently report that at 2 years' follow-up, RFA leads to more thorough tumor necrosis (90%) than percutaneous ethanol injection (80%), requires fewer treatments to achieve complete ablation, but is associated with more complications. In addition, RFA has been found to be comparable to percutaneous ethanol injection with respect to short-term survival, but superior to percutaneous ethanol injection with regard to both local and overall disease control. Despite these early reports on potential superiority of RFA over percutaneous ethanol injection, the use of RFA to treat HCC in patients awaiting a liver transplant must be undertaken with caution so as to not preclude the only long-term curative option for patients with advanced cirrhosis. The Barcelona Clinic Liver Cancer Group recently issued a warning about the use of RFA in patients with encapsulated HCC awaiting liver transplantation. In this report, needle track seeding or peritoneal metastases occurred in 12.5% of patients undergoing RFA compared to 0% of patients receiving percutaneous ethanol injection.[34] Neoplastic seeding was related to peripheral subcapsular location of tumors, poor tumor differentiation, or baseline AFP. The problem of local recurrence of HCC after RFA has not been thoroughly studied but is probably related to both technical and anatomic issues.[35,36] Technical limitations include the difficulty of accurate deployment of the RFA device into the tumor using two-dimensional imaging, accurate overlap of ablation zones to treat tumors larger than the RFA probe, difficulty in monitoring the margins of the ablation zone by ultrasound as a consequence of nitrogen gas release that occurs during tumor necrosis (outgassing), and the carbonization that occurs at the tumor/RFA probe interface. Carbonization interferes with the dispersion of the radiofrequency current uniformly into the tumor. Anatomic/physiologic limitations to effective RFA include size, consistency, and hypervascularity of the tumor, as well as proximity of the tumor to major blood vessels, bile ducts, and surface of the liver. When blood vessels larger than 3 mm are present in and/or near the tumor, they act as heat sinks preventing complete tumor necrosis.[37,38]

A number of technical advances and methods are actively being explored to overcome the current limitations of RFA.[35,36] Three-dimensional ultrasound transducers or CT can improve probe placement into the tumor. The manufacturers of RFA devices have made more powerful generators capable of delivering more power and better software that monitors the dispersion of energy and impedance of ablated tissue so as to avoid carbonization, and have also made larger probes with multiple deployable arrays to provide wider and denser ablation zones. Other means of improving radiofrequency current dispersion include the use of a cool tip electrode, pulsed delivery of energy, and the injection of hypertonic saline solution at the electrode tip. These factors reduce the problem of carbonization and enhance radiofrequency current dispersion and conduction through tissue. Reducing tumor and hepatic blood flow by way of pre-RFA tumor embolization, temporary application of a Pringle maneuver during the RFA, or pharmacologically induced hypotension will reduce the heat sink phenomenon. Collectively, all of these methods have enabled large zones of ablation to be made.

Recent reports suggest that RFA after superselective chemoembolization is effective for ablating HCCs larger than 5 cm.[39] At 6 months' follow-up, 75% of patients with 7 cm tumors had no evidence of recurrence.[40] In another study, 91% of patients with HCCs between 3 to 5 cm and 100% of patients with tumors larger than 5 cm had complete necrosis at follow-up.[41]

Despite the rapid development of RFA technology, it is currently unclear how this technology will affect patient outcomes, and this will require long-term meticulous study. Only prospective randomized clinical trials will determine whether specific advances in RFA technology over current systems will translate into improved outcomes for patients with cirrhosis and HCC. To more rapidly advance our understanding of the utility and limitations of RFA in the management of HCC, it is essential that patients be enrolled in well-designed prospective clinical trials by investigators who have the means and resources to accurately characterize liver tumors before and after RFA. For such clinical trials to be of any use, patients must be appropriately selected, treatment must be standardized and meticulously described, and follow-up must be complete and comprehensive.

In summary, RFA is a promising new therapeutic option for patients with cirrhosis and HCC. However, whether RFA is curative or improves survival of nontransplant patients with HCC or is able to maintain the transplant candidate on the liver transplant waiting list and improve overall survival awaits further study.

ADULT LIVING-DONOR TRANSPLANTATION FOR HEPATOCELLULAR CARCINOMA

For patients with early HCC and decompensated cirrhosis, the outcome after liver transplantation is universally accepted to be superior to other treatment alternatives and is therefore considered the treatment of choice.[4] The overall 5-year survival for patients undergoing transplantation approaches 50%

for all patients making it to transplantation[42] and is over 70% for patients with a single tumor less than 5 cm or with three tumors all smaller than 3 cm.[43–46] These outcomes are excellent when compared to the treatment of other common malignancies such as colon, lung, and pancreatic cancer. Unfortunately, almost 50% of patients listed for transplantation for HCC never receive an organ for one or more reasons.[47] First, there is a limited supply of cadaver organs for an increasing number of patients listed for orthotopic liver transplantation. This has resulted in progressively longer wait times, which in turn allows for local and/or distant tumor spread. The University of California at San Francisco transplant program recently reported that 2.3% of patients with HCC listed for transplantation were subsequently removed from the wait list per month because of tumor progression.[48] The Barcelona Clinic Liver Cancer Group reported that the actuarial 2-year survival of patients considered for orthotopic liver transplantation significantly decreased from the time periods between 1989–1995 (84%) and 1996–1997 (54%) and that this decrease in survival was related to a 21% dropout rate as a result of prolonged waiting times for transplantation.[46] Finally, patients who do not meet "restricted" listing criteria for HCC did not previously receive priority on the transplant list, which effectively ruled out cadaveric transplantation for them. New listing criteria according to the Mayo Clinic End-Stage Liver Disease system will change the allocation of cadaver livers based on need and thereby potentially change the waiting times for patients with HCC listed for orthotopic liver transplantation. The Mayo Clinic End-Stage Liver Disease system was only implemented in March of 2002. Hence it will not be known for several years how this new system will affect the outcome of patients with HCC awaiting orthotopic liver transplantation. Collectively, all of these events led to the recent development of adult living-donor liver transplant (LDLT) programs around the world.[49,50]

Factors that justify LDLT for patients with HCC and cirrhosis include the following: (1) better overall status of the recipient at the time of transplant; (2) better liver function of the graft as a result of optimal procurement conditions and less short cold ischemia times; (3) the ability of the patient to proceed expeditiously to transplant reduces the dropout rate from tumor progression and eliminates the risks, costs, and patient discomfort associated with pretransplant neoadjuvant therapies; (4) patients not meeting restricted listing criteria still have access to transplantation, which offers them the only chance for survival; (5) personal and psychological reward to the donor for saving the life of the recipient whose death without a LDLT would constitute a significant personal loss to the recipient; and (6) a potential 5-year survival greater than 50%, which is superior to any other form of treatment for patients with advanced cirrhosis and HCC. For example, at Mount Sinai Hospital, between 1991 and 1999, there were 43 patients with HCCs larger than 5 cm who received an orthotopic liver transplant with a mean follow-up of 55 months. In this group, the 5-year survival was 44% compared to a 0% 2-year survival for patients excluded from transplantation because of tumor progression despite neoadjuvant therapy. The University of California at San Francisco Liver Transplant group also reported a 57% 5-year survival for patients with HCC who exceeded the Milan criteria but were within the limits of an expanded criteria (solitary tumor < 6.5 cm, up to 3 tumors with the largest no more than 4.5 cm and the combined tumor diameter no more than 8 cm.[51]

During a 3-year period from 1998 to 2001, there were 98 LDLTs performed at Mount Sinai Hospital in New York and 34 of these were performed in patients with known HCC. The median wait time for all patients listed as status 2B by the United Network for Organ Sharing was 206 days in 2001. The mean wait times for patients with known HCC at Mount Sinai Hospital was 482 days for those receiving a cadaveric liver vs. 77 days for those undergoing LRDT. This reduction in mean wait time of a year not only reduces the risk of tumor progression but also saves money with respect to eliminating the cost incurred by monitoring every 3 months, neoadjuvant therapy, and managing other complications of end-stage liver disease.

Two groups have performed Markov models comparing LDLT to cadaveric liver transplants. Sarasin et al.[52] reported that in Barcelona there would be a substantial gain in life expectancy with LDLT when wait time on the list exceeds 7 months. Cheng et al.[53] reported that life expectancy would be expected to increase by 4.5 years with LDLT over cadaveric orthotopic liver transplantation for a 58-year-old patient, assuming an 82% 5-year survival, tumor doubling time of 204 days, and an average waiting time for United Network for Organ Sharing status 2B.

The United Network for Organ Sharing criteria for priority listing for orthotopic liver transplantation was developed in an effort to make the best use of a scarce resource. However, there is no a priori basis to assume that such restricted criteria should apply to LDLT because this operation does not draw on the limited pool of cadaveric livers. In fact, several centers have adopted expanded criteria for LDLT for HCC.[51] Criteria for LDLT at Mount Sinai Hospital are as follows: no extrahepatic disease as shown by

CT of the abdomen and chest and by bone scan; no regional lymph node involvement; patent main portal and hepatic veins; total tumor volume less than 75% of the liver; and if multicentric tumor exists in both lobes, then the tumor in the less involved lobe must be smaller than 5 cm. The Barcelona Cancer Liver Group has also adopted expanded criteria for LDLT that includes a single HCC larger than 7 cm; multinodular HCC, three nodules larger than 5 cm or five nodules smaller than 3 cm; partial response to any treatment for HCC lasting more than 6 months that achieves the conventional criteria for OLT.[4] Thirty-four patients undergoing LDLT at Mount Sinai Hospital exceeded the United Network for Organ Sharing criteria for status 2B. With a mean follow-up of 456 ± 269 days, recurrence has been observed in 4 out of 34 patients. Freedom from recurrence at 36 months of follow-up is 80%. Early nontumor-related deaths occurred in seven patients (20%). Five of the deaths (71%) were directly related to a bile leak.

The increasing public demand for adult LDLT coupled with the potential for significant 5-year survival has led to the proliferation of transplant centers performing adult LDLT. Unless there are substantial increases in the number of available cadaveric organs for orthotopic liver transplantation, patient demand will continue to fuel the growth of LDLTs for HCC, despite significant technical problems and complications associated with the procedure and a risk of donor mortality estimated between 0.5% and 1%. However, with additional experience and advances in technique, it is expected and hoped that donor safety and patient outcomes will be improved.

THE NEED FOR ADJUVANT THERAPY FOLLOWING RESECTION, ABLATION, OR TRANSPLANTATION FOR PATIENTS WITH CIRRHOSIS, HEPATOCELLULAR CARCINOMA, AND HEPATITIS B OR HEPATITIS C

Additional HCC develops in 70% to 100% of patients with hepatitis B– or C–induced cirrhosis within 5 years of liver resection or ethanol injection for a single, encapsulated HCC.[54–56] The rate at which tumors redevelop is estimated at 15% to 20% per year.[57] Thus, 5-year disease-free survival after surgical resection or ablation of HCC in the setting of cirrhosis and hepatitis B or C virus is unusual and justifies the need to identify effective adjuvant therapy.

The postulated causes for the high recurrence rate of HCC in patients with cirrhosis and hepatitis B or C are the presence of microscopic metastatic disease and/or undetected metachronous multicentric HCC within the liver at the time of initial treatment, postoperative exacerbation of hepatitis B virus, and ongoing necroinflammatory injury from chronic viral hepatitis. Recurrence of HCC following surgical resection is related to the severity of hepatic inflammation,[56] and recurrence-free survival is related to the severity of viral hepatitis.[58] It has also been reported that surgical resection leads to an exacerbation of hepatitis B virus infection,[59] thereby precipitating more hepatic injury.

Based on this background, strategies to prevent recurrent HCC include means of decreasing hepatic inflammation from hepatitis B and hepatitis C, and effective ways of killing early, undetectable metachronous tumors, as well as circulating metastatic disease. These efforts are hindered by the fact that, to date, there are no effective chemotherapeutic agents for HCC and cytotoxic and radiation therapy given early after liver resection impairs liver function and regeneration. Despite these obstacles, a number of clinical trials have been undertaken to identify adjunctive means of decreasing recurrent HCC following liver resection or ablation.

Controlling hepatitis C and B proliferation can be achieved with antiviral therapy. Several prospective, randomized clinical trials in Asia have reported that adjuvant antiviral therapy after surgical resection or ethanol ablation reduces recurrent HCC and improves disease-free survival.[60,61] Unfortunately, none of the trials performed in Asia have been independently replicated, and there is no evidence in the West that antiviral therapy prevents recurrent HCC in patients with virus-induced cirrhosis.[62]

A variety of chemopreventive agents have been investigated for their ability to prevent hepatocarcinogenesis.[63–65] When these agents are given to patients after treatment for primary HCC, they appear to decrease the risk of recurrent HCC. In a randomized clinical trial, polyprenoic acid, an acyclic retinoid, given postoperatively significantly reduced the incidence of new HCC in patients with cirrhosis following resection or ablation.[64] Sho-saiko-to, a mushroom extract, has been reported to improve overall survival and reduce HCC recurrence after treatment of primary HCC.[65]

Based on the premise that undetectable metachronous HCC exists in the liver at the time of surgical resection, investigators in China carried out a randomized clinical trial in which the effects of lipiodol coupled to 131-I given intra-arterially to the remnant liver after liver resection for HCC were compared to a control group that received no treatment.[66] The treated patients had decreased recurrence in the liver and improvement in overall median disease-free survival as well as 3-year survival compared to control subjects. In another randomized clinical trial in Japa-

nese patients, adoptive immunotherapy improved recurrence-free and overall survival compared to no change in control patients following resection of HCC.[67] Whether or not the benefits of this strategy were the result of an improved host defense against residual or new HCC or the consequence of improved antiviral defense is unknown. A nonrandomized, case-control study reported that postoperative systemic doxorubicin after liver resection improved the 1-, 3-, and 5-year survival compared to a no-treatment control group.[68]

The high recurrence rate for HCC after surgical resection and ablation substantiates the need for effective forms of adjuvant therapy. There is ample evidence, albeit confined to Asian patients, that adjuvant therapy reduces the incidence of HCC following resection and ablation. However, these results require independent confirmation through additional randomized clinical trials performed in non-Asian patients before wide adoption by clinicians in the West. In the future, new agents such as antiangiogenesis drugs and combined approaches such as intra-arterial 131-I lipiodol plus polyprenoic acid with or without antiviral therapy should be tested within the context of large randomized clinical trials.

There is a great need for effective neoadjuvant therapy for patients with cirrhosis and HCC who qualify for liver transplantation in order to prevent tumor progression while patients wait for increasing lengths of time before undergoing transplantation. It is estimated that between 2% and 4% of patients with HCC are removed from the waiting list each month.[48,52] Neoadjuvant therapy has the potential to impede tumor progression and distant spread, thus allowing patients to endure the increasing wait times for an orthotopic liver transplant. A number of prospective, nonrandomized clinical trials have evaluated the effect of transarterial chemoembolization, alone or in combination, with systemic chemotherapy prior to orthotopic liver transplantation.[16,69–71] The efficacy of this approach has not been tested within the context of a randomized clinical trial and thus solid evidence supporting this practice is lacking. Furthermore, transarterial chemoembolization is an expensive, potentially morbid and even lethal procedure in patients with advanced cirrhosis. Two retrospective studies reported that transarterial chemoembolization followed by percutaneous ethanol injection is associated with more tumor necrosis and improved disease-free survival in patients with HCC receiving an orthotopic liver transplant when compared to HCC patients undergoing orthotopic liver transplantation without neoadjuvant therapy.[72,73] Unfortunately, the nonrandomized nature of these trials prevents any conclusion as to the isolated neoadjuvant treatment effects of percutaneous ethanol injection and transarterial chemoembolization.

The Barcelona Clinic Liver Cancer Group published a Markov decision model on the cost-effectiveness of neoadjuvant therapy for patients with cirrhosis and HCC who are awaiting orthotopic liver transplantation. The analysis demonstrated that if a patient was expected to wait for an orthotopic liver transplantation for more than 6 months, percutaneous ethanol injection results in an increase in quality-adjusted life-years gained.[74] No study has compared the utility of percutaneous ethanol injection against RFA or any other form of ablation in patients awaiting liver transplant. Hence, randomized clinical trials should be performed comparing the relative safety, cost, and efficacy of percutaneous ethanol injection, RFA, and transarterial embolization, alone and in various combinations, in patients awaiting orthotopic liver transplantation.

OPTIMIZING TREATMENT STRATEGIES IN HCC: THE BARCELONA CLINIC LIVER CANCER GROUP

There are significant diagnostic and therapeutic controversies surrounding the management of patients with cirrhosis and HCC. This fact, coupled with the increasing interest paid to this disease led to a recent consensus conference on the clinical management of HCC.[5] Treatment strategies for patients with suspected or proved HCC should be based on the natural history of the disease, the prognosis for a given patient, the patient's expectations and wishes, the availability and costs of specific treatments, and the availability of clinical expertise. The prognosis for patients with HCC and cirrhosis is based on the severity of their liver disease, their clinical performance status, the presence of medical comorbidity, and the tumor stage. All of these factors must be taken into consideration before offering a specific plan of treatment for a given patient. General guidelines, established by consensus from experts in the management of patients with HCC and cirrhosis, are listed as follows[5]:

1. Screening and surveillance for HCC should be offered only to those patients with cirrhosis who would qualify for treatment if diagnosed with HCC. Patients not suitable for curative therapy should not be entered into surveillance programs. Child-Pugh class C patients should be considered for liver transplantation. But if this option is not available or a patient is not a candidate for liver transplantation, screening and surveillance are not justified because there are no data to indicate that survival is enhanced

with any treatment option in Child-Pugh class C patients with HCC. Improvement in outcome will result only from treating patients with early detected tumors, and this justifies the continued use of surveillance in patients with cirrhosis who are at risk for HCC.

2. Once a patient develops suspected HCC, as shown by either a suspicious nodule or an elevated AFP level, it is recommended that the patient be referred to a specialty center for further evaluation.
3. The diagnosis of HCC does not always require biopsy, and biopsy should be avoided when other predictive criteria are present. For example, the diagnosis of HCC can be confidently made when two imaging techniques (ultrasound, triple spiral CT, gadolinium MRI or angiography) identify a focal lesion larger than 2 cm with arterial hypervascularization or when one imaging technique shows a focal lesion larger than 2 cm with arterial enhancement combined with an elevated AFP level above 400 ng/ml. Lesions smaller than 1 cm should not be biopsied until they are observed to grow larger than 1 cm. If diagnosis of HCC between 1 and 2 cm is indicated, biopsy should be performed because imaging techniques are insensitive for accurate diagnosis of HCC.
4. Prognostic models should include four main factors: (1) the stage, aggressiveness, and growth rate of the tumor; (2) the general health of the patient; (3) the liver function of the patient; and (4) the specific treatment intervention.
5. Prospective randomized clinical trials are necessary to better define optimal treatment strategies for HCC. Phase III/IV studies should use survival as the primary end point. The results of liver transplant for patients with HCC should be analyzed from an intention-to-treat point of view. Sample size should be calculated according to the outcomes reported in modern series of patients with HCC whose disease stage corresponds to the target of intervention. For the evaluation of therapeutic effect(s), time zero should be the time of initial intervention, and this should be as close as possible to the time of diagnosis.
6. World Health Organization criteria for tumor response is inaccurate for assessing response of HCC to locoregional treatments because extensive tumor necrosis is usually not paralleled by a reduction in diameter of tumors.[75] The optimal technique for the assessment of locoregional treatments is currently by spiral CT scan.[76] Nonenhanced tumor areas reflect tissue necrosis, whereas viable cancer cells are recognized by enhanced areas inside treated areas.

In an effort to help guide clinicians in selecting a rational treatment option for a given patient with HCC, investigators from the Barcelona Clinic Liver Cancer Group recently published a staging classification and treatment schedule.[4,5,77] Staging and treatment schedules were established from the best available evidence generated from controlled clinical trials concerning patients with cirrhosis and HCC (Fig. 1). The Barcelona Clinic Liver Cancer Group established the following four stages of disease based on a combination of tumor characteristics, Okuda class, Child-Pugh class, and performance status: early, intermediate, advanced, and terminal.

BARCELONA CLINIC LIVER CANCER GROUP DISEASE STAGES

Early Stage

Surgical resection, liver transplantation, and percutaneous ethanol or RFA are potentially curative/effective treatments for patients with early HCC. Current evidence suggests that these approaches are assumed to improve the natural history of the disease prolonging the survival of patients with HCC smaller than 5 cm or three tumors smaller than 3 cm. There are no randomized clinical trials comparing these four treatment options, and thus the selection of a given approach must currently be individualized and based on analysis of prospective cohort studies.[46,74,78] However, some general consensus exists with regard to some aspects of disease management. Patients with no cirrhosis or compensated cirrhosis with predicted postresection adequate liver reserve should be considered for liver resection for HCC.[46] Patients with decompensated liver disease, significant portal hypertension, or multinodular early disease (e.g., 3 tumors < 3 cm) should not undergo resection; liver transplantation should be the first approach.[46,79] Percutaneous ethanol injection is highly effective for encapsulated HCC smaller than 3 cm[80] and should be considered the standard percutaneous technique for patients who are unable or unwilling to undergo operation until randomized clinical trials demonstrate equivalency or superiority of more expensive and invasive options such as RFA.[5,81] Recurrence after percutaneous treatments are as frequent as after surgical resection (50% at 3 years and 70% at 5 years).[82–84]

Intermediate Stage

Six randomized clinical trials found no survival benefit for transarterial embolization, alone or with

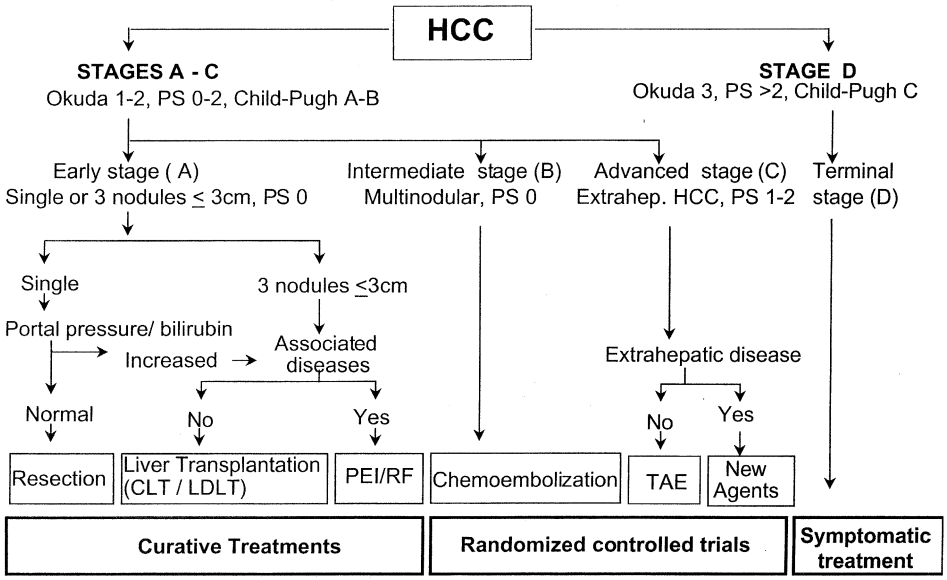

Fig. 1. Barcelona Clinic Liver Cancer Group staging and treatment strategy for hepatocellular carcinoma (HCC). CLT = cadaveric liver transplant; LDLT = living-donor liver transplant; PEI = percutaneous ethanol injection; PS = performance status; RF = radiofrequency ablation; TAE = transarterial embolization. (From Miller RA, et al. Reporting results of cancer treatment. Cancer 1981;47:207–214.)

chemotherapy (doxorubicin, cisplatin), with no treatment or suboptimal control therapies in patients with advanced, unresectable HCC.[85–90] However, two recent randomized clinical trials using more strict patient selection criteria for patients with intermediate HCC (no major portal vein invasion and preserved liver function) reported significantly improved survival in Europeans[91] and Asians[92] following transarterial embolization with either cisplatin or doxorubicin mixed with lipiodol when compared to a no-treatment control group. Based on the latter two randomized clinical trials, transarterial embolization appears to be a good treatment option available to patients with unresectable and nontransplantable, multinodular HCC and a good performance status.

Advanced Stage

At present, there are no adequate, effective chemotherapeutic agents for the treatment of advanced or extrahepatic HCC.[4,5] Patients with extrahepatic HCC or marginal performance status should be offered treatment with new agents within the context of randomized clinical trials. Large randomized clinical trials demonstrate that tamoxifen is not an effective therapy for HCC.[93,94] Chemotherapy has a negligible effect on HCC and should not be offered to patients outside of a clinical trial.

Terminal Stage

Patients with Child-Pugh class C, Okuda 3, and an impaired performance status should be offered only symptomatic treatment if they are not liver transplant candidates.

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Bile Duct Injuries 12 Years After the Introduction of Laparoscopic Cholecystectomy

William C. Chapman, M.D., Michael Abecassis, M.D., William Jarnagin, M.D., Sean Mulvihill, M.D., Steven M. Strasberg, M.D.

Laparoscopic cholecystectomy–associated bile duct injury continues to be an important clinical problem with significant long-term health implications for affected patients. Recent reports suggest that the incidence of this problem may have passed its peak, but the injury rate is still increased compared with the open cholecystectomy era. In addition, there appears to be a subset of patients who present with severe bile duct injury that may include injury to the bile duct bifurcation, the isolated right sectoral duct, and those that occur in conjunction with hepatic arterial ligation. A panel of hepatobiliary surgeons experienced in the management of these difficult surgical problems was convened at the annual meeting of the American Hepato-Pancreato-Biliary Association in Dallas, Texas, to address these unique problems and to suggest methods for injury prevention and management. This report outlines comments made by this panel.

MODERN PREVENTION OF INJURIES AT LAPAROSCOPIC CHOLECYSTECTOMY

Discussion by Steven M. Strasberg, M.D.

Biliary injury at laparoscopic cholecystectomy continues to occur at an increased rate compared with open cholecystectomy, despite the fact that more than 10 years have passed since the procedure was introduced in the United States.[1] A disturbing aspect of these injuries has been their occurrence even when cholecystectomy is performed by experienced, highly competent surgeons who are well informed of the risks of bile duct injury.[2] In reviews investigating possible contributing factors for such injuries, misidentification of the common bile duct for the cystic duct has been a common feature.[3,4] This technical error results in isolation and division of the common duct instead of the cystic duct. Thus, there appears to be a visualization error that occurs during the course of the operative dissection.[2]

One of the original methods described for identification of the cystic duct during laparoscopy involves the "infundibular" method of cystic duct isolation. In this technique, the cystic duct is dissected along its anterior and posterior aspects of the triangle of Calot. Confirmation of the anatomy is noted by seeing a "flair" as the cystic duct widens to become the infundibulum of the gallbladder neck, also described as seeing a "funnel shape." In a recent report, Strasberg et al.[2] performed a critical analysis of factors that possibly contributed to bile duct injury at laparoscopic cholecystectomy. Twenty-seven cases of common bile duct injury occurring at laparoscopic cholecystectomy were referred over a 7-year period. In this review, the "infundibular" technique was used by 80% of the operating surgeons sustaining a bile duct injury. Based on the operative reports, these surgeons thought they had correctly identified the cystic duct, when in fact the cystic duct was "hidden," and instead they had isolated the common duct. In cases of acute cholecystitis, the "hidden cystic duct" may be beneath the inflamed neck of a gallbladder inflammatory mass. Other factors that may contribute to the "hidden cystic duct" syndrome include large impacted stones, a short or absent cystic duct, and adhesions of the gallbladder to the common duct such that the cystic duct is obscured from view.

An alternative technique to the "infundibular" technique of dissection is the "critical view" technique, in which the purpose of the initial dissection is to clear the triangle of Calot completely of fibrous and fatty tissue so that the only two visible structures present in the triangle are the cystic duct and the cystic artery.[2,5] After this dissection, the base of the liver (segment IV) is visi-

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ble with no other structures seen traversing to the gallbladder. If this cannot be achieved, then the dissection is stopped and cholangiography is performed to define the anatomy and/or the procedure is converted to an open cholecystectomy.

Routine cholangiography has been suggested as a technique to eliminate or reduce the severity of bile duct injury.[6] However, it is possible that if the initial dissection incorrectly isolates the common duct or a right sectoral duct, then this misidentified duct will be ligated and a choledochotomy performed during the course of obtaining a cholangiogram.[7] In addition, the operating surgeon must correctly interpret the cholangiogram, including visualization of all proximal ducts. Although the correct interpretation of the cholangiogram may appear to be an obvious caveat, many reported series of bile duct injury include cases where only the distal common duct is visualized and the surgeon has inadvertently cannulated the common duct to achieve a "normal cholangiogram." If this technical error is not recognized, the next portion of the procedure will likely include division and/or excision of a portion of the common duct.

In summary, careful dissection of the gallbladder neck/cystic duct junction is the most important factor used to define the anatomy and prevent bile duct injury during laparoscopic cholecystectomy. Dissection of all tissue except for the cystic duct and cystic artery in the triangle of Calot allows confirmation of the true cystic duct. The "critical view" method of dissection is a safe technique to minimize the risk of inadvertent bile duct injury during laparoscopic cholecystectomy.

DIAGNOSIS AND STAGING OF REPAIRS
Discussion by William C. Chapman, M.D.

Proper management of bile duct injury during or after laparoscopic cholecystectomy is heavily influenced by the type of injury and the time of recognition. Intraoperative suspicion of bile duct injury should prompt conversion to open laparotomy with sufficient dissection and cholangiography to determine if an injury is present and to define the nature of the injury.[8] If bile duct injury is found, assistance should be sought from a surgeon with experience in bile duct repair, if possible, as this represents the best opportunity to achieve a satisfactory long-term result. Limited partial lacerations may be satisfactorily repaired with interrupted fine absorbable sutures or occasionally with end-to-end anastomosis over a T-tube. The advantage of end-to-end repair includes its simplicity and preservation of duct length. The disadvantage of this technique includes the approximately 50% rate of stricture during follow-up in most series.[9] More extensive injuries or thermal injury to the bile duct usually require Roux-en-Y reconstruction with hepaticojejunostomy, proximal to the level of the injury.

The majority of laparoscopic cholecystectomy–associated bile duct injuries, unfortunately, go unrecognized and may present weeks to months or even years later.[8] Patients with postoperative biliary fistula or subhepatic abscess usually have persistent symptoms of nausea and vomiting, abdominal distention, and mild abdominal pain. These symptoms are often inadequately evaluated by the original surgeon, so that by the time the bile duct injury is discovered, an abdominal abscess or infected biloma may be present. Although ultrasonography may demonstrate a fluid collection in the perihepatic region and ductal dilatation, if the bile duct has been ligated, more often this imaging study will not fully define the bile duct and associated injuries. Contrast-enhanced computerized tomography (CT) is probably a better initial study in the assessment of patients suspected to have bile duct injury, and may help to define the level of ductal injury and whether intra-abdominal fluid collections are present (biloma or abscess). In addition, more significant liver changes including lobar atrophy/hypertrophy from long-standing ductal obstruction or vascular injury may be defined.[8]

Initial management of patients with bile duct injury suspected in the post-cholecystectomy period is directed at establishing the diagnosis, drainage of bilomas or abscesses, and establishing the nature and extent of the bile duct injury.[9] This will usually require cholangiographic imaging of the biliary tree; although endoscopic retrograde cholangiopancreatograhy (ERCP) may provide this information and allow stent placement for cystic duct stump leaks or partial transection (Strasberg A and D injuries, respectively),[5] proximal injuries or those with complete ductal ligation or transection will usually require percutaneous transhepatic cholangiography (PTC). Suspected vascular injury should prompt performance of hepatic angiography to define the hepatic arterial and portal venous anatomy. Coexisting portal hypertension may be seen in 10% to 20% of patients, particularly those with repeated failed attempts at bile duct repair, and this finding significantly worsens the prognosis and perioperative mortality risks of surgical interventions.[10]

Although tangential bile duct injuries may be successfully managed with endoscopic or percutaneous stenting, almost all patients with complete transection or ligation of the bile duct will require operative reconstruction; however, there has been disagreement regarding the timing of such intervention. Many reports recommend initial nonoperative management

with biliary diversion via stenting and transabdominal drainage for a sufficient period (i.e., 8 to 12 weeks or longer) to allow resolution of localized inflammation associated with the injury and bile leak, followed by delayed repair. Although initial nonoperative management should be used in the unstable patient, we have found this strategy difficult to maintain in the outpatient setting; it is often associated with patient morbidity during the interval period because drains become occluded or incompletely drain the biliary tree.

Our group has used a strategy of operative reconstruction as soon as patients are stable and the necessary preoperative imaging studies have been completed. With the use of this strategy, we were able to perform reconstruction at a median of 2 days after referral,[9] resulting in an average length of stay of 11 days (median 9 days), compared with an average length of stay of 32 days in a recent report in which a strategy of delayed operative repair was used.[11] In our experience, 89% of patients have had no need for reintervention in follow-up, whereas 9% have required a single episode of balloon dilatation and 2% (1 patient) have required repeat operative bypass in the follow-up period.[9] Although we believe that such a selective approach can allow for earlier intervention with good or excellent long-term results, it cannot be overemphasized that the strategy of early repair of bile duct injury should be used only in stable patients without hemodynamic instability or ongoing sepsis.

THE ISOLATED ABERRANT RIGHT HEPATIC DUCT INJURY
Discussion by Sean Mulvihill, M.D.

Injury to aberrant right sectoral ducts appears to be a more frequent complication associated with laparoscopic cholecystomy than was seen during the era of open cholecystectomy.[7,12] These injuries (Bismuth V and Strasberg B, C, or E5) present a challenge because they can be difficult to diagnose and the small caliber of the involved duct often makes long-term repair unsatisfactory.[12] If the proximal and distal segments of these ducts are ligated, then the patient may remain asymptomatic after cholecystectomy (Strasberg B injury) and the involved liver may atrophy, with no apparent clinical sequelae. However, in many cases a fistula is present from a nonligated proximal segment (Strasberg C injury).[5] In this circumstance, ERCP images may be interpreted as "normal," because no obstruction or fistula site will be noted arising from the common bile duct. The correct diagnosis in this circumstance may be suggested by HIDA scanning, and can be confirmed with PTC demonstrating the involved segment(s) of the disconnected biliary tree.

Whenever any bile duct injury repair is undertaken, it is critically important to identify all involved segments of the biliary tree, and this is especially true for isolated aberrant right hepatic duct injuries.[8] This will usually require PTC. Under ideal circumstances, a biliary catheter is placed at the time of PTC, which may act as a technical aid at the time of operative exploration. Injury of a right sectoral duct occurring in combination with injury of the bile duct confluence can be very difficult to manage, because this results in widely separated ducts that must be reconstructed and the small-caliber right sectoral ducts often have only a short extrahepatic segment.[12]

The long-term results of reconstruction of an isolated right sectoral duct are less optimal than repairs of the main biliary tree, and higher rates of recurrent stricture formation and cholangitis are reported.[12] On this basis, construction of an access loop (see below) at the time of repair should be considered so that the radiologist can gain prompt access for cholangiography, if recurrent stricture is suspected in the follow-up period.[10]

Debate exists regarding the best management strategy when a small, 1 to 2 mm isolated sectoral duct injury is identified during the course of cholecystectomy. Many believe that such small ducts should be ligated because reconstruction of these ducts has a high likelihood of stricture formation with the risk of recurrent cholangitis. This strategy, although probably safe for the unmanipulated small sectoral duct, should not be used for a late-appearing biliary fistula or one that has been instrumented. In this circumstance, repair with a Roux-en-Y bypass to the sectoral duct should probably be performed, with the realization that stricture formation may develop in long-term follow-up.

Although some hepatopancreatic-biliary surgeons have reported the use of hepatic resection to treat the isolated bile duct stricture,[13,14] resectional approaches should probably only be used when patients are having significant symptoms of recurrent pain and cholangitis and no other satisfactory options exist for management. From this viewpoint, liver resection would be reserved for treatment failures of surgical bypass or attempts at stenting.

THE INJURY ABOVE THE BIFURCATION
Discussion by William Jarnigan, M.D.

Although the ongoing incidence of laparoscopic cholecystectomy–associated bile duct injury is not fully known, injuries to the proximal bile duct appear to occur at a higher rate than those that occur before the introduction of laparoscopy.[15,16] In the "classic" injury, the common bile duct is mistaken for the cys-

tic duct, ligated, and a significant length may be resected, often up to the level of the bile duct confluence.[3] During the course of this dissection, the right hepatic artery may be encountered with subsequent hemorrhage, and the operating surgeon may mistakenly ligate the right or common hepatic artery. Repair of these injuries can be extremely challenging, and ideally the surgeon should seek advice from a more experienced surgeon, because a poorly performed initial repair can greatly compromise an already difficult situation.

Unless the bile duct injury is recognized and an experienced surgeon is available at the time of the initial cholecystectomy, the extent of the injury should be fully investigated and coexisting conditions treated before a bile duct repair is attempted. Preoperative imaging studies should attempt to determine whether there is a subhepatic abscess or fluid collection, and these should be percutaneously drained prior to surgical repair. Only when the patient is fully stabilized should operative intervention be undertaken. The level of the injury will usually need to be defined with cholangiography (usually PTC) and associated vascular injuries determined.

There are three critical principles for a successful surgical repair of the proximal biliary confluence: dissection and identification of healthy bile duct mucosa proximal to the level of the injury or stricture; anastomosis to a Roux-en-Y limb of at least 70 cm in length, and direct mucosa-to-mucosa anastomosis with interrupted absorbable sutures.[15] The safest and most reliable approach for identifying the bile ducts at the confluence is to first expose the left hepatic duct by lowering the hilar plate at the base of segment IV.[17] In addition to its constant location, the left duct is rarely involved in the dense adhesions that may be present in the region of the initial injury. Once the left duct has been identified, the dissection proceeds to the right to identify the right ductal system. Whenever possible, a single anastomosis between the left and right ducts is constructed. A technical aid to performing the anastomosis for a proximal duct stricture involves initially placing the anterior row of sutures into the duct and these are secured on shods.[8] Next the posterior row of sutures is placed between the jejunum and bile duct. After the posterior row is placed and tied in place, the previously placed anterior row needles are passed through the jejunum and the anastomosis is completed.

Several additional points should be emphasized. Proximal bile duct injuries may sometimes separate the right anterior and posterior ducts. It is critically important to identify and drain all such ducts or serious complications may occur, including recurrent cholangitis, or persistent bile leakage. In rare circumstances, it may be necessary to incise the gallbladder fossa to gain access to the right ductal system or to perform a partial liver resection or segment III bypass, if there is no accessible left extrahepatic bile duct.[8,18] In cases where the repair is difficult or where the risk of recurrent stricture formation is likely, consideration should be given to establishing an access loop by tacking the end of the Roux limb to the peritoneal surface or subcutaneous tissue for future radiologic access, should this be needed.[15] With the use of these techniques, most proximal bile duct injuries can be approached and satisfactorily repaired at experienced centers.

COMBINED VASCULAR/BILIARY INJURIES: WHEN IS TRANSPLANTATION NEEDED? Discussion by Michael Abecassis, M.D.

Because of the close proximity of the right hepatic artery to the common hepatic duct, usually traversing posteriorly (80% to 90%) or anteriorly (10% to 20%) to the duct, the right hepatic artery is subject to injury in association with laparoscopic bile duct injury. Because laparoscopic cholecystectomy–associated bile duct injury appears to occur at a more proximal level than in previous open cholecystectomy–associated duct injury, it is not surprising that arterial injury may be a concurrent injury to bile duct injury. Although previous reports have recognized the risk of concomitant hepatic arterial injury, only recently has attention been directed toward the potential significance of arterial ischemia as a potential etiologic factor of failed initial repairs after bile duct injury.[19]

Several features of bile duct injury and stricture recurrence may suggest the possibility of arterial injury. High-level proximal injuries that involve misidentification of the common bile duct for the cystic duct may lead to dissection of the proximal hepatic duct with excision of a long segment of the duct itself, and this misdirected dissection can cause the surgeon to injure and subsequently ligate the adjacent hepatic artery. Concomitant vascular injury should be suspected in cases where significant hemorrhage is noted at the time of cholecystectomy, or when there is a significant increase in hepatic transaminases after cholecystectomy and should prompt performance of hepatic angiography to define the hepatic arterial and portal venous anatomy. Other clues to this possibility include the description of significant hemorrhage during the course of the cholecystectomy, and multiple clips on plain film images of the abdomen.

In a recent study of 18 consecutive cases of failed initial repair of bile duct injury referred to his center, Kaffron et al.[19] identified coexisting arterial injury in 11 patients (61%). All patients with failed initial repairs

underwent angiographic investigation. Duplex ultrasonography failed to identify the arterial injury in any case in which it was attempted, likely because of the presence of collateral flow that developed in the vicinity of the ligated right hepatic artery. There was a significant association between the proximal bile duct injury (Bismuth level III or IV, or Strasberg E3 or E4) and the presence of an arterial injury. There was also a longer time interval between stricture recurrence after the initial repair in patients with an arterial injury compared with those without an arterial injury, suggesting to these investigators that technical errors in reconstruction may have contributed to the failures without arterial injury, whereas ischemia in those with ligated hepatic arterial branches may have caused a technically successful reconstruction to fail.[20,21] Although the natural history of combined hepatic arterial and bile duct injury and reconstruction is not fully known,[22] it is well recognized that recurrent stricture formation is a risk that may occur for up to 5 to 10 years after successful biliary reconstruction, and this risk may be greater if arterial ischemia is also present.[19] From this perspective, identification of the full extent of associated injury, including arterial injury, is important to document, before embarking on reconstruction of the bile duct. This is particularly true for cases subject to medical-legal disputes, where identification of an arterial injury after several operative reconstructions potentially could place any of the operating surgeons under scrutiny as being responsible for the arterial injury.

Although the development of biliary cirrhosis and end-stage liver disease is a risk of recurrent cholangitis from failed reconstruction and persistent biliary obstruction, the need for liver transplantation from bile duct injury, even in association with hepatic arterial injury, is a very unlikely outcome, so long as a satisfactory reconstruction of the proximal biliary tree is able to be performed. Anecdotal reports of liver transplantation for laparoscopic bile duct injury have occurred, usually in the setting of very extensive bile duct and vascular injury, including portal venous injury. However, the need for liver transplantation after bile duct injury can occur after longstanding uncorrected bile duct obstruction with recurrent cholangitis and biliary cirrhosis, emphasizing the need to optimize the initial attempts at bile duct repair.

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Primary Cystic Neoplasms of the Pancreas: Neoplastic Disorders of Emerging Importance—Current State-of-the-Art and Unanswered Questions

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Primary cystic neoplasms of the pancreas have been recognized for years as curious but rare neoplasms. However, it was not until Compagno and Oertel,[1,2] in 1978, clearly characterized histopathologically both the features and differentiation of serous and mucinous cystic lesions that the importance of identifying the mucinous neoplasms became clear because of their overt or latent malignant potential. Since then, many groups have further refined the classification, diagnosis, differentiation, and appropriate management of these unusual neoplasms, recognizing their different biological behavior.

In 1982, Ohashi et al.,[3] in Japan, reported a seemingly new type of cystic neoplasm of the pancreas that they termed "mucous secreting cancer of the pancreas." Although this report went virtually unappreciated in the Western world for nearly 10 years, a dramatic increase in similar anecdotal reports of a spectrum of disorders began appearing in the world literature. These reports described a condition variably termed mucinous ductal ectasia, intraductal papillomatosis, intraductal adenoma (adenomatosis), intraductal mucin-secreting tumor, intraductal papillary mucinous tumor (IPMT), and most recently intraductal papillary mucinous neoplasm (IPMN).

Recent work has shown that IPMN is not a "new" entity secondary to a new mutation or environmental exposure, but rather a newly recognized disorder.[4] Better noninvasive imaging techniques, pathologic scrutiny of "atypical" mucinous ductal neoplasms, reanalysis of unusual clinical presentations of chronic pancreatitis, and a heightened interest in cystic diseases of the pancreas all contribute to increased recognition of this lesion. Although we acknowledge the referral bias of tertiary referral institutions, IPMN has represented 17% to 25% of resections for pancreatic neoplasms in the past 5 years at Mayo Clinic, Massachusetts General Hospital, and Johns Hopkins Hospital. Because IPMN harbors overt or latent malignancy at the time of diagnosis (about 40% of resected IPMNs have an invasive malignancy), we must recognize this disorder as an important and probably more common neoplasm than we have acknowledged in the past.

This review will present a focused discussion of the clinical presentation, spectrum of histopathologic findings, diagnosis, and appropriate treatment (or at least the controversies involved) of the primary cystic neoplasms of the pancreas, concentrating on and contrasting serous cystadenomas, mucinous cystic neoplasms (MCNs), and IPMNs. We will not address, in depth, the much less common solid pseudopapillary neoplasms of the pancreas, cystic islet cell neoplasms, or other cystic neoplasms involving the pancreas. Special emphasis will be placed on the differences between these unique disorders, clarifying past misunderstandings and confusion, and highlighting several still unanswered questions and controversies. This discussion stems from a state-of-the-art panel discussion of the spectrum of cystic neoplasms of the pancreas that was recently held at the American Hepato-Pancreato-Biliary Association Surgical Forum during the 2001 meeting of the Association for the Advancement and Study of Liver Disease.

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CLINICAL PRESENTATION

With the advent of better and more easily accessible noninvasive imaging modalities, cystic disorders of the pancreas are being diagnosed much more frequently. Patients with serous cystic neoplasms and MCNs have a mean age at presentation of 50 years with a distinct female predominance of 4 to 1; some groups even suggest that MCNs do not occur in men.[5] These patients are often asymptomatic (up to 75%) with the cystic mass identified incidentally during diagnostic investigation of an unrelated abdominal complaint or even at a "virtual physical examination" by "screening CT." When present, symptoms are secondary to the mass effect of these often large neoplasms. Jaundice is unusual (<15%), even when lesions are in the head of the pancreas, which is in stark contrast to ductal adenocarcinoma; likewise, weight loss, epigastric or back pain, gastric outlet obstruction, and severe malaise are usually absent. Although several reports relate a prior episode of (presumed) pancreatitis in 20% of patients,[6–8] the majority of these patients probably did *not* have a well-documented or characteristic episode of acute pancreatitis but instead had pain believed to be related to pancreatitis because of the now-recognized pancreatic mass. Nevertheless, rare patients have been diagnosed with MCN after a well-documented episode of acute pancreatitis.

Patients with IPMN are usually older (mean age approximately 65 years) and, in contrast to the serous and mucinous cystic neoplasms of the pancreas, have a slight male predominance. Although some patients (approximately 20%) with IPMN are asymptomatic (especially those with only branch duct disease, see below), most patients have vague abdominal pain and a clinical presentation of acute, recurrent, or more commonly chronic pancreatitis (steatorrhea, diabetes mellitus, weight loss). When invasive carcinoma coexists, as it does in up to 40% of patients, a symptom profile similar to that of ductal cancer of the pancreas (pain, jaundice, weight loss, malaise) may be seen.

PATHOLOGY

The most common cystic lesion of the pancreas is the pseudocyst, but surgeons and pathologists need to know that the epithelial lining of the cystic neoplasm is often (>40%) partly denuded of epithelium, and thus may be misdiagnosed as a pancreatic pseudocyst.[8] This problem is especially pertinent on a limited frozen section of a presumed "pancreatic pseudocyst." Neoplastic cysts of the pancreas can be categorized as true cysts (serous and mucinous cystic neoplasms), dilations of the pancreatic duct system (IPMN), and cystic degeneration of solid tumors (solid pseudopapillary tumor, cystic islet cell tumor). The latter subgroup will not be discussed here.

Serous Cystic Neoplasm

Serous "microcystic" adenoma, by far the most common serous neoplasm, forms a well-demarcated, spongy mass of small cysts filled with clear, watery fluid (Fig. 1). There is often a central stellate scar. The cysts are lined by a single layer of cuboidal cells with round nuclei and clear cytoplasm. The much less common serous oligocystic adenoma has fewer (sometimes only one) cystic spaces, but the histologic appearance is similar to that of the microcystic tumor. Serous cystadenocarcinomas have been described,[9] but they are so rare (fewer than 10 in the literature) that serous cystic neoplasms of the pancreas should be considered (and managed as) benign neoplasms.

Mucinous Cystic Neoplasm vs. Intraductal Papillary Mucinous Neoplasm

Gross and microscopic pathologic features usually allow one to distinguish between these two tumors. Tumor site is one helpful feature in that more than 90% of MCNs occur in the body or tail.[5,10] IPMNs can involve any part of the pancreatic ductal system; most arise in the pancreatic head, but body or tail involvement is not uncommon, and some IPMNs extend for many centimeters along the pancreatic duct, making it difficult to designate a primary site. In the Mayo Clinic series of 87 IPMNs,[11] 60% were in the head, 25% in the tail, and 15% showed diffuse involvement of the pancreas. Others describe a similar experience, with 73% of IPMNs in the head of the pancreas, 14% in the tail, and 25% diffuse.[12]

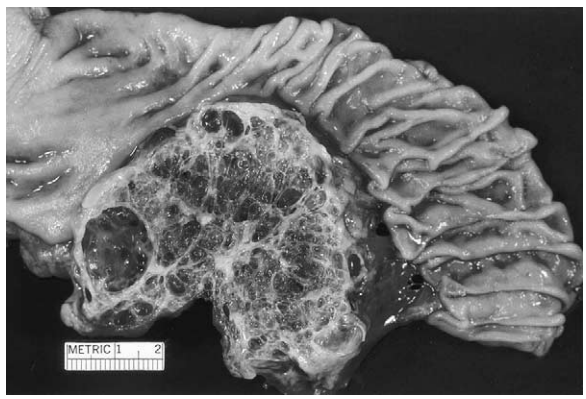


Fig. 1. Serous cystadenoma.

The gross contour of the two lesions is usually different. MCN has a round contour formed by a thick fibrous capsule that encircles cystic spaces,[6] and the cyst does not communicate with the pancreatic ductal system (Fig. 2). In contrast, one of the older synonyms for IPMN—mucinous pancreatic duct ectasia—is a useful reminder that IPMN results from cystic dilation of the duct system[12] (Fig. 3). As such, it is often poorly demarcated, particularly when the tumor involves the main pancreatic duct, presenting as an irregularly dilated, mucous-filled duct, with polypoid excrescences or solid areas in the duct wall. When involvement is limited to branch ducts only, as first described by Itai et al.[13] in 1986 as "ductectatic mucinous cystadenoma," branch duct IPMN is more sharply defined but still tends to form a lobulated mass, and the cystic areas communicate with the pancreatic ductal system. Awareness of this variant is important because surgeons might (and possibly have) mistaken the branch duct type of IPMN for a MCN. It has been proposed that the branch duct variant has a better prognosis than IPMN that involves the main duct.[14,15]

Histologic examination of the cyst lining does not necessarily help distinguish between IPMN and MCN. The epithelium of both lesions comprises pancreatic duct–like cells with mucin production and often exhibits a papillary architecture. The lining of MCN is usually a single layer of tall, mucin-producing columnar cells, but papillae and complex architectural patterns may be seen. The epithelium in IPMN usually resembles that of a colorectal villous adenoma—well-developed papillae with fibrovascular cores, covered by tall columnar cells, and occasional goblet cells. Sometimes the papillae are more complex and arborizing (the so-called pancreatobiliary pattern), and sometimes the epithelium is flat. A unique but rare variant is the oncocytic papillary tumor, in which complex papillae are lined by plump cuboidal cells with abundant pink cytoplasm.[16] A diverse metaplasia may be seen, including pseudopyloric, squamous, and osseous forms. All of the metaplastic changes described in IPMN can be seen in MCN.

Although examination of the epithelium may not help distinguish between MCN and IPMN, study of subepithelial stroma is useful. In IPMN, the involved ducts are surrounded by nondescript, loose fibrous stroma. In contrast, MCN features a spindle cell stroma strongly reminiscent of ovarian stroma. This stroma is commonly described as present in "most" or "almost all" MCNs, and recent reviews[5,10] suggest that ovarian stroma is required for the diagnosis of MCN. Extensive hyalinization of the cyst wall sometimes makes the stroma difficult to detect, but sufficient tissue sampling should reveal the characteristic stroma. The ovarian stroma also contains single epithelioid cells resembling luteinized ovarian hilar cells. The spindled stromal cells are often positive for estrogen and progesterone receptors, and the epithelioid cells are positive for inhibin, a marker of sex-cord stromal differentiation.

Pancreatic tissue adjacent to both IPMN and MCN can show changes of chronic pancreatitis with dense fibrosis and acinar atrophy. Changes in duct epithelium can be a part of this reactive process with the normal cuboidal or low columnar epithelium becoming taller and more mucin rich. In IPMN, nearby ducts often show dysplastic changes, with nuclear crowding, stratification, and hyperchromasia. These changes represent spread of the intraductal neoplasm along small duct branches (or multifocal involvement of the duct system). Dysplasia of ducts distant from the main lesion is not considered a feature of MCN, although a recent study described such changes in nearly half of carefully sampled MCNs.[17]

In summary, pathologic criteria distinguish between IPMN and MCN in nearly all cases. The most useful criteria include the following: site (head favors IPMN,

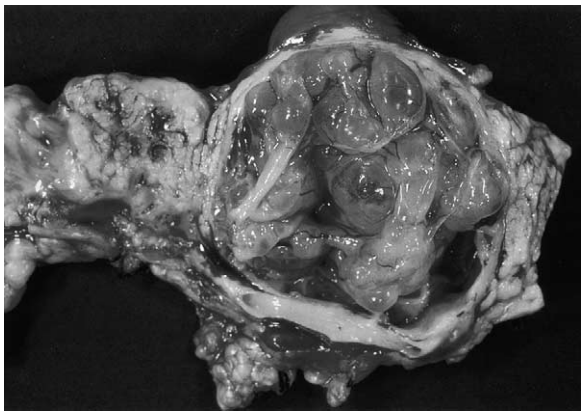


Fig. 2. Mucinous cystic neoplasm.

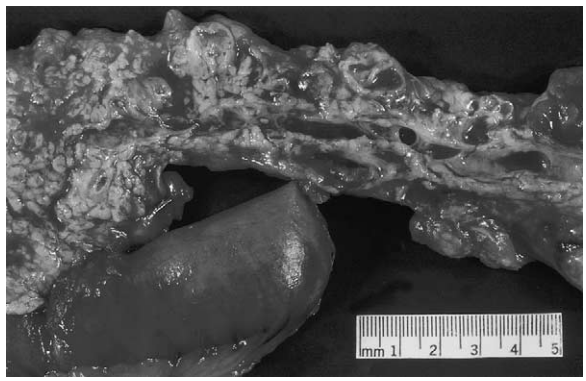


Fig. 3. Intraductal papillary mucinous neoplasm.

body or tail favors MCN); shape (poorly circumscribed or lobulated favors IPMN, round and well-circumscribed favors MCN); duct changes adjacent to the lesion (dysplasia seen commonly in IPMN), and ovarian stroma (seen only in MCN). Microscopic examination of the epithelium alone does not help with the differential diagnosis.

The differential diagnosis between IPMN and MCN can be difficult in two settings. One is a woman with a well-circumscribed tumor in the tail of the pancreas, without dysplasia in ducts adjacent to the lesion, but also without ovarian stroma. Although extensive sampling of the tumor will almost always reveal ovarian stroma, it is theoretically possible that hyalinization could obliterate all ovarian stroma. In this setting, if clinical and radiologic features support the diagnosis of MCN, a diagnosis such as "consistent with MCN" seems prudent. A second group of difficult-to-classify tumors occur in men with round, well-circumscribed cystic tumors in the head of the pancreas, with no changes in ducts adjacent to the mass, and no ovarian stroma. The diagnosis in this group is problematic, but branch duct IPMN is the most tenable diagnosis.

Pathologic Classification of Mucinous Cystic Neoplasm and Intraductal Papillary Mucinous Neoplasm: Invasive and Noninvasive

The most robust predictor of prognosis for MCN and IPMN is the presence or absence of invasive adenocarcinoma. Two recent series of MCN describe invasive carcinoma in 36% (47/130)[5] and 29% (16/56).[10] Seven of the 16 patients in the latter series were dead of disease at 2 to 45 months. The Mayo Clinic experience is slightly different, with only 7 (8%) of 84 MCNs having an invasive component. Six of the seven patients with invasive tumors died of disease. The prevalence of invasive carcinoma in IPMN appears to be higher, with rates ranging from 36% at the Mayo Clinic (unpublished data) to 53% in the Memorial Sloan-Kettering Cancer Center experience.[18] Crucial to recognizing invasive carcinoma is adequate tissue sampling, because the invasive component may be a small part of the lesion; in fact, it has been recommended that MCNs be completely embedded for histologic review.[19]

The noninvasive epithelium of MCNs and IPMNs is dysplastic, and the dysplasia can range from low to high grade. The World Health Organization recognizes three levels of dysplasia: adenoma, borderline, and carcinoma in situ (Table 1). Many lesions contain all three grades of dysplasia; grading should be based on the worst type of epithelium present. The World Health Organization classifies lesions with carcinoma in situ but no invasive carcinoma as "malignant, noninvasive." This designation is probably unfortunate with regard to MCNs, because complete excision of a noninvasive MCN appears to be curative regardless of the degree of atypia in the lining epithelium.[6,10] The designation "malignant, noninvasive" may be more significant in IPMN, because noninvasive IPMN can recur and can cause death.[11,18]

DIAGNOSIS—IMAGING

The suspicion of a cystic pancreatic neoplasm is almost always first evident after a noninvasive imaging procedure—ultrasonography, computed tomography (CT), or magnetic resonance imaging (MRI). Cystic pancreatic neoplasms often can be distinguished by their imaging morphology[20]; however, in some patients, accurate differentiation may be difficult, and cyst aspiration and biopsy may be necessary to determine appropriate treatment.

Serous Cystadenoma

The two most characteristic features of this neoplasm are the multiple small (<2 cm) and occasionally microscopic cysts and, when present, a central starburst calcification. These benign neoplasms occur most commonly in the body and tail of the pancreas but occasionally are seen to arise in the head of the gland. In the latter case, they only rarely cause extrahepatic biliary obstruction by compressing the adjacent intrapancreatic segment of the common bile duct. Serous cystadenomas have three morphologic patterns: polycystic, oligocystic, and honeycomb. These

Table 1. World Health Organization classification of cystic neoplasms of the pancreas*

Serous microcystic adenoma
Serous oligocystic adenoma
Serous cystadenocarcinoma
Mucinous cystadenoma
Mucinous cystic tumor—borderline
Mucinous cystadenocarcinoma
Noninvasive
Invasive
Intraductal papillary mucinous adenoma
Intraductal papillary mucinous neoplasm—borderline
Intraductal papillary mucinous carcinoma
Noninvasive
Invasive

*Compiled from Hamilton SR, Aaltonen LA, eds. World Health Organization Classification of Tumours. Tumours of the Digestive System. Lyon: IARC Press, 2000, pp 234–240.

neoplasms are best and most readily evaluated with CT and ultrasonography. Although MRI can be used, it rarely provides additional useful information. In contrast, endoscopic ultrasonography can be helpful for evaluation of the internal architecture of the cysts, particularly when the oligocystic or macrocystic variants are present.

The polycystic pattern is the most common, occurring in approximately 70% of patients. This pattern is characterized by a bosselated collection of cysts, usually greater than six in number, that range in size from a few millimeters to, on occasion, 2 to 3 cm in diameter (Fig. 4, *A*). A central fibrous scar with a characteristic stellate pattern of calcification occurs in up to 30% of these neoplasms and, when present, is considered to be virtually pathognomonic of serous cystadenomas (Fig. 4, *B*). The honeycomb pattern is seen in approximately 20% of patients and is characterized by numerous, subcentimeter cysts that cannot be depicted as individual cysts by cross-sectional imaging (Fig. 4, *C*). Thus they appear as solid masses, which are hypoechoic on ultrasound imaging, of low attenuation on CT, and have a high signal intensity on T2-weighted MRI. The oligocystic or macrocystic pattern is the least common, occurring in less than 10% of patients. These benign neoplasms may be difficult to differentiate from pancreatic pseudocysts or from mucinous cystic neoplasms on the basis of imaging alone. Biopsy and/or cyst aspiration may, on occasion, prove necessary.

Mucinous Cystic Neoplasm

MCNs predominantly are macrocystic (80%) (Fig. 5, *A*), but rarely they can be multilocular (20%) or have several adjacent cysts (Fig. 5, *B*). These neoplasms also occur predominantly in the body and tail of the pancreas, and although they do not communicate with the pancreatic duct, they can cause partial pancreatic ductal obstruction. MCNs usually range in size from 4 to 5 cm in diameter but may reach very large sizes of up to 20 cm in diameter. Cross-sectional imaging usually shows the cysts to have thick, irregular walls with papillary excrescences extending into the cysts. The complex internal architecture of the cysts is best depicted by ultrasonography, endoscopic ultrasonography, or MRI, allowing differentiation from serous cystadenomas. Although MCNs have often been misdiagnosed as pancreatic pseudocysts in the past, MCNs usually lack an extracystic, inflammatory component. Calcifications are uncommon but when present (<20%) tend to be located in an eggshell distribution within the peripheral cyst walls. It is believed that the likelihood of (invasive) cystadenocarcinoma increases if calcifications are seen.[21] The presence of an eccentrically located mass within a cystic area (Fig. 5, *C*), a recognizable pericystic mass/reaction (Fig. 5, *D*), extrahepatic biliary obstruction, associated metastatic cystic liver lesions, or ascites should raise the suspicion of a mucinous cystadeno-

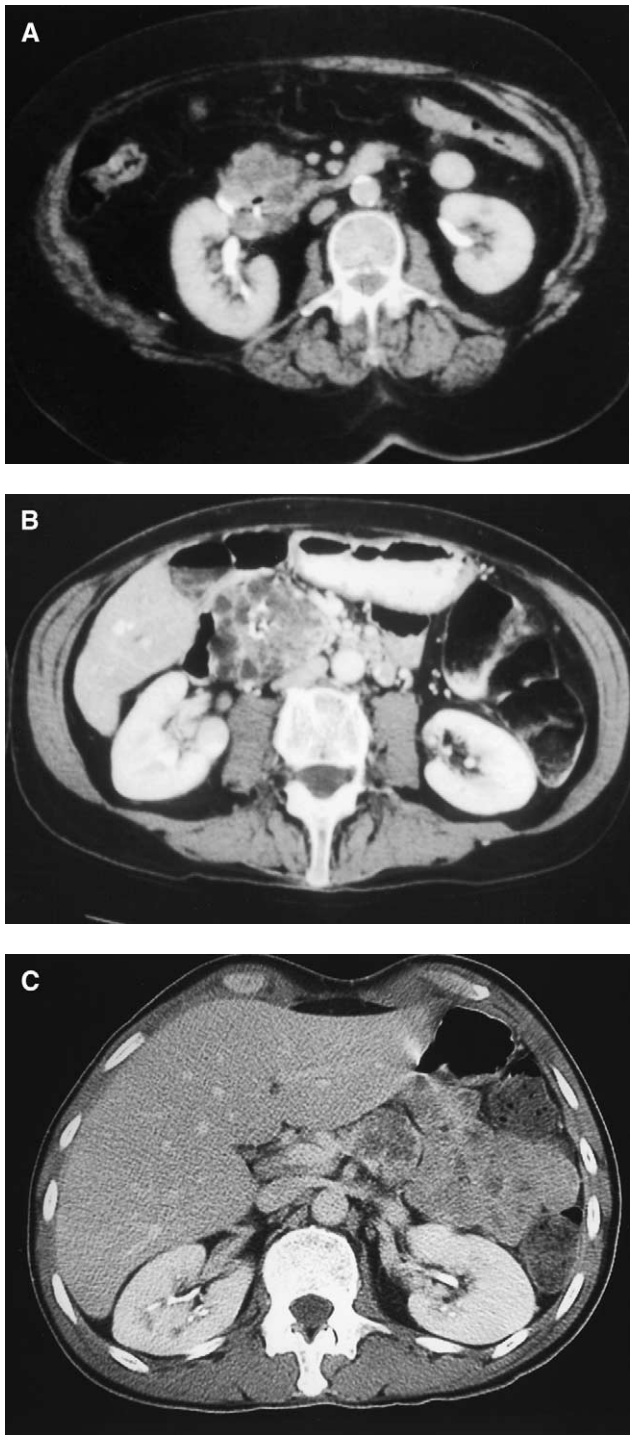


Fig. 4. Serous cystadenomas (CT appearance). **A**, Typical pattern; multiple small cystic areas. **B**, Central calcification. **C**, Solid-appearing mass.

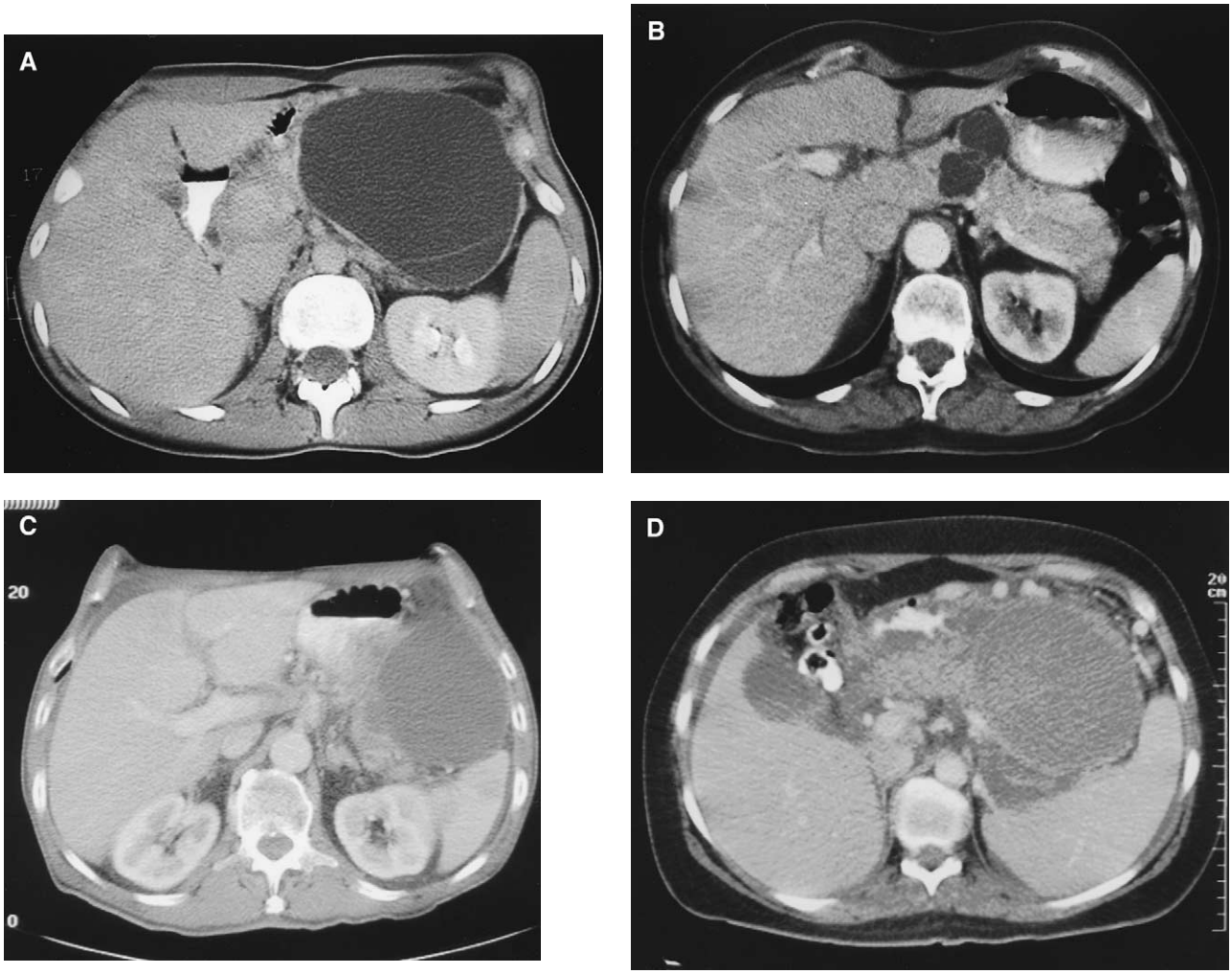


Fig. 5. Mucinous cystic neoplasms. **A**, Single cyst; note septum across the "cyst." **B**, Typical appearance, mid-body of pancreas; note lack of surrounding reaction. **C**, Eccentric mass within cystic area; characteristic of cystadenocarcinoma. **D**, Pericystic reaction, characteristic of cystadenocarcinoma.

carcinoma (invasive form). In the absence of these features, differentiation of benign mucinous cystadenoma from the noninvasive proliferative MCN may not be possible; however, multiple papillary invaginations on CT, ultrasonography, or endoscopic ultrasonography signify the proliferative nature of the mucinous epithelial lining.

Intraductal Papillary Mucinous Neoplasm

The characteristic feature of IPMNs is cystic dilatation of either the main pancreatic duct or a primary segmental side branch of the main duct. A feature differentiating serous or mucinous cystic neoplasms from IPMNs is the lack of a direct communication with the main pancreatic duct with the former and the presence of an obvious ductal communication in the latter.

Traditional imaging of IPMNs can be accomplished using a variety of modalities, including CT, MRI and magnetic resonance cholangiopancreatography (MRCP), transcutaneous or endoscopic ultrasonography, and endoscopic retrograde cholangiopancreatography (ERCP).[22–24] More recently, techniques of intraductal (within the pancreatic duct) ultrasonography and even intraductal pancreatoscopy have been described.[25–27] IPMN has four morphologic variations, which reflect the location of the small intraductal tumor and the amount of mucin secreted by the neoplasm (Fig. 6). The predominant patterns are (1) diffuse main pancreatic duct ectasia (see Fig. 6, *A*); (2) segmental main pancreatic duct ectasia (see Fig. 6, *B* and *C*); (3) side branch duct ectasia, usually located in the head or uncinate process of the pancreas (see Fig. 6, *D*); and (4) the much less common unifocal or multifocal cysts with pancreatic duct com-

munication (see Fig. 6, *E*). Although the degree of duct dilatation appears to be determined either by the amount of mucin production or the presence of proximal duct obstruction, a rare subtype of diffuse main pancreatic duct ectasia is caused by complete filling of the dilated main pancreatic duct by papillary tumor. The mucin may be seen as diffuse focal filling defects within the pancreatic duct during ERCP, which move about as contrast medium is injected into the duct. Mucus also has been seen "oozing" from a normal or patulous papilla during endoscopy.[28] Although the intraductal adenomas of IPMN may be recognized on ERCP, they often are obscured by the mucin. CT is probably the single best modality for evaluation of these patients, because it detects the location and degree of pancreatic duct dilatation and may be able to differentiate IPMN from other causes of duct dilatation, such as chronic pancreatitis or obstructing tumors. MRCP can display the pancreatic duct anatomy, and endoscopic ultrasonography (or more recently pancreatic ductoscopy) can detect the intraductal tumor component. However, the "gold standard" for diagnosis of IPMN is ERCP because of its ability to depict the communication between the cystic dilatation (or branch duct ectasia) and the main pancreatic duct, thus allowing differentiation from cystic neoplasms or other causes of duct dilatation.

In the combined series, preoperative discrimination of benign from malignant IPMN was believed by the authors to be difficult, if not impossible. Although it has been found that tumors confined to the side branch ducts are more often benign[14,15] and that tumors involving the main duct or causing main duct dilatation greater than 10 mm, or those associated with an intraductal tumor mass greater than 10 mm, are more often malignant, considerable overlap exists.[23,28,29] However, most investigators agree that accurate differentiation of benign from malignant tumors can usually not be made even by the surgeon at the time of pancreatectomy but rather can be made only at histopathologic examination of the resected segment of pancreas.

DIFFERENTIATION OF CYSTIC NEOPLASMS

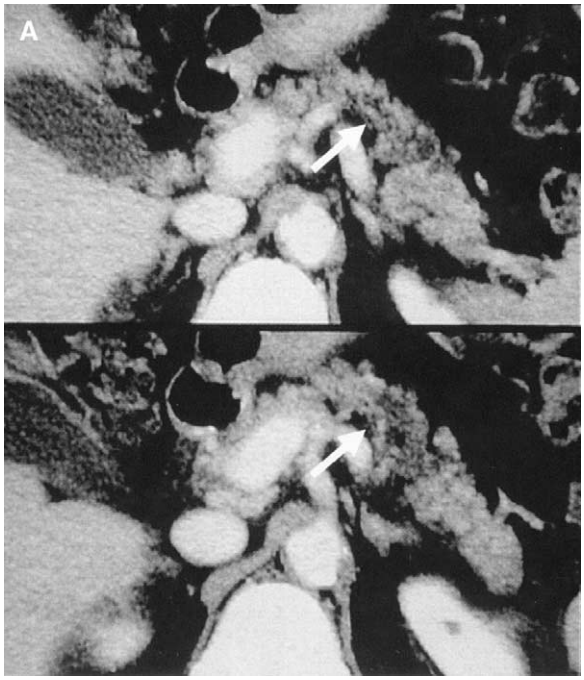
Because cystic lesions of the pancreas often masquerade as one another, it may prove very important to differentiate the following: pancreatic cystic neoplasms from pancreatic pseudocysts; pancreatic from peripancreatic cystic lesions; serous from mucinous neoplasms; IPMN from obstructive chronic pancreatitis; and maybe even MCN from branch duct IPMN. Much of this differentiation can be made on clinical presentation and by the characteristics of imaging described earlier in this report, but still a select few of these lesions remain uncharacterized. For this reason, much work has been devoted to the analysis of pancreatic enzymes, cytologic findings, and tumor markers within intracystic fluid obtained by percutaneous or more recently endoscopic ultrasound–guided aspiration.

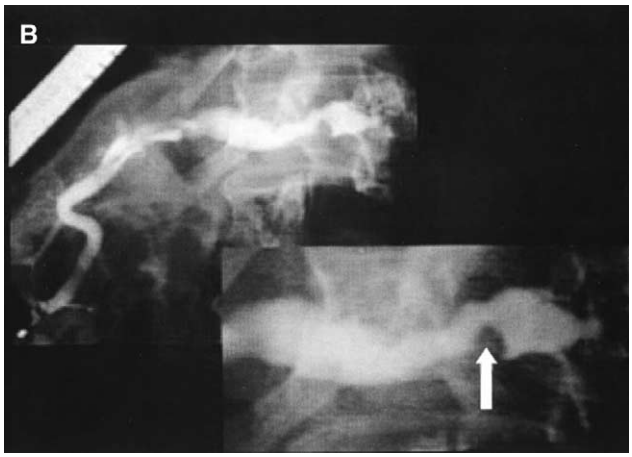
Initially, the presence of increased amylase or lipase activity in the cystic fluid suggested a benign, nonneoplastic etiology (pancreatic pseudocyst). However, a small percentage of MCNs also contained increased amylase or lipase activity[30,31]; in retrospect, these "MCNs" were possibly (probably) branch duct IPMNs, which communicated with the main pancreatic duct. Nevertheless, in a patient with a history of well-documented acute pancreatitis and a well-demarcated, unicystic lesion without septae, increased amylase or lipase activity is virtually pathognomic of a pancreatic pseudocyst; conversely, absence of increased amylase activity excludes a pancreatic pseudocyst.

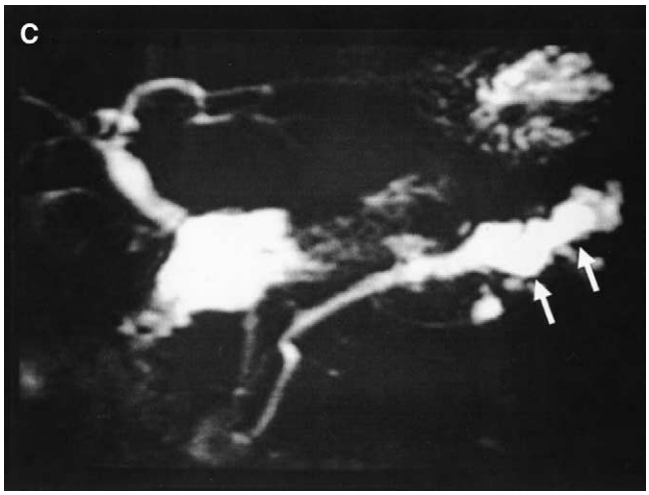
The sensitivity of cystic fluid cytology for recognition of cystic neoplasms[30,31] is not yet defined. Cystic fluid cytology will not reliably differentiate pseudocysts from neoplasms; however, the presence of a positive stain for mucin or an increased viscosity is highly sensitive for a MCN but will not reliably differentiate invasive from noninvasive neoplasms. The ability to obtain "minibiopsies" from the solid component of a cystic neoplasm or of the cyst wall itself increases the ability to make a differential diagnosis. The presence of cuboidal cells or mucinous epithelium, when present, has a very high specificity for the diagnosis of serous or mucinous cystic neoplasms, respectively.

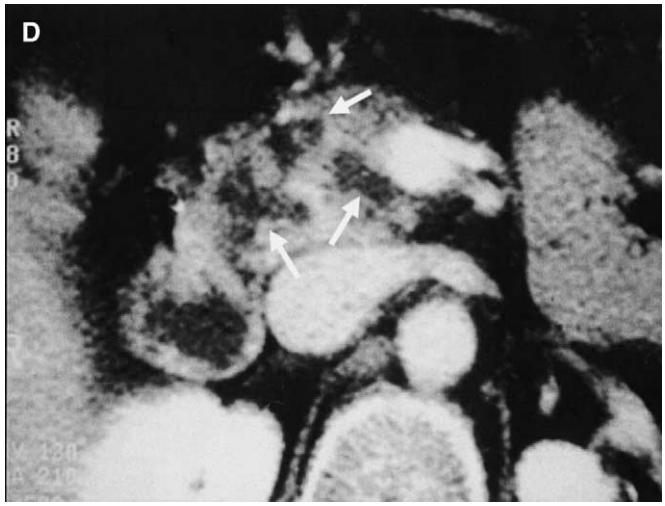
Although many tumor markers have been evaluated (carcinoembryonic antigen [CEA], CA 19-9, CA-125, CA 72-4, and CA 15.3 protein), the most discriminating markers across several studies are a positive stain for mucin and an increase in CEA. A positive mucin stain reliably identifies the mucinous (premalignant or overtly malignant) neoplasms from serous neoplasms or pseudocysts.[30,32] Likewise, an intracystic CEA concentration greater than 250 ng/ml reliably identifies a mucinous neoplasm, whereas a value less than 5 ng/ml is quite sensitive for ruling out a mucinous neoplasm. The discriminatory value of CA 19-9 in the cystic fluid is controversial.[30,32,33]

Probably the most important clinical situations in which analysis of cystic fluid is helpful are the following: (1) a patient with a persistent multicystic lesion after a poorly documented or questionable episode of acute pancreatitis; (2) an elderly woman with a radiologically indeterminate (serous vs. mucinous) cystic mass in the head of the pancreas that would require a pancreatoduodenectomy to resect and in whom ob-









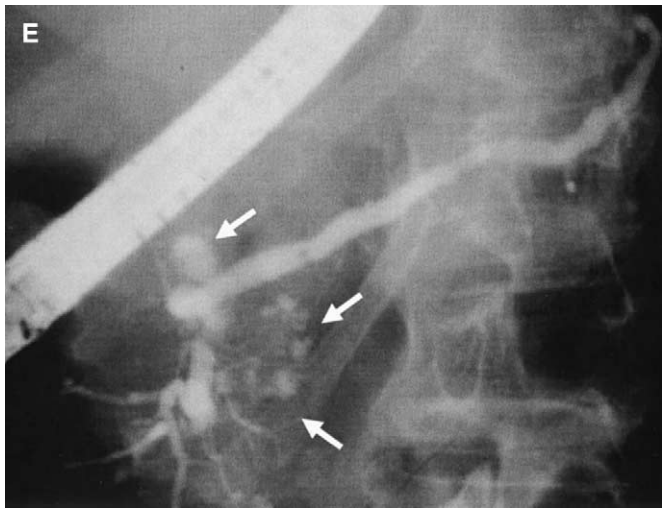


Fig. 6. Intraductal papillary mucinous neoplasm. **A**, CT showing dilatation of entire main pancreatic duct. **B**, ERCP showing segmental dilatation of main pancreatic duct in body and tail with a nodule seen within duct (*arrow*). **C**, MRCP showing findings similar to Fig. 3, *B*. **D**, Side-branch IPMN with second branch dilatation limited to head of pancreas. **E**, ERCP showing continuity of focal-appearing "cysts" with the main pancreatic duct.

servation is the plan if a serous neoplasm is present; and (3) a patient with a presumed mucinous neoplasm who requests objective evidence of such prior to resection. Use of cystic fluid markers to differentiate invasive from noninvasive neoplasms has proved futile. Whether positron emission tomography and/or endoscopic ultrasonography will be able to reliably differentiate benign from malignant disease is unknown.[34]

TREATMENT

Treatment varies with the type (and even subtype) of cystic neoplasm of the pancreas. Each will be discussed separately.

Serous Cystic Neoplasm

Because these neoplasms are virtually always benign (<10 cases of serous cystadenocarcinoma have been reported[9]), resection should probably be reserved for mass-related symptoms (jaundice, pain, early satiety, etc.) or when differentiation from a mucinous neoplasm cannot be made confidently.[35] Small lesions (<2.5 cm) that do not change in size can be followed with serial imaging and managed nonoperatively.[36] Resection should be at least discussed with younger patients (<60 years of age), especially when the lesion is located in the body/tail region, because the group at Mayo Clinic has followed several patients in whom the neoplasms have increased markedly in size. When resection is planned, a conservative, nonradical resection should be used. There is no need for a lymphadenectomy or any "extended" resection; although enucleation may be possible technically, the incidence of a postoperative pancreatic fistula appears to be increased.[35,37] Segmental "central" pancreatectomy and spleen-preserving distal pancreatectomy are very reasonable procedures. In summary, complete resection is curative, and postoperative surveillance imaging is probably neither cost-effective nor necessary.

Mucinous Cystic Neoplasm

In contrast to serous cystic neoplasms, almost all MCNs should be considered premalignant or overtly malignant and, whenever safe, resected. The unpredictable spectrum of multicentric metaplasia, dysplasia, carcinoma in situ, and tissue invasion implies that these lesions can dedifferentiate and transform into a life-threatening malignancy.[6] For MCNs in the head of the pancreas, a formal pancreatoduodenectomy is usually indicated. The benefit of an extended lymphadenectomy in this setting is untested and unproved, and most surgeons would not perform an extended resection unless an invasive MCN was highly suspected. Lesser nonanatomic resections, such as enucleation or duodenum-preserving subtotal pancreatic head resections, although technically feasible, would not be appropriate if the final pathology report demonstrates a true invasive malignancy. Moreover, others have questioned whether MCN is associated with a "field defect" in the surrounding parenchyma,[38] and a subtotal or nonanatomic resection might jeopardize the chance of cure. As questioned earlier, several presumed MCNs in the past may actually have been branch duct IPMNs, and a nonanatomic resection might have left residual IPMN, not evident on gross inspection in the operating room.

For MCNs in the body or tail region, a segmental central resection or a spleen-preserving resection can be considered if there are no indications that the neoplasm has an invasive component; yet this decision is taken at a small calculated risk. Indeed, one study has suggested the presence of K-ras mutations in non-neoplastic ducts adjacent to the neoplasm.[38] In many patients, a classical distal pancreatectomy with splenectomy may be the best treatment.

The long-term outcome of surgical treatment of MCNs is well-established.[2,6,7,8] Complete anatomic resection of MCNs lacking any invasive component is curative, and surveillance imaging is probably not necessary, even for the Armed Forces Institute of Pathology subclassification "mucinous cystadenocarcinoma of low-grade malignant potential,"[5] or the subclassification of Zamboni et al.[10] of "noninvasive mucinous cystadenocarcinoma," or the World Health Organization classification of "noninvasive mucinous cystadenocarcinoma." More controversial is the long-term survival of patients with MCN with tissue invasion, that is, overt or invasive mucinous cystadenocarcinoma. In the past, numerous articles have claimed survival rates above 50% for "mucinous cystadenocarcinoma"[8,39,40]; however, many pathologists in the past (and some even today) consider all MCNs to be low-grade malignancies. When one closely examines the series that clearly identify and segregate out the survival of patients with MCNs containing tissue invasion, 5-year survival rates appear to be somewhat better than those for typical ductal cancer of the pancreas and range from 15% to 33%,[6,41] but are not the 60% to 70% reported in the past. Whether adjuvant or neoadjuvant chemoradiation therapy is of benefit is unknown because of the obligate, limited, single-center experience with MCN.[42]

Intraductal Papillary Mucinous Neoplasm

The appropriate surgical treatment of IPMN remains as yet unclear, because long-term analysis of

outcomes is still not clearly defined. Although surgical experience with this entity has skyrocketed in the past decade, with reports of individual series of more than 40 patients,[11,15,43–46] nevertheless, the number of patients who are more than 5 years post resection remains small.

The basic and as yet unanswered question is whether or not IPMN represents a localized process or a field defect with the potential to affect all of the pancreatic ductal epithelium. If this is a localized process, as with typical ductal cancer of the pancreas, then a focused resection of the involved anatomic region of the gland would be indicated. In contrast, if IPMN is a global disorder (field defect) of all the pancreatic ducts, then a total pancreatectomy would be indicated. Total pancreatectomy with its obligate apancreatic state, of course, has its own potential problems (brittle diabetes, exocrine insufficiency) and may not be appropriate for many patients.

Currently, most experts have gravitated toward an image-guided localized resection. When the IPMN involves branch duct disease, a localized but formal anatomic, oncologic procedure is carried out—pancreatoduodenectomy for head/uncinate tumors and distal pancreatectomy for body/tail regions. Most pancreatic surgeons would argree that there is no place for nonanatomic resections for IPMN.

Main duct IPMN is more controversial. When the dilatation of the main duct involves only the body and tail (much less common, approximately 10% of patients), most pancreatic surgeons advocate a distal pancreatectomy with immediate frozen-section analysis of the proximal pancreatic margin.[11,14,43,44] If the frozen section is negative for clearly neoplastic cells, most surgeons do not advocate total pancreatectomy in the absence of objective evidence that the proximal duct is involved. In contrast, if the margin is positive for invasive or noninvasive IPMN, then most surgeons would advocate a further pancreatic resection; if a tumor-free margin is not attainable without completing the pancreatectomy, most would proceed with total pancreatectomy, provided the patient is an appropriate candidate.

When the entire pancreatic duct is dilated, the assumption is that the disease is, at the least, in the head of the gland. Because of this assumption (provided no intraluminal or extraluminal solid mass is evident elsewhere in the duct outside the boundaries of a pancreatic head resection), a pancreatoduodenectomy is undertaken with immediate frozen-section analysis of the distal margin. A positive margin necessitates further resection and, if necessary and appropriate, a total pancreatectomy is completed. Few, if any, pancreatic surgeons would advocate a nonanatomic resection for main duct IPMN.

WHAT ARE THE OUTCOMES OF THIS SURGICAL PHILOSOPHY FOR INTRADUCTAL PAPILLARY MUCINOUS NEOPLASM?

Very few long-term, outcome-based studies are available with follow-up of more than 3 years.[11,14,15,43,45–48] Overall, 5-year survival rates average about 60 % to 70%. For branch duct IPMN, several studies strongly suggest that a local, anatomic resection is essentially curative, with no recurrences in any of the 13 patients of Terris et al.[15] and only one recurrence in the 13 patients of Kobari et al.,[14] albeit with short follow-ups (none longer than 10 years). In contrast, with main duct IPMN, occurrence in the remnant gland has been found with variable rates (0% to 10%),[11,14,15,48] provided that the frozen-section margin is negative and the resected specimen lacks invasive IPMN.[44] In a recent report, the findings of noninvasive or invasive IPMN at the resection margin did not appear to influence the overall prognosis in short follow-up.[43] However, when the resection specimen shows invasive disease, even if the margin is negative, recurrent IPMN, either in the remnant gland or more commonly in extrapancreatic sites, occurs in 50% to 90% of patients,[11,14,15,43,44,48] thereby decreasing the 5-year survival to less than 50%; multivariate analysis in one study[45] showed that lymph node status and type of resection (partial vs. total pancreatectomy) were independent predictors of survival with patients who underwent total pancreatectomy having a significantly improved survival. Not all centers, however, concur with this last observation; indeed, the opposite was found by the group from Johns Hopkins Hospital. [43] Thus invasive IPMN is an aggressive malignancy that behaves, in many respects, similar to ductal cancer of the pancreas, but with a slightly better prognosis.

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Roux-en-Y Gastric Bypass After Previous Unsuccessful Gastric Restrictive Surgery

To the Editors:

An interesting study on the Roux-en-Y gastric bypass after unsuccessful gastric restrictive surgery was published in the March/April 2002 issue of the JOURNAL OF GASTROINTESTINAL SURGERY.[1] This study confirms the results of prior studies done in the United States more than 15 years ago.[2] A recent survey from the American Society for Bariatric Surgery shows that less than 5% of bariatric surgeon members of this association are currently performing any type of restrictive procedure. However, with the widespread use of laparoscopic adjustable gastric banding in Europe, one might see a resurgence of reoperations for failed restrictive procedures, and most of them will probably involve a second banding, which may fail again. We agree with the authors that a Roux-en-Y gastric bypass is the best choice in many patients because the weight loss results are better and quality of life is improved in comparison to other methods,[3] but not all of them. These revisions may need to be done in two stages, where the band is removed first and the bypass is then performed at a later date, allowing for inflammation and gastric distortion to decrease after several months. This may, in fact, be the best strategy for the gastric banding that has been positioned very high near the gastroesophageal junction, in the so-called "esophagogastric position" or "Pars Flaccida technique." When the silicone band is removed, it leaves a scarred tract with full-thickness fibrous replacement or serosal fibrosis in the area where the intention actually is to create a small gastric pouch.[4] The surgeon may be faced with acceptance of the fact that he or she has created a pouch that is too large by transecting too low. Transecting above the tract will appear too dangerous or will frankly be transesophageal. Another possibility that the authors have not entertained is to perform a biliopancreatic diversion with or without a duodenal switch, which will not allow an anastomosis to be performed much lower than the actual site where the band was located, thereby decreasing the risk of a leak from poor tissue approximation. The authors have quoted an abstract from my group concerning the role of laparoscopy in revisional surgery. An update was published recently on 27 patients in whom laparoscopic Roux-en-Y gastric bypass was used to convert prior restrictive procedures.[5] Although the operative time appeared to be longer than in this study (232 minutes vs. 155 minutes, average vs. median), the hospital stay was decreased by 2 days (3.7 days vs. 6 days), and there were no deaths, complication rates appeared similar, and an initial weight loss with a similar trend emerged. This experience parallels or even improves on the open surgery data[6,7] and clearly we do see a place for laparoscopy in revisional bariatric surgery. Perhaps this may come in selected groups with sufficient laparoscopic experience with the primary operation and open experience in revisional surgery.

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Reply

We share Dr. Gagner's concerns about what will happen to the more than 70,000 patients who have undergone silicone adjustable gastric banding (SAGB) in recent years.[1,2] We hope this operation will not be introduced as widely in the United States as it has been in Europe before prospective controlled studies have proved its long-term safety and efficacy.

The Roux-en-Y gastric bypass (RYGBP) is well established as a remedial procedure for failures after classic gastric restrictive procedures and, in our view, also after SAGB. However, we believe that the con-

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version to RYGBP preferably should be undertaken in one session for several reasons. In our experience, the scarred tract with fibrous replacement and the full-thickness gastric wall fibrosis after simple band removal will remain for a long time if not forever. If the RYGBP procedure is delayed for a couple of months, the patient will have regained weight and the surgeon will encounter some of the same difficulties in creating the proximal pouch as if the operation was done in one session. Of course, dissections of the cardiac region tend to be more dangerous and difficult with every new attempt.

Similar to Dr. Gagner, we stress the importance of constructing a very small proximal pouch to avoid future stomal ulcers.[3] We believe that, in redo cases, this is best accomplished in an open approach. Clearly, patients will derive some benefits from having laparoscopic surgery in terms of earlier mobilization and decreased risk of incisional hernias. However, the RYGBP procedure comprises a large number of important technical details to ensure an excellent long-term result including small gastric pouch, safe stapling of the scarred gastric wall, closure of all mesenteric defects, to mention a few. The prime interest for the patient must be to have all these maneuvers carried out as precisely as possible. One limitation with laparoscopy is that intra-abdominal structures cannot be directly palpated and manipulated by the surgeon's hand as in open surgery or in the hand-assisted laparoscopic technique.[4,5] We believe that only very specialized laparoscopic surgeons working in high-volume hospitals should attempt these extremely challenging laparoscopic procedures. Dr. Gagner has shown that it can be done.

In a few cases of band erosion, we have only removed the band and sutured the gastric defect in the first session because of severe local inflammation with abscess formation. The RYGBP was then performed electively at a later time. In one of these patients with superobesity, the bileopancreatic diversion with duodenal switch proved to be an excellent alternative.

We agree with Dr. Gagner that leaks might occur from the gastrojejunostomy due to poor tissue approximation, but there might also be other causes such as tension and poor blood supply. In our experience, leaks are more common after redo procedures (4 leaks in 94 patients; 4%) than after primary operations (1 leak in 191 patients; 0.5%). Again, we believe that it is safer to perform the anastomosis and check its perioperative airtightness in open surgery.

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