Western Blot Analysis

Cells were lysed in RIPA buffer containing 50 mmol/L HEPES (pH 7.0), 250 mmol/L NaCl, 0.1% Nonidet P-40, 1 mmol/L phenylmethylsulfonylfluoride (PMSF), and 20 μg/mL gabexate mesylate and incubated on ice for 10 minutes; then the lysate was sonicated for 10 seconds. Total extracts were cleaned by centrifugation at 15,000 rpm for 10 minutes at 4°C, and the supernatants were collected. Protein concentration was measured by a BCA protein assay reagent (Pierce, Rockford, IL). The lysates were resuspended in 1 volume of the gel loading buffer that contained 50 mmol/L Tris-HCl (pH 6.7), 4% SDS, 0.02% bromophenol blue, 20% glycerol, and 4% 2-mercaptoethanol and then heated at 95°C for 5 minutes. The extracted protein was subjected to Western blotting. In brief, 50 μg aliquots of protein was size-fractionated to a single dimension by SDS-PAGE (6%–10% gels) and transblotted to a 0.45-μm polyvinylidene difluoride membrane (IPVH304F0, Millipore, Billerica, MA) in a semi-dry electroblot apparatus (Bio-Rad, Richmond, CA). The blots were then washed 3 times in TBS with 0.1% Tween-20 (TBST) and incubated for 1 hour at room temperature in blocking buffer (Block Ace, Dainipponseiyaku, Osaka, Japan). Subsequently, the blots were immunoblotted with an appropriate primary antibody for 1 hour at room temperature or overnight at 4°C. Unbound antibody was removed by washing the membrane with TBST 3 times, each 10 minutes. The membrane was then incubated with a horseradish peroxidase–conjugated secondary antibody for 1 hour at room temperature followed by an addition of TBST. Antibody reaction was detected by the enhanced chemiluminescence system (Amersham, Buckinghamshire, UK). The membranes were treated with enhanced chemiluminescence reagents according to the manufacturer's protocol and were exposed to x-ray films for 5–120 seconds.

Immunoprecipitation

For immunoprecipitation of cadherin-catenin complexes, total cell lysate were obtained as described above. To remove nonspecifically bound material, cell lysates (about 1 mg of protein) were precleared by incubation with 0.25 μg of normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA.), together with 20 μL of Protein G PLUS-Agarose (Santa Cruz) for 30 minutes at 4°C. After centrifugation, the supernatants were incubated with primary antibody for 2 hours at 4°C and then with 20 μL of protein G PLUS-Agarose at 4°C overnight on a rotating device. The immunocomplexes were collected by centrifugation, washed 4 times with extraction buffer, and eluted by boiling the beads in 40 μL of electrophoresis sample buffer (sc-24945, Santa Cruz) for 3 minutes. Immunoprecipitates were separated on SDS-8% polyacrylamide gels and transferred onto membranes. Western blots were further processed as described above.

Immunocytochemistry

Pancreatic cancer cells were seeded on coverslips and incubated for 24 hours at 37°C in a humid atmosphere of 5% CO_2/95% air. The coverslips with cells were then fixed with 4% paraformaldehyde in PBS for 10 minutes, washed with PBS, permeabilized in 1% Triton X-100 in PBS for 15 minutes, washed, and blocked with TBST with 1% BSA. Fixed and permeabilized cells were incubated with an appropriate primary antibody diluted in 1% BSA-TBST (v/wk) for 2 hours at 37°C, and rinsed 3 times with TBST, and then incubated for 30 minutes with Cy3-conjugated Affinipure goat anti-mouse IgG diluted in 1% BSA-TBST (v/wk). After the final wash, coverslips were mounted on the slide glass with Vectashield mounting medium (Vector Laboratories, Burlingame, CA).

TABLE 1. Expression of E-, N-cadherin, α-, β-, and γ-Catenin Expression in Human Pancreatic Adenocarcinoma

	AsPC-1	BxPC-3	Capan-2	CFPAC-1	HPAC	MIA PaCa-2	PANC-1
E-cadherin							
RT-PCR	+	+	+	+	+	−	+
Western blotting	+	++	+	++	++	−	+
Immunocytochemistry	+	+	+	+	+	−	+
N-Cadherin							
RT-PCR	−	±	+	+	−	−	+
Western blotting	−	±	++	++	−	−	+
Immunocytochemistry	−	−	+	+	−	−	+
α-catenin							
RT-PCR	+	+	+	+	+	+	+
Western blotting	+	+	+	+	+	±	+
β-catenin							
RT-PCR	+	+	+	+	+	+	+
Western blotting	++	++	++	++	++	−	++
Immunocytochemistry	+	+	+	+	+	UT	+
γ-catenin							
RT-PCR	+	+	+	+	+	±	+
Western blotting	++	++	++	++	+	±	++
Immunocytochemistry	+	+	+	+	+	UT	+

++, strong expression; +, positive expression; ±, weak expression; −, negative expression; UT, untried.

The cells were examined under a fluorescein microscope (Olympus, Tokyo, Japan).

RESULTS

The expression of cadherins in human pancreatic cancer cell lines was performed by 3 accepted techniques for gene expression. Figure 1 shows the mRNA expression of E-cadherin and N-cadherin. E-cadherin mRNA was detected in all cell lines except for MIAPaCa-2. N-cadherin mRNA was detected in BxPC-3, Capan-2, CFPAC-1, and PANC-1. The protein expression of E-cadherin and N-cadherin was measured by Western blot analysis (Fig. 2). The results of protein expression were identical to the mRNA expression for each protein. Figure 3 shows the typical staining of E-cadherin and N-cadherin. Both E-cadherin and N-cadherin were detected as a membranous protein. Immunocytochemical detection of E-cadherin and N-cadherin was consistent with the expression of mRNA and protein.

We next measured the expression of catenins in human pancreatic cancer cells. The mRNA expression of α-, β-, and γ-catenins was detected in all cell lines that we examined. However, the expression of γ-catenin in MIAPaCa-2 was extremely low (Fig. 4). Protein expression of catenins was similar to the mRNA expression in these cells except MIAPaCa-2. No protein expression of β-catenin was found in MIAPaCa-2, although it expressed β-catenin at the mRNA level (Fig. 5). Immunocytochemical detection of α-, β-, and γ-catenins showed membranous expression (Fig. 6). The results were summarized in Table 1. E-cadherin/β-catenin interaction was detected in all cell lines except MIAPaCa-2 (Fig. 7A), whereas N-cadherin/β-catenin interaction was detected in N-cadherin–expressing cells (Fig. 7B).

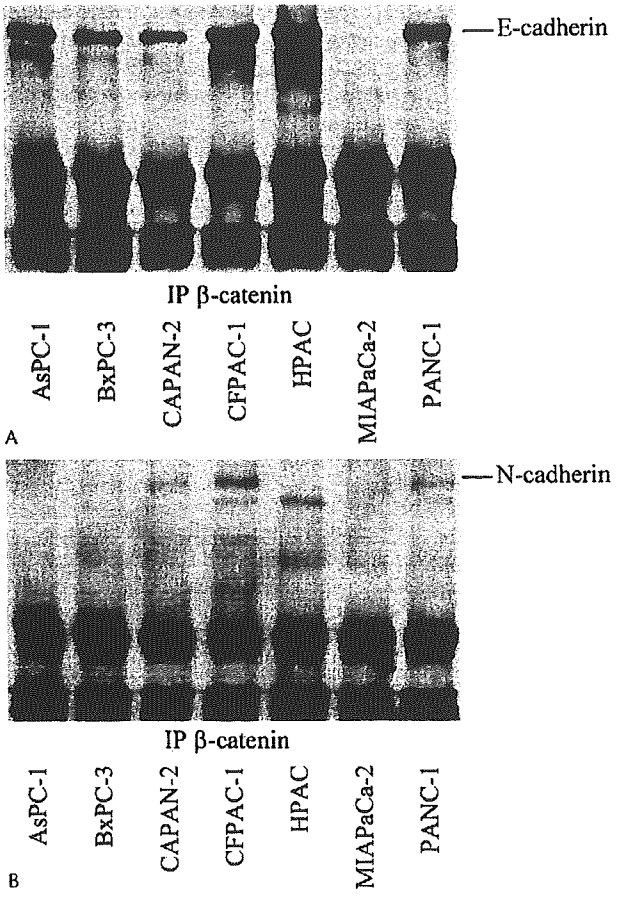

FIGURE 7. Immunoprecipitation of β-catenin with E- and N-cadherin. Equal amounts of proteins from each cell line were immunoprecipitated (IP) with antibody against β-catenin. Immunoprecipitates were separated by SDS-PAGE, transferred onto membranes, and immunoblotted with E-cadherin (A) and N-cadherin (B). Efficiency of immunoblot reaction and molecular weight of each detected protein were verified by loading 25 μg of CFPAC protein lysate. E-cadherin/β-catenin interaction was detected in all cell lines except MIAPaCa-2, whereas N-cadherin/β-catenin interaction was detected in N-cadherin–expressing cells. The IgG heavy and light chains recognized by secondary antibodies are visible at the bottom of the gel.

DISCUSSION

Pignatelli et al[18] reported that loss of normal surface E-cadherin expression was found in 19 of 36 (53%) pancreatic cancer tumors compared with the adjacent normal ductal cells. Abnormal E-cadherin expression was found more frequently in poorly differentiated than in well-differentiated tumors. Membranous E-cadherin expression was also lost more frequently in primary tumors with lymph node and distant metastasis compared with lymph node–negative tumors. Based on these observations, they suggested that the loss of membranous E-cadherin expression is associated with high grade and advanced stage in pancreatic cancer. Therefore, we thought that a considerable number of cell lines would show a decreased or loss of E-cadherin expression. However, contrary to our expectation, we found that E-cadherin is expressed in 6 of 7 pancreatic cancer cell lines at levels of both mRNA and protein.

Among the pancreatic adenocarcinoma cell lines that were tested in this study, AsPC-1 is derived from the ascites of a patient with cancer of the pancreas,[20] and CFPAC-1 is from the liver metastasis of ductal carcinoma.[21] Other cell lines were derived from human pancreatic ductal carcinomas. We found decreased expression of E-cadherin in MIAPaCa-2. However, there were no differences in expression tendency between cell lines from primary tumors and those from ascites and metastasis. Therefore, we speculate that the decreased or loss of E-cadherin expression in excised pancreatic cancer tissue could be an in vivo event that is affected by surrounding tissues such as the extracellular matrix component.

N-cadherin expression was detected in 4 cell lines (BxPC-3, Capan-2, CFPAC-1, PANC-1). CFPAC-1, derived from liver metastasis, demonstrated the strongest expression of N-cadherin. The result is in accordance with the cadherin switch theory. However, AsPC-1 that is derived from ascites did not express N-cadherin mRNA or protein. Capan-2 derived from the primary pancreatic cancer tumor showed strong

N-cadherin expression. Therefore, we did not find E-cadherin and N-cadherin conversion, at least, in our observations in vitro. Therefore, similar to the results of E-cadherin, the cadherin switch from epithelial to mesenchymal cadherins would be an in vivo phenomenon.

The cytoplasmic domain of E-cadherin interacts with intracellular proteins called α-, β-, and γ-catenins, which make contact with the microfilament network.[22] Catenins are essential for E-cadherin function, and alterations in expression or structure of the catenins may lead to the disassembly of adherens junctions and the generation of more invasive cells. In some human cancers, such as that of the breast, esophagus, and prostate, decreased expression of α-catenin has been noted.[23] In addition, alterations in β- and γ-catenin expression and phosphorylation have been described for some tumor cell lines.[24,25] Finally, we showed that the expression of catenins was found in all cultured pancreatic cancer cell lines. Therefore, the expression of catenins in pancreatic cancer cells was not altered in in vitro conditions. In conclusion, as suggested previously, the decreased or loss of cadherins and catenins expression could be involved in the tumor progression and metastasis, although these events may occur in in vivo conditions by interaction between cancer cells and extracellular matrices.

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Impact of Reconstruction Methods on Outcome of Pancreatoduodenectomy in Pancreatic Cancer Patients

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Abstract. Local recurrence is one of the most frequent forms of pancreatic cancer recurrence, although local recurrence is rare for other periampullary cancers. Because the type of recurrence and outcome differ depending on the type of cancer, these factors should be considered when the type of reconstruction is chosen. Fifty-four pancreatoduodenectomies were performed in patients with ductal adenocarcinoma of the pancreas from 1994 to 2001. Billroth I reconstruction was performed in 27 consecutive patients before 1999, and thereafter Billroth II reconstruction was performed in another consecutive 27 patients. Postoperative nasogastric intubation and the duration before oral ingestion were longer for Billroth I patients than Billroth II patients. Seven complications occurred in Billroth I patients, whereas there were two complications in Billroth IIpatients. Disease-free survival and overall survival were not different between the two groups; however, bypass operations were required in nine patients of the Billroth I group and in one patient of the Billroth II group. Percutaneous transhepatic cholangio-drainage (PTCD) procedures were required in six patients of the Billroth I goup and in two patients of the Billroth II group. The Billroth II reconstruction may have some advantages over the Billroth I reconstruction in terms of postoperative oral ingestion and avoiding bypass surgery and PTCD at the time of recurrence.

The recent remarkable progress in diagnostic screening tests, such as computed tomography, magnetic resonance imaging, and fluorodeoxyglucose positron emission tomography, have made it possible, to a certain extent, to detect a relatively early stage of pancreatic cancer, although the 5-year survival rates after resection of this cancer have been reported to be as low as 10% to 30% [1, 2], with more than half the patients dying of cancer relapsing within 2 years postoperatively. Local recurrence is one of the most frequent forms of pancreatic cancer recurrence, even after margin-negative resection [3, 4]. In contrast, it has been reported that local recurrence is rare in patients who undergo pancreatoduodenectomy for distal duct cancer [5–8], ampullary cancer [9, 10], or duodenal cancer [11–14]. Based on these reports, we can easily speculate that the type of reconstruction (the root of the alimentary tract) may affect the postoperative course of the patients. It is therefore of great significance to investigate whether the type of reconstruction affects short-term and long-term morbidity and the overall outcome of the pancreatic cancer patients. The current study was conducted to compare Billroth I and Billroth II reconstructions after pancreatoduodenectomy.

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Methods

Patients with ductal adenocarcinoma of the head of the pancreas who underwent pancreatoduodenectomy in the Department of Surgery and Surgical Basic Science, Kyoto University Hospital from 1994 to 2001 were analyzed retrospectively. A single team of the same surgeons performed all the operations. Fifty-four patients with microscopically margin-negative resection (R0) with D2 lymph node dissection were included; and patients with microscopically or macroscopically margin-positive resection (R1/R2) were excluded. Billroth I reconstruction (Imanaga method [15]) was performed in 27 consecutive patients before 1999. Thereafter, Billroth II reconstruction (Whipple method [16]) was employed in another consecutive 27 patients. The Billroth I reconstruction was performed as described by Imanaga [15]. Here, end-to-end gastrojejunostomy, end-to-side pancreatojejunostomy, and choledochojejunostomy were performed, in that order (Fig. 1). The Billroth II reconstruction was performed as described by Whipple [16]: end-to-side choledochojejunostomy, end-to-side pancreatojejunostomy, and retrocolic end-to-side gastrojejunostomy, in that order. Table 1 shows the characteristics of the patients.

Parameters such as pancreatic leakage, biliary leakage, complications, removal of the nasogastric tube, postoperative fasting duration, hospital stay, disease-free survival duration, and overall survival duration were evaluated. The results are expressed as the mean ± SD. Clinicopathologic characteristics were compared with the types of reconstruction using the chi-squared test or Fisher's exact probability test. Disease-free and overall survival curves were plotted according to Kaplan-Meier's method [17], and the statistical differences were analyzed by the log-rank test. The statistical analyses were performed with JMP software (version 3.1.5; SAS Institute, Cary, NC, USA). A probability value of <0.05 was considered statistically significant.

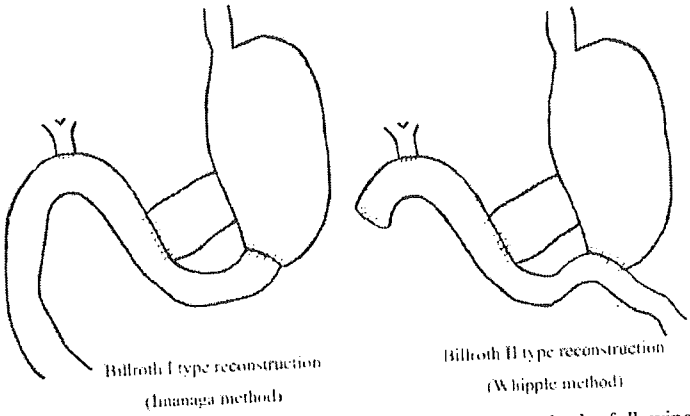

Fig. 1. Billroth I and Billroth II reconstruction methods following pancreatoduodenectomy.

Table 1. Characteristics of pancreatic cancer patients after Billroth I and Billroth II reconstructions.

Characteristic	Billroth I (n = 27)	Billroth II (n = 27)	p
Age (years)	63.0 ± 8.9	65.2 ± 8.9	0.36
Sex (no.)			
Male	15	18	0.40
Female	12	9	
Size of tumor (cm)	3.6 ± 1.4	2.8 ± 1.4	0.06
Tumor grading (no.)			
Well	2	2	1.00
Moderately	23	24	
Poorly	1	1	
UICC stage (no.)			
I + II	3	6	0.27
III + IVa	24	21	

Results

The characteristics of patients, such as age and sex, and the clinicopathologic factors, including the size of the tumor, tumor grading, and UICC stage, were not different between the two groups (Table 1). The early postoperative complications in both groups are recorded in Table 2. Seven complications occurred in Billroth I patients (two pancreatic fistulas, two anastomotic strictures, two anastomotic failures not related to the pancreas, one ileus), whereas two anastomotic failures occurred in Billroth II patients.

The duration of nasogastric intubation was 11.1 ± 9.2 days for Billroth I patients and 4.0 ± 2.5 days for Billroth II patients (p = 0.0003) (Table 3). The time before oral ingestion of a normal diet was 26.9 ± 12.0 days for Billroth I patients and 18.6 ± 8.4 days for Billroth II patients (p = 0.004). The postoperative hospital stay was longer for Billroth I patients than Billroth II patients (p = 0.01)

The patterns of recurrence were evaluated. As shown in Table 4, patterns of failure were not significantly different between the two groups, although a bypass operation was performed for gastrointestinal obstruction due to recurrence in nine Billroth I patients but in only one Billroth II patient (p = 0.01) (Table 5). In addition, percutaneous transhepatic cholangio-drainage (PTCD) was performed for biliary obstruction owing to recurrence in six Billroth I patients but in only two Billroth II patients.

Table 2. Early postoperative complications of pancreatic cancer patients after Billroth I and Billroth II reconstructions.

Postoperative complications	Billroth I (n = 27)	Billroth II (n = 27)
Total no.	7	2
Pancreatic leakage	2	0
Biliary leakage	0	1
Anastomotic stenosis	2	0
Gastrointestinal leakage	2	1
Ileus	1	0
Relaparotomy	0	0
In-hospital mortality	0	0

Table 3. Nasogastric intubation, fasting period, and hospital stay after Billroth I and Billroth II reconstructions.

Parameter	Billroth I (n = 27)	Billroth II (n = 27)	p
Nasogastric intubation (days)	11.1 ± 9.2	4.0 ± 2.5	0.0003
Start of normal diet (days)	26.9 ± 12	18.6 ± 8.4	0.004
Hospital stay (days)	66.5 ± 18	51.8 ± 23	0.01

Table 4. Site of recurrence of pancreatic cancer patients after Billroth I and Billroth II reconstructions.

Site of recurrence	Billroth I (n = 23)	Billroth II (n = 20)
Local	13	15
Liver	15	12
Peritoneum	10	9
Lung	3	1
Bone	0	2

Table 5. Palliative procedures for recurrence and survival time of pancreatic cancer patients after Billroth I and Billroth II reconstructions.

Parameter	Billroth I (n = 27)	Billroth II (n = 27)	p
Palliative procedure (no.)			
Gastrojejunostomy	9	1	0.01
PTCD	6	2	0.25
Disease-free survival (months)			
Median	7.9	8.1	0.74
95% CI	6.1–10.8	6.7–16.1	
Overall survival (months)			
Median	11.5	19.8	0.11
95% CI	8.3–12.5	12.9–28.4	

PTCD: Percutaneous transhepatic cholangio-drainage; CI: confidence interval.

Disease-free survival times and overall survival times were not significantly different between the two groups (Table 5).

Discussion

Pancreatoduodenectomy or pylorus-preserving pancreatoduodenectomy is a standard surgical method for periampullary adenocarcinoma. The principle of the surgical technique for this one-stage pancreatoduodenectomy was established by Whipple [16]. There have been two types of reconstruction procedure for the remnant alimentary tract after pancreatoduodenectomy: Billroth I reconstruction with gastrojejunostomy, pancreatojejunostomy, and choledocholejejunostomy, in that order [15, 18]; and Billroth

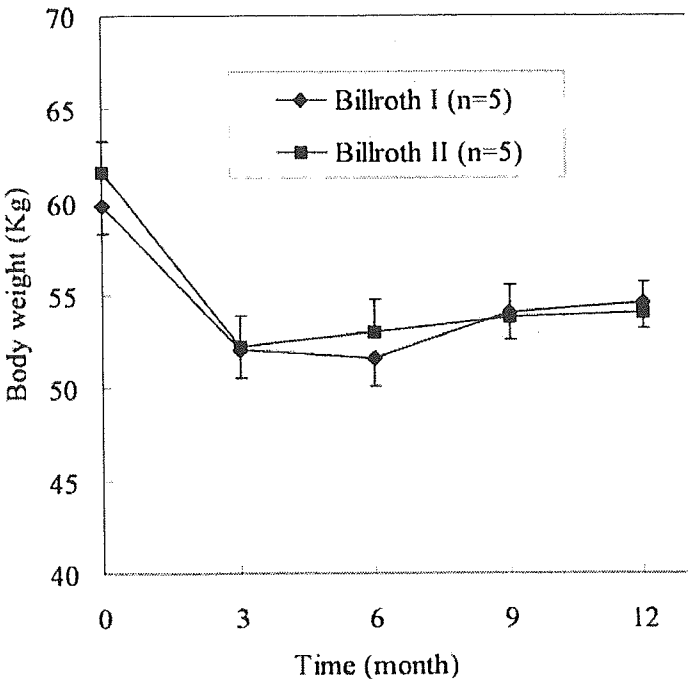

Fig. 2. Body weight changes after pancreatoduodenectomy with Billroth I or Billroth II reconstruction.

II type reconstruction with pancreatojejunostomy, choledocholejejunostomy (or choledocholejejunostomy, pancreatojejunostomy), and gastrojejunostomy, in that order [16, 19].

We have been performing Billroth I and Billroth II anastomoses after pancreatoduodenectomy. The Billroth I type of reconstruction has been most commonly performed in our department for the last two decades. We also apply it test after pylorus-preserving pancreatoduodenectomy (PPPD) [20] because it is conceivable that the passage of food through the entire remnant upper small intestine is more physiologic than food bypassing the upper portion of the remnant. Because this method leaves no blind intestinal segment, it simulates the normal anatomic arrangement and provides, theoretically, a physiologic mixture of food and fresh bile in the jejunum similar to that seen in normal subjects. A previous report demonstrated satisfactory mixing of fresh bile and food after Billroth I reconstruction using hepatobiliary and gastrointestinal dual scintigraphy [21, 22]. The nutritional indicators such as D-xylose absorption and bile secretion were better in Billroth I patients than Billroth II patients [23]. In addition, Billroth I reconstruction enables endoscopic examination of the patency of the orifices of the pancreatic and bile ducts and evaluation of the exocrine function of the remnant pancreas [24].

In the current study we did not have complete data regarding body weight and nutrition from 54 pancreatic cancer patients because it is difficult to assess eating parameters of pancreatic cancer patients, in whom recurrence could occur as early as 2 months after surgery and when the patients died from recurrence even 6 months after surgery. Instead, in bile duct cancer patients who underwent pancreatoduodenectomy and who were observed at least 1 year without recurrence, there were no differences in body weight after pancreatoduodenectomy in patients with Billroth I or Billroth II reconstructions (Fig. 2).

As for the early postoperative complications, we noted more frequent anastomotic failure with the Billroth I reconstruction than with Billroth II. The reason for this difference is not clear, but we speculate as follows: When the Billroth I reconstruction is used, the lining of the jejunal loop is curved sharply, even rectangularly, from the gastrojejunostomy to the pancreatoduodenostomy *and* from the pancreatoduodenostomy to the choledochojejunostomy. In contrast, with the Billroth II reconstruction, the lining of the jejunal loop is curved rather gently (Fig. 3). We think this difference could affect the attachment or the tension at each anastomosis and could be one of the causes of early postoperative complications.

We noted that after a Billroth I reconstruction there was a significantly longer nasogastric intubation period than with a Billroth II reconstruction (Table 3). Accordingly, the postoperative fasting time and hospital stay after a Billroth I reconstruction was significantly prolonged compared to that with a Billroth II reconstruction. Our results are in accordance with the previous report that the nasogastric intubation lasted 13 days for Billroth I patients and 6 days for Billroth II patients who underwent PPPD [25]. It has been believed, however, that these disadvantages of the Billroth I reconstruction can be balanced with the nutritional advantages after long period of time.

We have been adopting Billroth I reconstruction after pancreatoduodenectomy irrespective of the kind of periampullary disease. Patients with a benign lesion, distal bile duct cancer, ampullary cancer, and duodenal cancer have enjoyed the advantages of this reconstruction; however, the postoperative follow-up data have forced us to recognize that local failure occurred frequently in patients with pancreatic cancer before they could benefit from the Billroth I reconstruction. The root of the alimentary tract connected in series adversely affected food passage once the local recurrence appeared at the pancreatic bed.

It has been reported that the poor outcome of pancreatoduodenectomy for pancreatic ductal cancer is largely attributed to a high incidence of local failure even after margin-negative resection has been performed [3, 4]. The recurrence pattern in the current series of pancreatic cancer patients was similar to those already reported [3, 26]. In addition, the pattern of the recurrence was not significantly different for Billroth I and Billroth II patients. The disease-free survival time was not significantly different between the two groups.

Importantly, palliative secondary procedures were necessary more frequently in Billroth I patients than in Billroth II patients. In Billroth I patients, obstruction of the upper jejunum by local recurrence easily brought on gastric dilatation, and gastrojejunal bypass surgery was needed. In contrast, in Billroth II patients, gastrojejunal flow was secured even after the local recurrence was evident. Similarly, the PTCD procedure was necessary in six Billroth I patients and two Billroth II patients. The reason PTCD was frequently needed in Billroth I patients may be explained as follows. Once local recurrence occurs in Billroth I patients, the jejunal loop for reconstruction is involved and obstructed. Gastric juice and even food are trapped at the obstruction, which would deteriorate the obstructed jejunum mechanically by inducing mucosal edema in the Billroth I reconstruction. As a result, the flow of bile fluid also would be blocked. The current cohort study did not use a questionnaire to evaluate the quality of life of the patients, although it is suggested that avoidance of the secondary

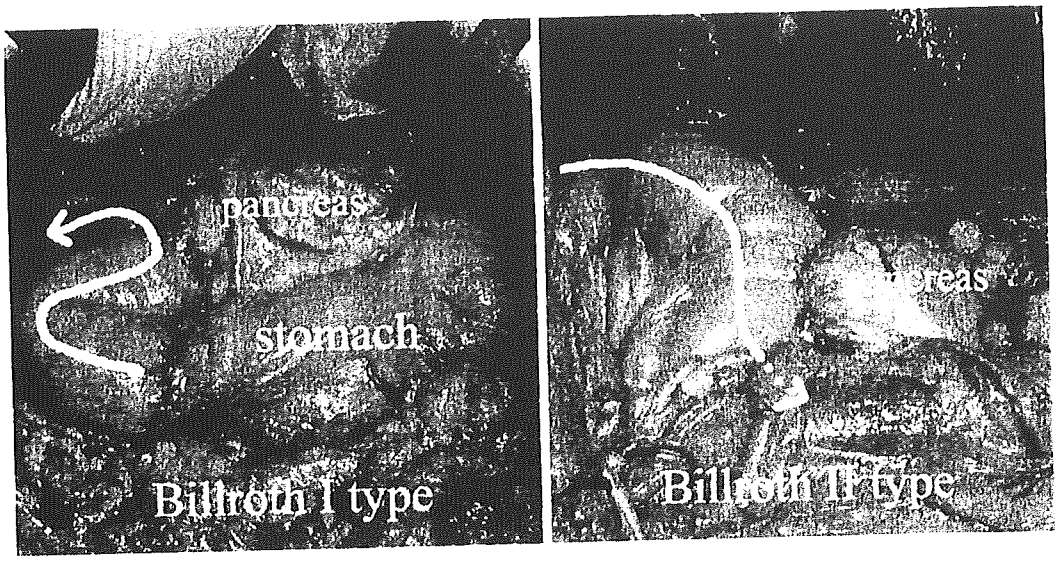

Fig. 3. Lining of the jejunal loop in Billroth I (**left**) and Billroth II (**right**) reconstructions after pancreatoduodenectomy.

operation or PTCD is a good reason to select Billroth II construction for pancreatic cancer patients.

The 5-year survival rates after pancreatoduodenectomy for distal bile duct cancer varies between 20% and 40% [5–8], which is not a lot better than that for pancreatic ductal cancer, although the type of the recurrence is much different. The cause of death for resected distal bile duct cancer was mainly due to liver metastases and peritoneal dissemination, and local recurrence appeared only in patients with positive resection margins. As for ampullary cancer, it has been reported that the 5-year survival rate is 46% to 55% after pancreatoduodenectomy, and the local control rate is 69% at 5 years [9, 10]. Similarly, duodenal cancer was apparently much better controlled, with 5-year survival rates of 50% to 60% [11–14]. In most patients with duodenal cancer whose disease recurred, the initial pattern included distant failure [11]. We think that the Billroth I reconstruction might still have advantages among patients with benign lesions and malignant diseases other than pancreatic cancer. Therefore the Billroth I procedure may be considered as one of the reconstruction methods after pancreatoduodenectomy for such patients.

Conclusions

Our results suggest that, in pancreatic cancer patients, the Billroth II reconstruction may have some advantages over the Billroth I reconstruction. It allows faster postoperative oral ingestion and prevents secondary procedures such as a bypass operation and PTCD at the time of recurrence.

Acknowledgments

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Regulation of the resistance to TRAIL-induced apoptosis as a new strategy for pancreatic cancer

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Background. *Tumor necrosis factor–related, apoptosis-inducing ligand (TRAIL) is a potent inducer of apoptosis in a wide variety of tumor cells, but it does not cause toxicity in the majority of normal cells. Therefore, TRAIL could become a suitable agent for anticancer therapies. However, a number of tumor cell lines are known to be resistant to TRAIL-induced apoptosis. The purpose of this study was to determine the mechanisms of resistance to TRAIL in pancreatic cancer cells.*

Methods. *In human pancreatic cancer cell lines, the sensitivity to TRAIL-induced apoptosis was tested. The expression of TRAIL receptors (DR4, DR5, DcR1, and DcR2) and the expression of death signal–transducing proteins were investigated. In the TRAIL-resistant pancreatic cancer cells, effects of cycloheximide, a protein synthesis inhibitor, on death signal–transducing proteins were tested. Finally, the effects of the combined treatment with cycloheximide and TRAIL on the induction of apoptosis and on the expression of death signal–transducing proteins were examined.*

Results. *Pancreatic cancer cells responded to TRAIL in a different way. Resistant cell lines, AsPC-1, Suit-2, and CFPAC-1, expressed higher levels of FLIP-S protein, one of the splice variants of FLIP. Cycloheximide reduced the expression of FLIP in the resistant cells. Combined treatment with cycloheximide and TRAIL induced cleaved forms of caspases and simultaneously restored the sensitivity to TRAIL-induced apoptosis in the resistant cells.*

Conclusions. *Pancreatic cancer cells are resistant to TRAIL-induced apoptosis via strong expression of the anti-apoptotic protein FLIP-S. Suppression of FLIP-S by cycloheximide restored sensitivity to TRAIL-induced apoptosis in resistant cancer cells. These findings may provide useful information for the development of TRAIL-based therapeutic strategies aimed at restoring the functionality of apoptotic pathways in pancreatic cancer cells. (Surgery 2005;138:71-7.)*

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PANCREATIC CANCER is one of the most malignant tumors with a low survival rate. The majority of patients with diagnosed pancreatic cancer present with an already unresectable tumor stage and frequently need chemotherapy and radiotherapy. One of the reasons for the low survival rate is the insensitivity of pancreatic cancer to most present oncologic therapies such as chemotherapy, radiotherapy, and immunotherapy. Tumor development and progression, as well as resistance to most oncologic therapies, result mainly from lack of response to apoptotic stimuli. Therefore, novel therapeutic strategies that overcome the resistant mechanisms in pancreatic cancer would be crucial in improving the survival rate of the patients.

Tumor necrosis factor–related, apoptosis-inducing ligand (TRAIL), also called Apo-2 ligand (Apo-2L), is a member of the tumor necrosis factor (TNF) family that preferentially triggers apoptosis in various tumor cells via 2 death domain–containing agonistic receptors, DR4 and DR5. Both DR4 and DR5 contain a cytoplasmic death domain that is required for TRAIL receptor–induced apoptosis.[1,2] TRAIL also binds to DcR1 and DcR2, which act as decoy receptors by inhibiting TRAIL signaling. Unlike DR4 and DR5, DcR1 does not have a cytoplasmic domain, and DcR2 retains a cytoplasmic fragment containing a truncated form of the consensus death domain motif.[1]

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Recent studies have shown that TRAIL triggers apoptosis by recruiting the initiator, procaspase-8, through the adaptor protein FADD.[3,4] Caspase-8 can directly activate downstream effector caspases, including procaspase-3, procaspase-6, and procaspase-7. In an alternative death signal pathway, caspase-8 cleaves Bid and triggers mitochondrial damage that in turn leads to cytochrome c release. Cytochrome c in the cytoplasm then binds to Apaf-1 and activates caspase-9. Subsequently, caspase-9 activates caspase-3, which induces poly(ADP-ribose) polymerase (PARP) cleavage. Therefore the availability of caspase-8 may be the key step of the TRAIL-induced apoptosis.

The TRAIL pathway represents a potentially promising target for anticancer therapy. However, we have noted that several cell lines from human pancreatic cancer are resistant to TRAIL-induced apoptosis. Several endogenous intracellular proteins could potentially inhibit TRAIL-induced apoptosis.[1,2] Because TRAIL can be an effective tool as a cancer treatment, the mechanism of resistance to TRAIL must be clarified before it can be used clinically for pancreatic cancer. Thus, the current study was conducted to investigate the signaling pathway that is responsible for the resistance in pancreatic cancer.

MATERIAL AND METHODS

Cell lines and culture conditions. Human pancreatic cancer cell lines (CFPAC-1, HPAC, AsPC-1, PANC-1, BxPC-3, and Suit-2) were cultured in the following media at 37°C in a humid atmosphere of 5% CO_2 /95% air. CFPAC-1 cells were cultured in Iscoves modified Dulbecco medium with 10% fetal bovine serum (FBS). PANC-1 and Suit-2 were cultured in Dulbecco modified Eagle medium with 10% FBS. BxPC-1 cells, HPAC, and AsPC-1 were cultured in RPMI 1640 medium with 10% FBS. Each medium contained 100 U/mL penicillin and 100 μg/mL streptomycin.

Antibodies, recombinant proteins, and other reagents. Antibodies, recombinant proteins, and other reagents were purchased as follows: mouse anti-DR4 antibody and rabbit anti-DcR1antibody (IMGENEX, San Diego, Calif); rabbit anti-DR5 antibody (Cayman Chemical, Ann Arbor, Mich); rabbit anti-DcR2 antibody (Oncogene, San Diego, Calif); mouse anti-FLIP antibody (Apotech Corp, Lausen, Switzerland); rabbit anti-Smac antibody (IMGENEXf); mouse anti-Bcl-2 antibody, rabbit anti-Bcl-XL antibody, rabbit anti-Bax antibody (Santa Cruz Biotechnology, Santa Cruz, Calif); rabbit anti-caspase-9 antibody and rabbit anti-caspase-3 antibody (Cell Signaling Technology, Beverly, Mass); mouse anti-XIAP antibody and mouse anti-FADD antibody (MBL, Nagoya, Japan); rabbit anti-caspase-8 antibody, mouse anti-cytochrome c antibody, and mouse anti-PARP antibody (BD PharMingen, San Diego, Calif); mouse β-actin antibody, cycloheximide, and Hoechst 33342 (Sigma Chemical Co, St. Louis, Mo); and recombinant human TRAIL (Pepro Tech EC Ltd, London, United Kingdom).

Western blot analysis. Cells were lysed in ice-cold RIPA buffer (10 mmol/L phosphate-buffered saline, pH 7.4, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mmol/L EDTA) supplemented with 1% phenylmethylsulfonyl fluoride and 20 μg/mL gabexate mesilate (FOY; Ono Pharmaceutical, Osaka, Japan) for 30 minutes at 4°C. The lysate was homogenized and centrifuged at 15,000 rpm for 30 minutes at 4°C at 12,000 *g* to remove debris; the protein concentration was measured with the use of a BCA protein assay kit (Pierce, Rockford, Ill). Equal amounts of protein were loaded onto sodium dodecylsulfate-polyacrylamide gels (8%-12%), and the proteins were transferred to a polyvinylidene difluoride membrane (IPVH304F0; Millipore, Billerica, Mass). The blots were blocked at 4°C overnight with 5% (w/v) nonfat milk in TBST buffer (10 mmol TRIS-HCl, 150 mmol/L NaCl, 0.5% Tween-20). Subsequently, the blots were immunoblotted with an appropriate primary antibody overnight at 4°C. Unbound antibody was removed by washing the membrane with TBST 3 times, each for 10 minutes. The membrane was then incubated with a horseradish peroxidase–conjugated or alkaline phosphatase–conjugated secondary antibody for 1 hour at room temperature, followed by the addition of TBST described above. Antibody reaction was visualized by ECL Western Blotting Detection Reagent (Amersham, Buckinghamshire, United Kingdom) or alkaline phosphatase solution supplemented with 100 mmol/L TRIS-HCl, 100 mmol/L HCl, 5 mmol/L $MgCl_2$, 0.03% nitroblue tetrazolium, and 0.017% 5-bromo-indolylphosphate P-toluidine salt.

Cell viability assay. Cells were seeded at a density of 5000 cells per well into 96-well plates in culture medium containing 10% FBS. After 24 hours, the cultures were washed and treated with designated agents. After 24 hours, the number of viable cells was counted with the use of the Cell Counter Kit 8 (Dojindo Co, Kumamoto, Japan) according to the manufacturer's instructions. The assay reagent is a tetrazolium compound (WST-8) that is reduced by live cells into a colored formazan product measured at 450 nm. The quantity of formazan product

measured at 450 nm is directly proportional to the number of live cells in the culture.

Reverse transcriptase-polymerase chain reaction. Total cellular RNA was prepared with the use of TRIZOL Reagent (Life Technologies, Rockville, Md). Complementary DNA was prepared by random priming from 1 μg of total RNA with the use of a First-Strand cDNA Synthesis kit (Pharmacia Biotech, North Peapack, NJ) according to the manufacturer's instructions. The reverse-transcriptase polymerase chain reaction (RT-PCR) analysis was performed. The sequences for the primers used in this study are as follows:

β-actin: (sense) atggatgacgatatcgct, (antisense) atgaggtagtctgtcaggt,
DR4: (sense) ctgagcaacgcagactcgctgtccac, (antisense) aaggacacggcagagcctgtgccat,
DR5: (sense) ctgaaaggcatctgctcaggtg, (antisense) cagagtctgcattaccttctag,
DcR1: (sense) accctaaagttcgtcgtcgtcatc, (antisense) tcaaacaaacacaatcagaagcac,
DcR2: (sense) cttttccggcggcgttcatgtccttc, (antisense) gtttcttccaggctgcttccctttgtag.

The products were 569 (β-actin), 506 (DR4), 583 (DR5), 140 (DcR1), and 464 (DcR2) base pairs, respectively.

Hoechst 33342 staining. Cells were seeded on chamber slides and incubated for 24 hours at 37°C in a humid atmosphere of 5% CO_2/95% air. The cells were pretreated with cycloheximide (3 μmol/L) for 1 hour before treatment with TRAIL (100 ng/mL) for 6 hours. Subsequently, the cells were incubated for 15 minutes at 37°C with 10 μg/mL of Hoechst 33342 dye in culture medium. The stain was washed with PBS 3 times, and the cells were examined under a fluorescent microscope.

RESULTS

Sensitivity of pancreatic cancer cells to TRAIL. Sensitivity to TRAIL was investigated in 6 pancreatic cancer cell lines (AsPC-1, CFPAC-1, Suit-2, PANC-1, BxPC-3, and HPAC) and HUVEC (Fig 1). The cells were treated with various concentrations of TRAIL for 24 hours; the number of viable cells was determined by WST-8 assay. Susceptibility to the cytotoxic effect of TRAIL was variable in different pancreatic cancer cells. AsPC-1, CFPAC-1, Suit-2, and HUVEC were resistant to TRAIL treatment. On the contrary, BxPC-3 and HPAC cells were highly sensitive, and PANC-1 was moderately sensitive to TRAIL. According to the cell viability, the cell lines were designated as TRAIL-resistant (AsPC-1, Suit-2, and CFPAC-1), moderately sensitive (PANC-1), and highly sensitive (BxPC-3 and HPAC), respectively.

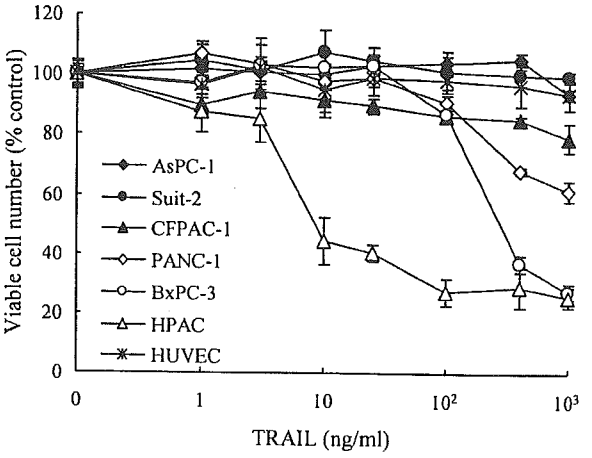

Fig 1. Sensitivity of pancreatic cancer cells to TRAIL. Cells were treated with various concentrations of TRAIL for 24 hours. Cell viability was determined by WST-8 assay. Cell survivals represent the mean percentage survivals, compared with untreated cells. According to the cell viability, the cells were designated as TRAIL-resistant (AsPC-1, Suit-2, and CFPAC-1), moderately sensitive (PANC-1), and highly sensitive (BxPC-3 and HPAC), respectively. Data are expressed as the mean ± SD of 3 repeated experiments. *TRAIL,* Tumor necrosis factor–related, apoptosis-inducing ligand.

Expression of TRAIL receptors in pancreatic cancer cells. To identify whether expression of TRAIL receptors is linked with TRAIL sensitivity in pancreatic cancer cells, we determined the expression of DR4, DR5, DcR1, and DcR2 by RT-PCR and Western blot analysis (Fig 2). DR4, DR5, and DcR2 were detected in all human pancreatic cancer cell lines at different levels, but DcR1 was expressed only in AsPC-1, CFPAC-1, and BxPC-3 at a very low level by RT-PCR. Receptor expressions detected by Western blot analysis were similar to those detected by RT-PCR. The expression of the death domain–containing agonistic receptors (DR4, DR5) was not related to the TRAIL sensitivity.

Expression of death signal–transducing proteins in pancreatic cancer cells. To clarify whether TRAIL sensitivity was influenced by the pro- and anti-apoptotic molecules, we investigated the expression of FLIP, Bcl-2, Bcl-XL, Bax, FADD, caspase-3, caspase-8, caspase-9, cytochrome c, Smac, and XIAP (Fig 3). These molecules are known to play important roles in the death receptor–induced apoptotic pathway. The pro-apoptotic molecules, FADD, caspase-3, caspase-8, caspase-9, Bax, Smac, and cytochrome c, were detected in all cell lines although expression levels did not show any

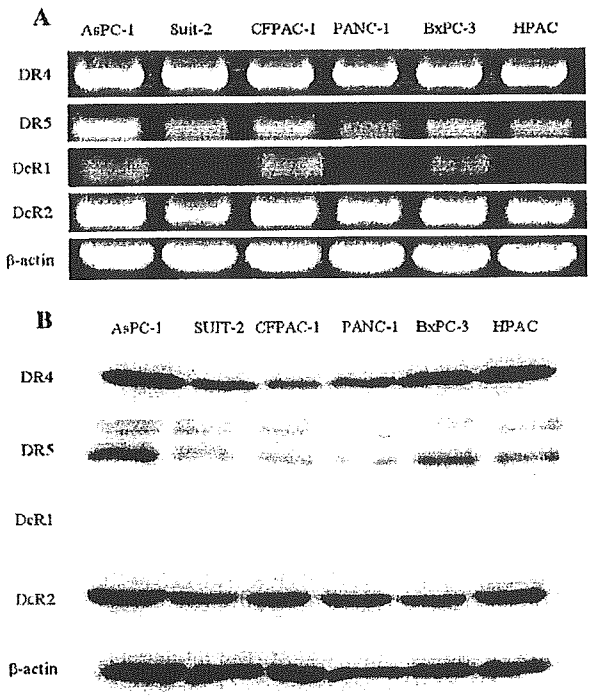

Fig 2. Expression of TRAIL receptors in pancreatic cancer cells. The expression of DR4, DR5, DcR1, and DcR2 mRNA was assessed by RT-PCR analysis (**A**) and Western blot analysis (**B**). The expression level of DR4 and DR5, the responsible receptors to TRAIL-induced apoptosis, did not differ significantly among cell lines. This experiment were performed 3 times. *TRAIL,* Tumor necrosis factor–related, apoptosis-inducing ligand.

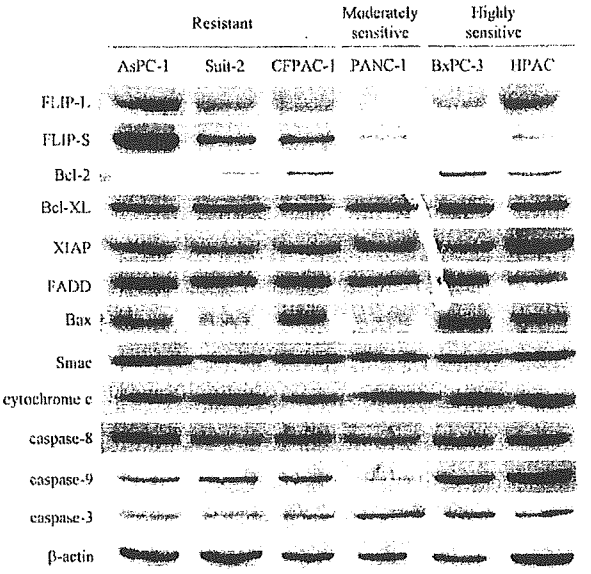

Fig 3. Expression of death signal–transducing proteins in pancreatic cancer cells. The expression of death signal–transducing proteins was analyzed by Western blot analysis in pancreatic cancer cells. β-actin was used to verify equal loading of proteins. Strong expression of FLIP-S is noted in TRAIL-sensitive cell lines, compared with the insensitive cell lines. The expression of caspase-8 was not related to the TRAIL-sensitivity. In contrast, the expression of caspase-3 was weak in TRAIL-resistant cell lines, compared with TRAIL-sensitive cell lines. Expression of other anti-apoptotic proteins did not show any significant difference among cell lines. This experiment was performed 3 times. *TRAIL,* Tumor necrosis factor–related, apoptosis-inducing ligand.

significant difference. Among anti-apoptotic proteins, FLIP-S, one of the splice variants of FLIP, was strongly expressed in only resistant cell lines and weakly expressed in the other sensitive cell lines. The expression of caspase-8 was not related to TRAIL sensitivity. In contrast, the expression of caspase-3 was weak in TRAIL-resistant cell lines, compared to TRAIL-sensitive cell lines. Expression of other anti-apoptotic proteins did not show any significant difference among cell lines.

Effect of cycloheximide on death-transducing signals in pancreatic cancer cells. Cycloheximide was reported to enhance and restore the sensitivity to death receptor–induced apoptosis, presumably by reducing the expression of anti-apoptotic proteins with rapid turnover.[5] Therefore, we investigated the inhibitory effects of cycloheximide on the synthesis of the anti-apoptotic proteins FLIP, Bcl-2, Bcl-XL, XIAP, and other necessary molecules for signal transduction (Fig 4). The expression of FLIP and XIAP were most evidently reduced by cycloheximide in all resistant cell lines. In contrast, the levels of other anti-apoptotic proteins, such as Bcl-2 and Bcl-XL, were not affected by cycloheximide.

Effect of TRAIL and cycloheximide on expression of caspases. We examined whether combined treatment with TRAIL and cycloheximide affects the activation of caspases in resistant cells (Fig 5). In particular, we assessed the effects of 24-hour treatment with cycloheximide on caspase-3, caspase-8, caspase-9, and PARP in the presence or absence of TRAIL. TRAIL alone did not induce a significant level of proteolytic-processing caspase-8, whereas the combined treatment with TRAIL and cycloheximide induced the significant proteolytic processing of pro-caspase-8. Similar results were observed for activation of caspase-9 and caspase-3, and for PARP cleavage. These results consistently showed that cycloheximide significantly enhanced TRAIL-induced apoptosis through the activation of caspases.

Effect of cycloheximide on TRAIL-induced apoptosis. We tested the effect of cycloheximide on TRAIL sensitivity in resistant cells and found

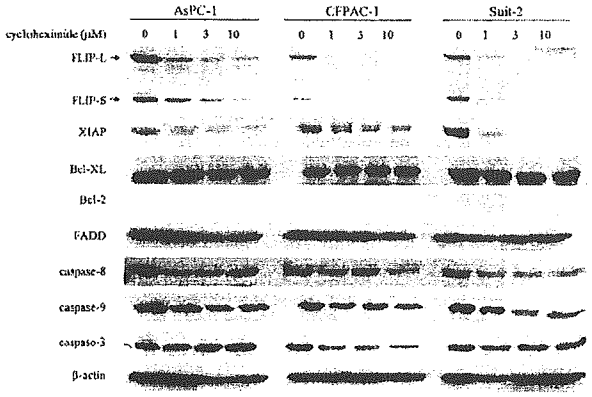

Fig 4. Effect of cycloheximide on death-transducing signals in pancreatic cancer cells. Cells were treated with various concentrations of cycloheximide for 6 hours. The expression of death-transducing proteins was analyzed by Western blot analysis. β-actin was used to verify equal loading of proteins. The expression of FLIP-L, FLIP-S, and XIAP was significantly reduced by cycloheximide. Other signals were not significantly changed by cycloheximide. This experiment was performed 3 times. *TRAIL,* Tumor necrosis factor–related, apoptosis-inducing ligand.

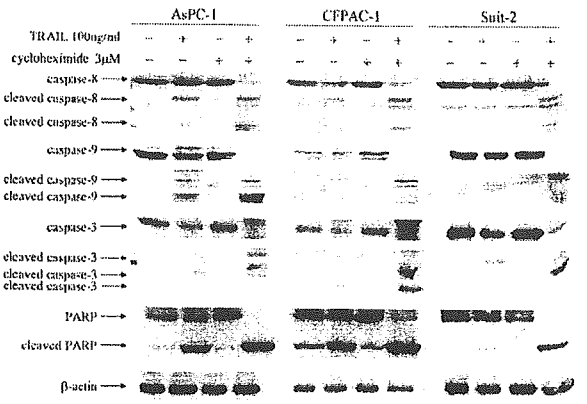

Fig 5. Effect of cycloheximide and TRAIL on expression of caspases. The effect of cycloheximide on the activation of caspase-3, caspase-8, caspase-9, and PARP cleavage were determined by Western blot analysis in TRAIL-resistant cells. Treatment with cycloheximide and TRAIL showed that the expression of caspase-3, caspase-8, caspase-9, and PARP was significantly suppressed, and that the cleaved forms of caspase-3, caspase-8, caspase-9, and PARP became apparent. This experiment was performed 3 times. *TRAIL,* Tumor necrosis factor–related, apoptosis-inducing ligand.

that cycloheximide significantly enhanced their sensitivity to TRAIL (Fig 6). A treatment with cycloheximide restored TRAIL sensitivity in all resistant cells. Combined treatment with TRAIL and cycloheximide showed cells undergoing apoptosis, which was detected by intense fluorescence corresponding to chromatin condensation and fragmentation on Hoechst 33342 staining only (Fig 7).

DISCUSSION

In the present study, we found that pancreatic cancer cells responded to TRAIL in a different way. Several cell lines were very sensitive to TRAIL treatment, but other cell lines were not sensitive to TRAIL. Because the expression levels of death domain–containing agonistic receptors and decoy receptors did not differ among cell lines, the TRAIL sensitivity of pancreatic cancer cells may be primarily regulated at the intracellular level, rather than at the receptor level. We found the difference of the sensitivity could be attributed to the expression level of FLIP-S, 1 of the splice variants of FLIP. The results suggest that FLIP-S is an important determinant in TRAIL resistance of the pancreatic cancer cells.

FLIP is an intracellular apoptosis suppressor protein.[6] Although the inhibitory effects of FLIP on apoptosis induced by a variety of stimuli have been demonstrated, its role in the regulation of TRAIL sensitivity in pancreatic cancer is unknown. Previous studies have shown that FLIP interrupts apoptotic signaling by interacting with FADD and caspase-8, and by blocking the activity of caspase-8, suggesting that the intracellular level of FLIP may determine the sensitivity of tumor cells to a variety of pro-apoptotic stimuli.[7,8] In agreement with the current results, increased cellular levels of FLIP have been shown to enhance the resistance of cancers to TRAIL-induced apoptosis, whereas a reduction in FLIP levels is associated with increased sensitivity to TRAIL in other cancer cells.[9,10]

FLIP exists in 2 spliced isoforms derived from FLIP gene. The long isoform (FLIP-L) is a 55-kDa protein containing two N-terminal death effector domains (DEDs) and an inactive, C-terminal caspaselike domain, whereas the short isoform (FLIP-S) contains only two DEDs.[6] Both forms of FLIP can interact with FADD and caspase-8 through DED-DED interaction, and can potently block caspase-8 binding to DISC.[7,8] We showed that FLIP-S was very strongly expressed in resistant cells, but not expressed in sensitive cells. It has been reported that FLIP-L and FLIP-S inhibit different steps of caspase-8 activation. FLIP-L allows partial cleavage of procaspase-8, generating a p10 subunit, which is an active fragment of caspase-8. In contrast, FLIP-S completely inhibits the cleavage of caspase-8.[11] These findings taken together with our current results indicate that the

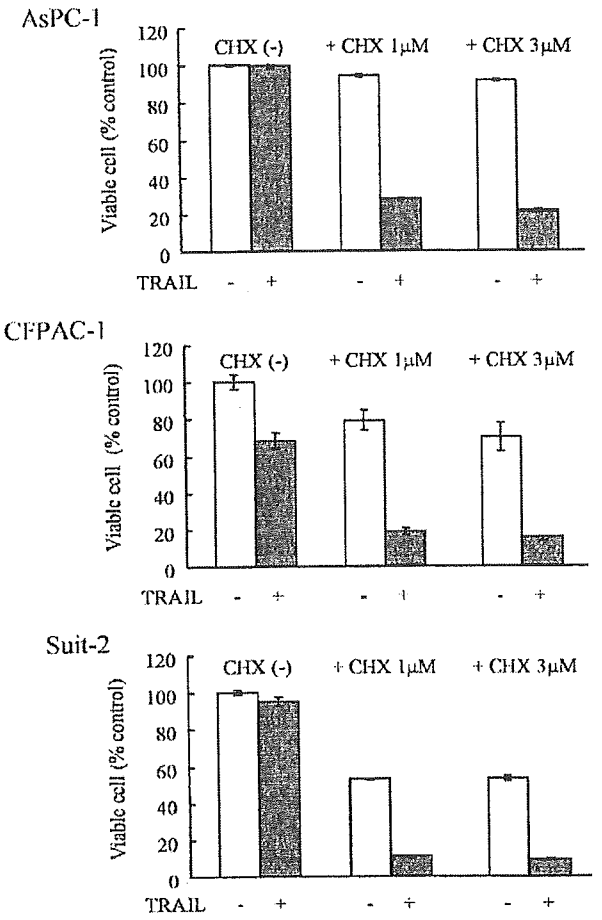

Fig 6. Effect of cycloheximide and TRAIL on cell viability. Cells were treated with various concentrations of cycloheximide and 100 ng/mL TRAIL for 24 hours. Cell viability was determined by WST-8 assay. The addition of cycloheximide restored the TRAIL sensitivity in the TRAIL-resistant pancreatic cancer cells. Cell viability represents the mean percentage survivals, compare with untreated cells. Data are expressed as the mean ± SD of 3 repeated experiments. *TRAIL,* Tumor necrosis factor–related, apoptosis-inducing ligand; *CHX,* cycloheximide.

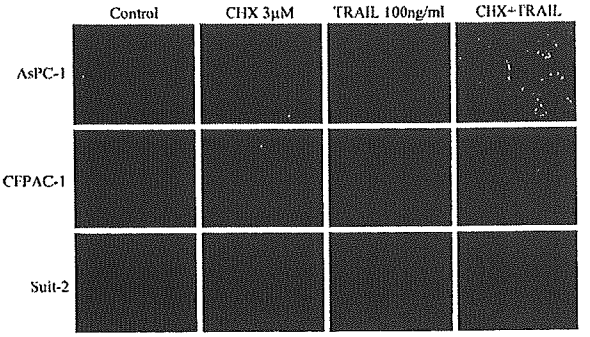

Fig 7. Effect of cycloheximide on TRAIL-induced apoptosis in pancreatic cancer cells. Cells were treated with 3 μmol/L cycloheximide and/or 100 ng/mL TRAIL for 24 hours and then stained with Hoechst 33342. Significant chromatin condensation and fragmentation are noted in only cells treated with the combined use of cycloheximide and TRAIL (original magnification ×100). This experiment was performed 3 times. *TRAIL,* Tumor necrosis factor–related, apoptosis-inducing ligand; *CHX,* cycloheximide.

anti-apoptotic function of FLIP-S may be more impotent than that of FLIP-L in TRAIL-induced apoptosis in resistant pancreatic cancer cells.

We found that, in all resistant cells, cycloheximide could restore the sensitivity to TRAIL and reduced the expression level of FLIP-S in resistant cells. In addition, the treatment with cycloheximide could enhance and restore caspase-8 activation in TRAIL-resistant cells, which indicates that the death machinery was intact and functional. The activation of caspase-8 might be masked under the negative regulation by anti-apoptotic proteins; expression of the anti-apoptotic proteins could be inhibited by cycloheximide treatment. We found that the expression of FLIP and XIAP were significantly decreased by cycloheximide treatment in resistant cells. Thus, it is conceivable that these anti-apoptotic proteins should be the major inhibitors in TRAIL-induced apoptosis in pancreatic cancer cells.

We found that the expression of XIAP was decreased by treatment with cycloheximide in resistant cells. Therefore, this protein also could play such a role in enhancing or restoring TRAIL-induced apoptosis of pancreatic cancer cells. XIAP has been identified as one of the potent inhibitors of caspase and apoptosis.[12] Overexpression of XIAP in tumor cells has been shown to cause an inhibitory effect on cell death induced by a variety of apoptotic stimuli and, thus, induce resistance to chemotherapy.[12-14]

Overexpression of active Smac has been reported to render resistant tumor cells sensitive to TRAIL treatment in several cancer cells.[15,16] In response to apoptotic stimuli, Smac is released into the cytoplasm from mitochondria and binds to IAPs, thereby relieving IAP inhibition of caspases. Although cycloheximide decreased the expression levels of XIAP, the exogenous regulation of XIAP expression by IAP inhibitors would not affect the sensitivity because, as shown in our study, the basal expression levels were not related to the sensitivity of pancreatic cancer cells to TRAIL.

The recombinant TRAIL has been reported to kill normal human hepatocytes in vitro.[17] However, this finding was not reproduced in subsequent studies using clinical-grade recombinant human

TRAIL; the finding is now attributed to non-optimized recombinant ligand preparations. Moreover, preclinical studies in mice and primates have shown that administration of TRAIL can induce apoptosis in human tumors, but not induce cytotoxicity in normal organs or tissues.[18] Therefore, anticipation of the clinical use of TRAIL-induced apoptosis as an anticancer strategy would be a reality.

CONCLUSION

We have demonstrated that pancreatic cancer cells are resistant to TRAIL-induced apoptosis via overexpression of the anti-apoptotic protein FLIP-S. Suppression of FLIP-S by cycloheximide restored the sensitivity to TRAIL-induced apoptosis in resistant cancer cells. We believe that these findings may provide useful information for the development of TRAIL-based therapeutic strategies aimed at restoring the functionality of apoptotic pathways in pancreatic cancer cells.

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Downregulation of survivin by siRNA diminishes radioresistance of pancreatic cancer cells

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Background. *Survivin is a member of the inhibitor of apoptosis protein family, which inhibits apoptosis and regulates cell division. Survivin is expressed by the majority of human cancers, including pancreatic adenocarcinoma. We have reported that its expression is correlated with shorter survival of pancreatic cancer patients, so regulation of this molecule could be a new strategy for fighting pancreatic cancer.*
Methods. *In 3 pancreatic cancer cell lines (AsPC-1, SUIT-2, and Panc-1), survivin promoter activity was determined by the luciferase reporter assay, and survivin messenger RNA (mRNA) expression was examined by quantitative reverse transcriptase-polymerase chain reaction. The dose-dependent cytotoxity of radiation was also assessed, while caspase-3 activity and induction of DNA fragmentation were evaluated. Furthermore, the effect of silencing or nonsilencing short interfering RNA (siRNA) expression plasmids directed against the survivin gene on AsPC-1 cells, the most radioresistant cell line, was evaluated.*
Results. *Pancreatic cancer cell lines expressed varying levels of survivin mRNA in association with transcriptional activity of the survivin promoter. Both survivin promoter activity and mRNA expression were correlated with tumor cell radiosensitivity. Radiation significantly increased survivin promoter activity and survivin mRNA expression in all cell lines. Radiation induced a significant increase in caspase-3 activity and DNA fragmentation in AsPC-1 cells. After silencing siRNA treatment of AsPC-1 cells (AS-S cells), there was a significant decrease in survivin mRNA expression and increase in caspase-3 activity, compared with the effect of nonsilencing scramble siRNA on AsPC-1 cells (AS-NS cells). AS-S cells were more radiosensitive than AS-NS cells. Radiation induced higher caspase-3 activity and more DNA fragmentation in AS-S cells, compared with AS-NS cells.*
Conclusions. *Survivin may play an important role as 1 of the radioresistance factors. Downregulation of survivin by siRNA can diminish the radioresistance of pancreatic cancer cells, so combined therapy with survivin inhibition and radiation may be useful for the treatment of pancreatic cancer. (Surgery 2005;138:299-305.)*

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SURVIVIN is a member of the inhibitor of apoptosis protein (IAP) family, which inhibits apoptosis and regulates cell division.[1] Survivin usually is undetectable in most normal adult tissues, except for the testis, thymus, and placenta.[2] In contrast, survivin is widely expressed during fetal development[3] and is found in most human carcinomas, including pancreatic cancer.[1,4-6]

Survivin expression is known to be correlated with shorter survival in the majority of cancers. We previously reported that survivin is a prognostic marker for pancreatic cancer patients.[6] Survivin also is thought to be involved in tumor cell resistance to radiation therapy as well as to several anticancer agents. Previous reports have demonstrated that inhibition of survivin expression by antisense oligonucleotides, dominant negative mutation,[7] or ribozymes[8] could reduce the growth and radioresistance of pancreatic cancer cells.

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RNA interference (RNAi) involves post-transcriptional gene silencing via a process in which double-stranded RNA (dsRNA, short interfering RNA [siRNA]) inhibits gene expression in a sequence-dependent manner through degradation of the corresponding mRNA. RNAi is a new technique that is effective for suppressing gene expression.[9,10] Recent reports have indicated that siRNA has advantages over antisense oligonucleotides in vitro and in vivo,[11,12] partly because of the greater resistance of siRNA to degradation by nuclease.

In the present study, we examined the transcriptional activity of the survivin promoter and the expression of survivin mRNA in pancreatic cancer cells. Then, we examined the relationship between survivin expression and radioresistance of pancreatic cancer cells. Finally, we evaluated the effect of survivin siRNA on radioresistance of pancreatic cancer.

MATERIAL AND METHODS

Cells. Three human pancreatic cancer cell lines (AsPC-1, SUIT-2, and Panc-1) were purchased from the American Type Culture Collection (Manassas, Va). AsPC-1 cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, Calif) with 10% heat-inactivated fetal calf serum. SUIT-2 cells and Panc-1 cells were cultured in Dulbecco modified Eagle medium (Invitrogen) with 10% heat-inactivated fetal calf serum.

Irradiation. A PANTAK X-ray irradiation system (Shimadzu Co, Kyoto, Japan) was employed to irradiate the cells. Irradiation was performed at room temperature at a dose rate of 1.7 Gy/min with the use of a 250 kV X-ray generator unit operating at 25 mA and equipped with an external 0.5-mm copperfilter. The culture medium was replaced immediately after irradiation. At 24 hours after irradiation, the luciferase assay was done; the other experiments outlined below were performed at 48 hours.

Luciferase reporter gene constructs. Transcriptional analysis of the human survivin gene was performed as reported previously.[13] A 397-bp survivin promoter region was generated by the polymerase chain reaction (PCR) and subcloned into the pCR2.1 vector (Invitrogen) to generate the plasmid 397-2.1A. This promoter sequence represents nucleotides 2376 to 2772 of the human survivin gene (accession no. U75285). The nucleotide sequence of this region was confirmed by DNA sequence analysis. The 414-bp blunt-ended EcoRI fragment of 397-2.1A was subcloned into the blunt-ended KpnI site of the promoter-less luciferase basic vector pGL3 (Promega Corp, Madison, Wis) to generate the plasmid pGL3-surv for use in transfection and the luciferase assay.

Transient transfection and luciferase assay. Pancreatic cancer cells were seeded into 6-well dishes at 2×10^5/well in the indicated medium at 24 hours before transfection. Then the cells were transfected with 1 μg/well of firefly luciferase reporter vector pGL3-surv and 0.1 μg/well of a reference Renilla luciferase vector with thymidine kinase (tk) promoter pRL-tk (Promega KK, Tokyo, Japan) with the use of Lipofectamine 2000 (Gibco, Gaithersburg, Md) according to the manufacturer's protocol. Twenty-four hours after transfection, the cells were irradiated with X-rays (0, 5, or 10 Gy), and the culture medium was changed immediately after irradiation. After another 24 hour of incubation, the cells were lysed, and the luciferase assay was performed with the use of a Dual-Luciferase Reporter Assay System (Promega KK) according to the manufacturer's protocol. Luciferase activity was normalized for the total protein level as well as for Renilla luciferase activity.

RNA interference. Validated human survivin siRNA was purchased from Cell Signaling Technology (Beverly, Mass). The silencing of survivin expression with survivin siRNA has been reported previously.[13] A control scramble (nonsilencing) siRNA was purchased from Takara (Seta, Japan). Cells were seeded at 24 hours before transfection with either survivin siRNA or control scramble siRNA by using the TransIT-TKO Transfection Reagent (Mirus, Madison, Wis) according to the manufacturer's instructions. The final concentration of siRNA duplex in the culture medium was 100 nmol/L.

Determination of cell survival. Cells were seeded at a density of 3,000/well in a 96-well plate. The number of viable cells was estimated at 48 hours after irradiation with the use of a Cell Counting Kit 8 from Dojindo (Kumamoto, Japan) according to the manufacturer's instructions. The assay reagent was a tetrazolium compound (WST-8) that is reduced to a colored formazan product by live cells and detected at 450 nm. The quantity of formazan product measured at 450 nm is directly proportional to the number of live cells in the culture.

Quantitative reverse transcriptase-polymerase chain reaction. Total RNA was isolated from cells with the use of the RNeasy Kit from Qiagen (Valencia, Calif) according to the manufacturer's protocol. After isolation of RNA, complementary DNA (cDNA) was prepared from each sample with the use of the First-Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol. Quantification

Table I. Survivin expression and survival curve parameters in pancreatic cancer cell lines*

	Survivin promoter activity	*Survivin mRNA expression*	*SF_2 (%)*	*LD_{50} (Gy) (95% CI)*
Panc-1	5.68 ± 0.65†	0.018 ± 0.002†	67.1 ± 6.1	6.61 (4.10-9.42)
SUIT-2	16.7 ± 0.40†	0.033 ± 0.004†	79.7 ± 5.5	41.8 (28.4-74.9)
AsPC-1	39.9 ± 0.81†	0.084 ± 0.018†	87.6 ± 5.9	79.5 (50.7-164)

mRNA, Messenger RNA; *SF_2*, sodium difluoride; *LD_{50}*, median lethal dose.
*Lucifease activity of the survivin promoter is indicated as relative values to that of the thymidine kinase promoter. Survivin mRNA expression is indicated as relative values to β-actin mRNA expression.
†$P < .05$ between each cell line.

of cDNA was carried out as described previously.[14,15]

Quantitative PCR for survivin and β-actin was performed as 40 cycles in triplicate with the use of fluorescence-based real-time detection with the ABI PRISM 7700 (Applied Biosystems, Foster City, Calif) as described previously.[14,16] The following specific primers and fluorescent hybridization probe for survivin were used in the quantitative PCR:

Forward primer: 5′-GGGCTGCCACGTCCAC-3′;
reverse primer: 5′-GTCGTCATCTGGCTCCCA-3′
Probe: 5′-TTCATCCACTGCCCCACTGAGAACGA-3′.[16]

The primers and fluorescent hybridization probe for β-actin were purchased from Applied Biosystems. The ratio of the number of copies of survivin to that of β-actin was calculated and standardized by using a standard human DNA sample.

DNA fragmentation. To evaluate apoptotic DNA fragmentation, an ELISAPLUS cell death detection ELISA kit (Roche, Mannheim, Germany) was used according to the manufacturer's instructions. Briefly, cells were seeded at a density of 1×10^4/well in 96-well plates. At 48 hours after irradiation, the cells were lysed in 200 μL of lysis buffer and incubated at room temperature for 30 minutes. Aliquots of the supernatant (20 μL) were transferred to an enzyme-linked immunosorbent assay microtiter plate for analysis; the absorbance was measured at 405 nm with a microplate reader. This method is based on the detection of mono- and oligonucleosomes in the cytoplasmic fraction of cell lysates by using biotinylated antihistone- and peroxidase-coupled antiDNA antibodies. The absorbances at 405 nm per minute and per microgram of protein were used as indexes of DNA fragmentation.

Caspase-3 activity. Caspase-3 activity was measured with the use of the ApoAlert Caspase 3 assay kit (Clontech Laboratories, Palo Alto, Calif). Briefly, cells were seeded at a density of 2×10^6/well in a 6-cm dish. At 48 hours after irradiation, 2×10^6 cells were lysed in 50 μL of the lysis buffer provided in the assay kit and were incubated at 4°C for 30 minutes. Enzyme activity in the supernatant was detected by cleavage of the substrate acetyl-Asp-Glu-Val-Asp-p-nitroanilidine (DEVD-pNA) to yield pNA. Relative caspase-3 activity was measured as the absorbance at 405 nm with the use of a microplate reader. Results were calibrated relative to known concentrations of pNA and expressed as picomoles of substrate cleaved per minute and per microgram of protein.

Statistical analysis. Data are expressed as the mean ± SD. Statistical analysis was performed by analysis of variance followed by the Fisher protected least significant difference test, the unpaired *t* test, or by the Kruskal-Wallis test followed by the Mann-Whitney *U* test with Bonferroni's correction for nonparametric data, if applicable. The analyses were done with Stat View software version 5.0 (Abacus Concepts, Berkeley, Calif). A *P* value of less than .05 was regarded as statistically significant.

RESULTS

Transcriptional activity of the survivin promoter and survivin mRNA expression in pancreatic cancer cells. Figure 1, *A* shows transcriptional activity of the 397-bp survivin promoter; Figure 1, *B* shows survivin mRNA expression by the 3 human pancreatic cancer cell lines. Transcriptional activity and survivin mRNA expression were high in AsPC-1 cells (0.084 ± 0.018), intermediate in SUIT-2 cells (0.033 ± 0.004), and low in Panc-1 cells (0.018 ± 0.002). As shown in Figure 1, *A* and *B*, and Table I, transcriptional activity of the 397-bp survivin promoter was correlated with the level of expression of survivin mRNA transcripts by each cell line.

Survivin expression and radiosensitivity. Radiation inhibited the growth of the 3 human pancreatic cancer cell lines in a dose-dependent manner (Fig 1, *C*) although the actual sensitivity to radiation varied. Panc-1 was the most-sensitive cell line, and AsPC-1 was the most radioresistant cell line (Fig 1, *C*). For each cell line, survivin promoter activity and mRNA expression were correlated with

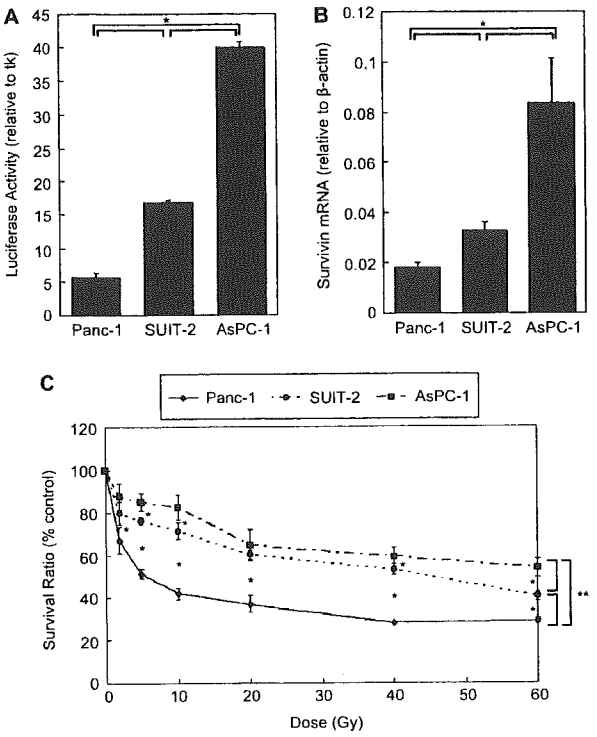

Fig 1. Expression of survivin promoter activity and survivin mRNA, and radiosensitivity of pancreatic cancer cell lines. **A,** Transcriptional activity of the 397-bp survivin promoter in human pancreatic cancer cell lines. Luciferase activity is expressed relative to that for pRL-tk containing the tk promoter. $*P < .001$ between each cell line. **B,** Survivin mRNA expression measured by the quantitative TaqMan RT-PCR in pancreatic cancer cell lines. Survivin mRNA expression is shown relative to that of β-actin. $*P < .05$ between each cell line. **C,** Dose-dependent cytotoxicity of radiation for human pancreatic cancer cell lines. At 48 hours after the indicated dose of radiation, viable cells were counted by the WST-8 assay. $*P < .05$ between cultures at each dose; $**P < .001$ between cultures. Panc-1 was the most-radiosensitive cell line, while AsPC-1 was most resistant to radiation. Survivin promoter activity and mRNA expression were associated with the radiosensitivity of these cell lines. Experiments were performed in triplicate; data are the mean ± SD. *mRNA,* Messenger RNA; *tk,* thymidine kinase.

sodium difluoride (SF_2) and median lethal dose (LD_{50}), respectively (Table I).

Survivin promoter activity (Fig 2, *A*) and survivin mRNA expression (Fig 2, *B*) were significantly increased by either 5 Gy or 10 Gy of radiation in all cell lines.

A radiation dose of 10 Gy significantly increased caspase-3 activity (Fig 3, *A*), while either 5 Gy or 10 Gy significantly augmented DNA fragmentation (Fig 3, *B*) in radioresistant AsPC-1 cells.

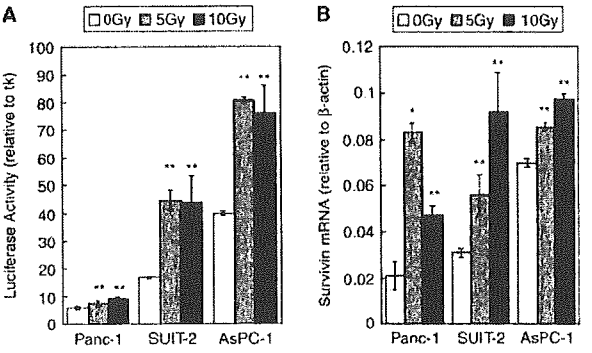

Fig 2. Radiation-induced survivin promoter activity and survivin mRNA expression. **A,** Transcriptional activity of the survivin promoter in nonirradiated cells (0 Gy) and 24 hours after irradiation with doses of 5 or 10 Gy. Luciferase activity is expressed as relative to that for pRL-tk containing the tk promoter. Survivin promoter activity was increased significantly by irradiation. **B,** Survivin mRNA expression measured by quantitative TaqMan RT-PCR in nonirradiated cells (0 Gy) and at 48 hours after irradiation with 5 or 10 Gy. Survivin mRNA expression is shown relative to that of β-actin. Survivin mRNA expression was significantly increased by radiation. The experiments were performed in triplicate; data are the mean ± SD. $*P < .05$ and $**P < .01$ vs 0 Gy for each cell line. *mRNA,* Messenger RNA; *tk,* thymidine kinase.

Downregulation of survivin mRNA with siRNA and radioresistance of AsPC-1 cells. We monitored the suppression of survivin mRNA expression with siRNA by quantitative RT-PCR (Fig 4, *A*). At 72 hours after transfection, AsPC-1 cells treated with survivin-specific siRNA (AS-S cells) showed a significant decrease in survivin mRNA expression by 38%, compared with AsPC-1 cells treated with control scramble (nonspecific) siRNA (AS-NS cells). The decrease in survivin mRNA expression in AS-S cells was observed at 5 Gy as well as at 10 Gy of irradiation (Fig 4, *B*). AS-S cells were significantly more sensitive to radiation from 5 Gy to 40 Gy, compared with AS-NS cells (Fig 4, *C*).

Caspase-3 activity and DNA fragmentation were significantly greater in AS-S cells than in AS-NS cells when radiation was not given as a proapoptotic stimulus (Fig 5). In AS-NS cells, caspase-3 activity was not increased by irradiation, whereas AS-S cells showed a significant increase in caspase-3 activity at both 5 Gy and 10 Gy (Fig 5, *A*). DNA fragmentation was significantly increased by 5 Gy or 10 Gy of radiation in both AS-S and AS-NS cells (Fig 5, *B*).

Table II summarizes relative survivin mRNA expression, caspase-3 activity, and DNA fragmentation in AS-S cells when the levels for AS-NS cells

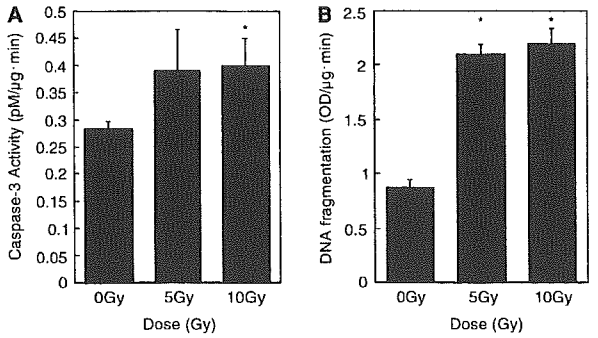

Fig 3. Basal and radiation-induced caspase-3 activity and DNA fragmentation. **A,** Basal and radiation-induced caspase-3 activity in AsPC-1 cells. Caspase-3 activity was expressed as picomoles of substrate cleaved per minute and per microgram of protein in nonirradiated cells (0 Gy) and at 48 hours after radiation with 5 or 10 Gy. **B,** Basal and radiation-induced DNA fragmentation in AsPC-1 cells. Apoptotic DNA fragmentation is expressed as absorbance per minute and per microgram of protein in nonirradiated cells (0 Gy) and at 48 hours after radiation with 5 or 10 Gy. The experiments were performed in triplicate; data are the mean ± SD. $*P < .01$ vs 0 Gy.

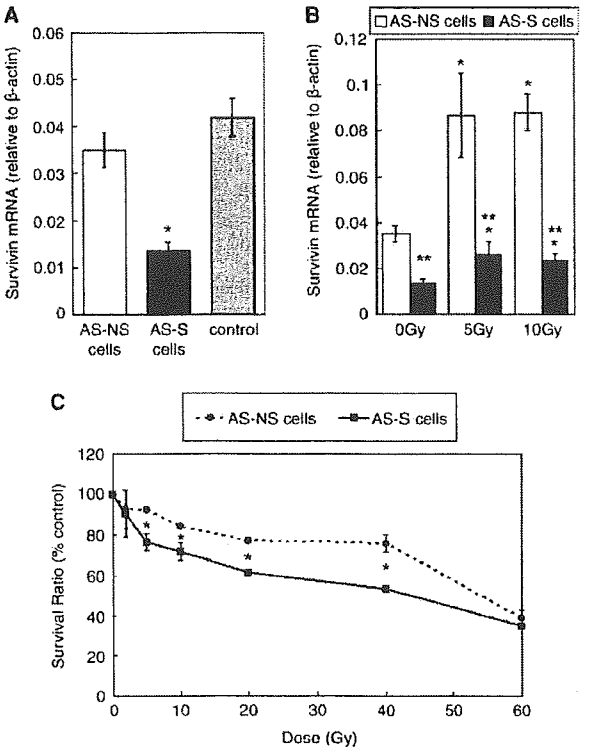

Fig 4. Effect of survivin siRNA on survivin mRNA expression and cell viability. **A,** Survivin mRNA expression in AS-NS cells or AS-S cells 72 hours after treatment. Survivin mRNA expression is shown relative to that of β-actin. Scramble siRNA (AS-NS) did not affect survivin expression, compared with the control (transfection reagent without siRNA), whereas survivin-specific siRNA (AS-S) induced significant suppression of survivin expression. $*P < .05$ vs scramble siRNA. **B,** Survivin mRNA expression by nonirradiated AS-NS cells and AS-S cells (0 Gy) and at 48 hours after irradiation with 5 or 10 Gy. Survivin mRNA is shown relative to that of β-actin. Radiation induced an increase in survivin mRNA expression in both AS-S cells and AS-NS cells, but the increase in survivin mRNA expression was significantly smaller in AS-S cells, compared with that in AS-NS cells. $*P < .05$ vs 0 Gy; $**P < .05$ vs AS-NS cells. **C,** Dose-dependent cytotoxicity of radiation for AS-S cells and AS-NS cells. At 48 hours after the indicated dose of radiation, viable cells were counted by the WST-8 assay. The experiments were performed in triplicate; data are the mean ± SD. $*P < .05$ between groups at each dose. *mRNA,* Messenger RNA; *AS-NS cells;* AsPC-1 cells treated with nonsilencing scramble siRNA; *AS-S cells,* AsPC-1 cells treated with silencing survivin siRNA.

were set as 1. AS-S cells showed a significant decrease in survivin mRNA, increase in caspase-3 activity, and increase in DNA fragmentation after exposure to radiation at 0 and 10 Gy. A dose of 10 Gy significantly augmented the changes of survivin mRNA expression and csapase-3 activity in AS-S cells, compared with AS-NS cells. However, the augmentation of DNA fragmentation by radiation was not significantly different between AS-S cells and AS-NS cells.

DISCUSSION

Previous reports have demonstrated that survivin is 1 of the radioresistance factors in a variety of human cancers, including pancreatic cancer. It has been reported that survivin acts as a constitutive and inducible radioresistance factor in pancreatic cancer.[17] Pancreatic cancer cells transduced with the wild-type survivin gene show increased radioresistance; pancreatic cancer cells transduced with a dominant-negative mutant survivin gene show a decrease in radioresistance via modulation of caspase-3 activity.[7] Therefore, regulation of survivin expression could be a possible new treatment for this refractory tumor.

Reported strategies for targeting survivin include antisense oligonucleotides,[18] ribozymes,[8] dominant-negative mutation,[7] an anticancer vaccine,[19] and siRNA.[18,20,21] Among these methods, siRNA may be the simplest and most powerful tool since it is used widely for studying gene function in human cancers and allows highly specific suppression of target gene expression. It has been reported that adenovirus-mediated transfer of survivin siRNA induces apoptosis and attenuates glioma cell growth in vitro and in vivo.[22] Sarcoma cells expressing wild-type p53 show decreased radioresistance when combined treatment is