Table 1. Patient characteristics

Patient number	Sex	Age (years)	GEM dose (mg/m^2)	Number of GEM courses	WBC before GEM administration	Surgerical resection
1	F	54	729	2	3.9	NR
2	F	76	604	1	4.1	NR
3	M	63	625	2	6.3	NR
4	M	60	743	2	4.9	NR
5	F	59	675	1	4.7	NR
6	M	73	722	2	7.4	NR
7	F	51	566	2	3.2	NR
8	F	65	878	2	3.9	NR
9	M	62	726	1	4.7	NR
10	F	60	795	2	5.6	NR
11	F	66	709	2	4.4	NR
12	M	55	778	2	3.9	NR
13	F	64	634	2	3.9	NR
14	M	72	663	2	4.4	NR
15	F	76	567	1	3.6	NR
16	M	73	594	1	4.1	NR
17	M	72	890	2	5.2	NR
18	M	72	845	2	6.6	NR
19	F	61	850	2	6.4	NR
20	F	75	746	2	6.2	NR
21	M	55	748	2	7.2	NR
22	M	67	832	2	6.2	NR
23	M	77	523	2	6.2	NR
24	F	61	767	2	4.6	NR
25	M	54	851	2	7.0	PPPD
26	F	60	699	1	5.0	PPPD
27	F	71	863	2	4.8	DP
28	M	69	572	2	4.4	PPPD
29	F	58	784	2	7.3	PD
30	M	73	626	1	4.7	DP
31	M	46	601	2	4.6	PD
32	F	55	840	2	3.0	PPPD
33	M	55	552	2	5.3	PD
34	M	50	770	1	2.8	PD
35	M	73	699	2	4.9	MP
36	M	66	724	2	7.1	DP
37	F	51	705	2	3.3	PD
38	M	62	722	2	7.5	DP

GEM, gemcitabine hydrochloride; PD, pancreatoduodenectomy; PPPD, pylorus-preserving pancreatoduodenectomy; DP, distal pancreatectomy; MP, middle pancreatectomy; NR, no resection

GEM in detail, and analyzed the association between the patients' backgrounds and leukopenia.

Patients and methods

Patients

Thirty-eight patients who received one or two courses of GEM treatment for pancreatic cancer between April 2001 and June 2003 at the Kyoto University Hospital were included in this study. They consisted of 22 men and 16 women with a mean age of 63.5 ± 8.5 years. The mean administered dose of GEM was 716.2 ± 101.6 mg/m^2, and the leukocyte count prior to administration of GEM was 5100 ± 1300/μl. The patients' characteristics are summarized in Table 1. When grade 3 or 4 leukopenia occurred, 100 μg of lenograstim (Neutrogin; Chugai Pharmaceuticals, Tokyo, Japan) was administered subcutaneously. Written informed consent was obtained from all patients before initiation of the cancer chemotherapy.

Evaluation of leukopenia

Leukopenia was classified in accordance with the common toxicity criteria established by the National Cancer Institute.

Statistical analysis

Values are expressed as means ± SDs. For statistical analyses, the χ^2 test or unpaired *t*-test was performed. $P < 0.05$ was regarded as significant.

Results

Correlation between the dose of GEM and the leukocyte nadir

We analyzed leukopenia after the administration of GEM. The grade of leukopenia was evaluated as 3 or 4 in 36.8%,

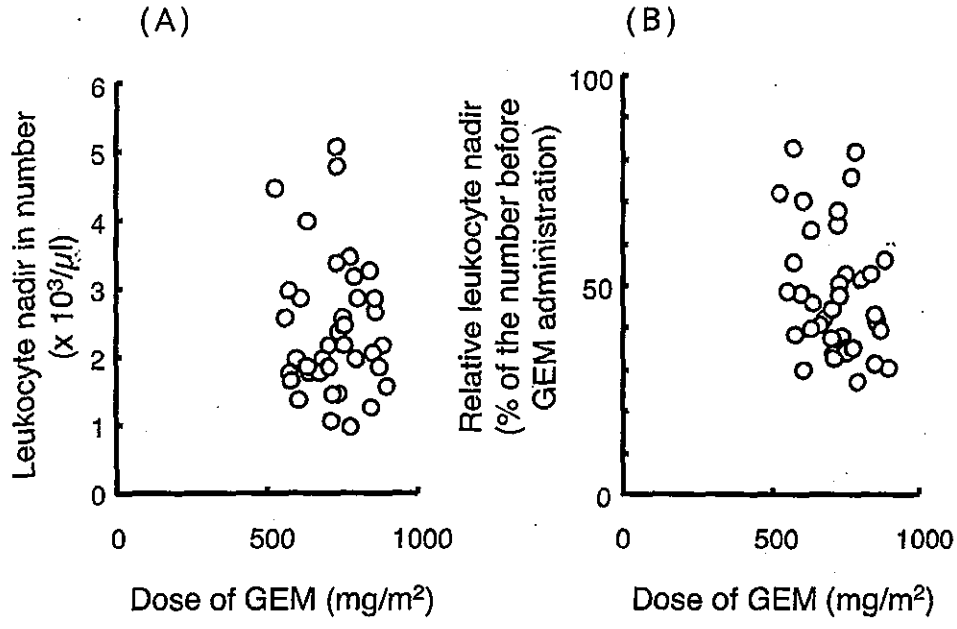

Fig. 1A,B. Correlation between dose of gemcitabine hydrochloride (*GEM*) and leukocyte nadir. **A** Leukocyte nadir, in number. **B** Relative leukocyte nadir (percentage of leukocyte count before GEM administration)

1 or 2 in 52.7%, and 0 in 10.5% of the patients. When the relationship between the grade of leukopenia and the number of GEM courses was analyzed, there were no significant difference in the grade of leukopenia between one and two courses of GEM treatment ($P = 0.97$ by the χ^2 test).

Figure 1 shows the correlation between the dose of GEM and the leukocyte nadir. The dose of GEM ranged from 523 to 890 mg/m^2 in the patients. Administration of GEM decreased the leukocyte count from 5100 ± 1300/μl to 2500 ± 1000/μl as an average in all patients. But there was no correlation between the dose of GEM and the leukocyte nadir, in number (Fig. 1A), nor was there a correlation between the dose of GEM and the relative leukocyte nadir (Fig. 1B). When we analyzed the background of patients with a leukocyte nadir of less than 2000/μl (equivalent to grade 3 or 4 leukopenia), we found that 8 of these 14 patients had undergone surgical resection of the pancreas. Therefore, we classified the patients into two groups for the further analyses: non-resected patients and resected patients.

Comparison of leukopenia after GEM administration between resected and non-resected patients

Table 2 shows the grade of leukopenia in the resected and non-resected patients. The incidences of grade 3 or 4 leukopenia were 25% in the non-resected patients and 57% in the resected patients ($P = 0.048$ by the χ^2 test). We then calculated the percentages of the leukocyte counts before and after administration of GEM (relative leukocyte nadir) in the resected and non-resected patients (Fig. 2). In the non-resected patients, the percentage was calculated as 52.6 ± 16.0%. However, in the resected patients, a more severe decrease in this percentage (41.3 ± 9.9%) was observed. These two percentages showed a significant difference ($P = 0.023$ by t-test). There were no significant differences between resected and non-resected patients in the GEM dose, leukocyte count before GEM administration, leukocyte nadir in number, or days to reach the leukocyte nadir after GEM administration (Table 3).

Table 2. Comparison of leukopenia between non-resected and resected patients

Patients	Leukopenia		
	Grade 0–2	Grade 3–4	
Non-resected	$n = 18$ (75.0%)	$n = 6$ (25.0%)	$P = 0.048$
Resected	$n = 6$ (42.8%)	$n = 8$ (57.1%)	

Discussion

In cancer chemotherapy, the most important issue is how to gain the maximal pharmacologic effect; at the same time, the appearance of adverse effects should be considered. Adverse effects of anticancer agents may lead not only to the patients' pain and anxiety but also to death in some cases. Antiemetic agents, such as 5-HT$_3$ receptor antagonists, and the development of G-CSF have greatly improved the therapeutic efficacy of anticancer chemotherapy.[7–10] In particular, the development of G-CSF analogues as medicines has facilitated more potent cancer chemotherapy than before; however, leukopenia is still a dose-limiting factor in many regimens of anticancer chemotherapy.

In a clinical study of GEM conducted in Japan, the incidence of leukopenia was reported to be 90%, whereas the incidence of grade 3 or 4 leukopenia was approximately 20%;[5] a low incidence of other serious adverse effects was

Table 3. Comparison of various parameters between resected and non-resected patients

	Non-resected	Resected	*P*
GEM dose (mg/m²)	717.0 ± 104.1	714.9 ± 101.0	0.95
Leukocyte count before GEM administration (/μl)	5100 ± 1200	5100 ± 1600	0.91
Leukocyte nadir in number (/μl)	2600 ± 900	2200 ± 1100	0.18
Number of days to reach leukocyte nadir after GEM administration	17.5 ± 9.4	17.1 ± 13.3	0.90

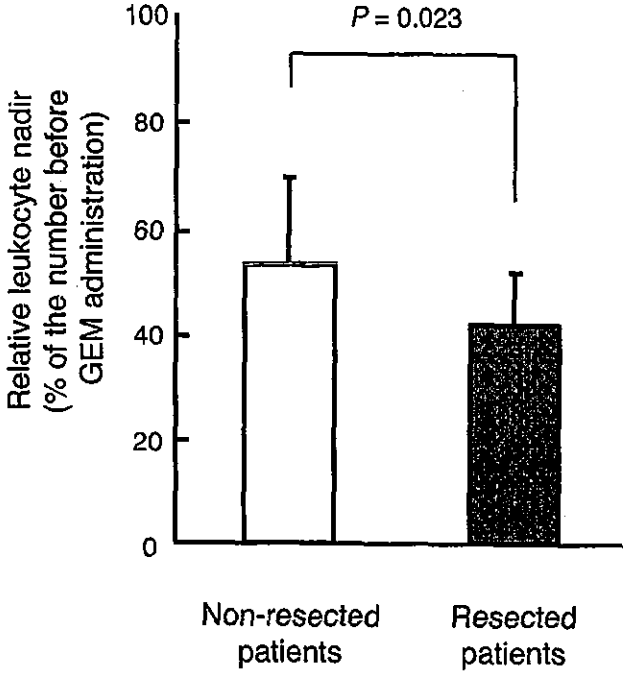

Fig. 2. Comparison of the relative leukocyte nadir between resected and non-resected patients. Data values are expressed as means ± SDs. The numbers are as follows: non-resected patients (n = 24) and resected patients (n = 14)

also demonstrated in the study. However, this study in Japan included only 11 patients with pancreatic cancer who did not undergo pancreatectomy. Considering the frequent use of GEM in radiation chemotherapy or adjuvant chemotherapy, sufficient information regarding the adverse effects of GEM should be provided, in addition to its therapeutic effects. In the present study, we investigated the backgrounds of pancreatic cancer patients who developed leukopenia from the administration of GEM, and found that preceding surgical resection affected the grade and the frequency of GEM-induced leukopenia.

In the present study, the incidence of grade 3 or 4 leukopenia was 36.8%, which was higher than that in the results of the previous reports.[3,5] We found that those patients who had undergone surgical resection for pancreatic cancer had a significantly higher incidence of grade 3 or 4 leukopenia than those patients with unresected primary pancreatic cancer. The mechanisms for the effect of surgical resection on leukopenia remain to be clarified; however, reduction of systemic immunity in postoperative patients may be involved in the increased frequency of the severe leukopenia. Steube et al.[11] measured cytokine secretion in several kinds of cancer cells, and reported excessive secretion of granulocyte macrophage colony-stimulating factor (GM-CSF) by four kinds of pancreatic cancer cells. In addition, Blumenthal et al.[12] reported that culture media which was incubated with two kinds of pancreatic cancer cells (TGP51, TGP47) promoted proliferation of myelocytes. They speculated that the excessive secretion of GM-CSF or G-CSF derived from pancreatic cancer cells may be related to the stimulation of myelocyte proliferation. It is, therefore, speculated that the excessive secretion of pancreatic cancer cell-derived GM-CSF or G-CSF in patients without surgical resection may attenuate the severe reduction of leukocytes induced by GEM administration. Further analyses are needed to clarify the mechanisms inducing a higher rate of leukopenia in patients who undergo surgical resection, particularly in regard to immunological conditions and sensitivity to GEM.

In conclusion, we found that severe leukopenia induced by GEM administration developed readily in patients who had undergone preceding surgical resection for pancreatic cancer. In patients treated with GEM, monitoring of the leukocyte count should be performed with extreme care, and the interval between doses, and the dose of GEM, should be adjusted, especially in patients with preceding pancreatic resection.

Acknowledgments This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

References

1. Warshaw AL, Castillo CF-D (1992) Pancreatic carcinoma. N Engl J Med 326:455–465
2. Laethem J-LV, Demols A, Gay F, et al. (2003) Postoperative adjuvant gemcitabine and concurrent radiation after curative resection of pancreatic head carcinoma: a phase II study. Int J Radiat Oncol Biol Phys 56:974–980
3. Burris HA III, Moore MJ, Andersen J, et al. (1997) Improvements in survival and clinical benefit with gemcitbine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. J Clin Oncol 15:2403–2413
4. Haller DG (2003) Chemotherapy for advanced pancreatic cancer. Int J Radiat Oncol Biol Phys 56:16–23
5. Okada S, Ueno H, Okusaka T, et al. (2001) Phase I trial of gemcitabine in patients with advanced pancreatic cancer. Jpn J Clin Oncol 31:7–12

6. Onoue M, Kato T, Terada T, et al. (2003) Pharmaceutical care on pancreatic cancer chemotherapy. Appropriate usage of G-CSF (granulocyte colony-stimulating factor) for gemcitabine-induced leukopenia (in Japanese). Jpn J Pharm Health Care Sci 28:391–396
7. Marty M, Pouillart P, Scholl S, et al. (1990) Comparison of the 5-hydroxytryptamine3 (serotonin) antagonist ondansetron (GR 38032F) with high-dose metoclopramide in the control of cisplatin-induced emesis. N Engl J Med 322:816–821
8. Italian Group for Antiemetic Research (1992) Ondansetron + dexamethasone vs metoclopramide + dexamethasone + diphenhydramine in preventation of cisplatin-induced emesis. Lancet 340:96–99
9. Gabrilove JL, Jakubowski A, Scher H, et al. (1988) Effect of granulocyte colony-stimulating factor on neutropenia and associated morbidity due to chemotherapy for transitional-cell carcinoma of the urothelium. N Engl J Med 318:1414–1422
10. Goncalves A, Viret F, Ciccolini J, et al. (2003) Phase I and pharmacokinetic study of escalating dose of docetaxel administrated with granulocyte colony-stimulating factor support in adult advanced solid tumors. Clin Cancer Res 9:102–108
11. Steube KG, Meyer C, Drexler HG (1998) Secretion of functional hematopoietic growth factors by human carcinoma cell lines. Int J Cancer 78:120–124
12. Blumenthal RD, Reising A, Leon E, et al. (2002) Modulation of marrow proliferation and chemosensitivity by tumor-produced cytokines from syngeneic pancretic tumor lines. Clin Cancer Res 8:1301–1309

Survivin expression is a prognostic marker in pancreatic cancer patients

Kazuhiro Kami, MD, Ryuichiro Doi, MD, Masayuki Koizumi, MD, Eiji Toyoda, MD, Tomohiko Mori, MD, Daisuke Ito, MD, Koji Fujimoto, MD, Michihiko Wada, MD, Shin-Ichi Miyatake, MD, *and* **Masayuki Imamura, MD,** *Kyoto and Osaka, Japan*

Background. *In this study, we assessed survivin expression in pancreatic cancer specimens from patients who underwent either pancreatic resection alone or pancreatic resection plus postoperative radiation therapy (PORT) to evaluate whether survivin expression is predictive of sensitivity to PORT and outcome in pancreatic cancer patients.*

Methods. *Fifty-two patients who underwent pancreatic resection for ductal adenocarcinomas were included in this study. Forty-seven pancreatic ductal adenocarcinoma and 5 normal pancreatic tissues were evaluated for survivin expression by immunohistochemistry. Then the relationship between survivin expression and clinicopathologic data were analyzed.*

Results. *Sixty-eight percent (32/47) of pancreatic cancer tissues were positive for survivin expression; 32% (15/47) were negative. Normal pancreatic exocrine tissues were negative for survivin expression (0/5). Survival of the patients with positive survivin was significantly shorter than those with negative survivin (P = .02). Survivin was an independent variable that correlated with overall survival (P = .01). There was no difference in survival time between patients with and without PORT. Likely, PORT showed no impact on survival time in survivin-positive patients (P = .12) as well as in survivin-negative patients (P = .95).*

Conclusions. *The results suggest that survivin expression in pancreatic cancer tissues could be a useful prognostic marker in pancreatic cancer patients. (Surgery 2004;136:443-8.)*

From the Department of Surgery and Surgical Basic Science, Kyoto University, Kyoto, Japan; and Department of Neurosurgery, Osaka Medical College, Osaka, Japan

SURVIVIN IS A MEMBER of the inhibitor of apoptosis protein (IAP) family, which counteracts apoptosis and regulates cell division.[1] Survivin shows unique features when compared with other apoptosis inhibitors. Survivin expresses in a cell cycle–regulated manner and shows highest expression in the G2/M phase.[2] Therefore, it is usually undetectable in most normal adult tissues except for the testis, thymus, and placenta.[3] In contrast, survivin is widely expressed in human tissues during fetal development,[4] and in most transformed cell lines and human carcinomas[1] tested to date, including bladder, blood, brain, breast, colon, esophagus, kidney, liver, lung, pancreas, prostate, and stomach.[5-16]

Survivin expression correlates with poor survival in the majority of cancers reported to date.[8-12] Further, survivin is considered to be a radio-resistant factor in several human cancer cell lines, such as pancreatic cancer,[17] colorectal cancer,[18] and melanoma.[19] However, in pancreatic ductal adenocarcinoma, it has been reported that survivin expression of tumor cells was not a prognostic marker. In this study, we investigated survivin expression in human pancreatic ductal adenocarcinoma and examined whether survival time of pancreatic cancer patients who underwent curative pancreatic resection correlated with survivin expression. The relationship between survivin expression and postoperative radiation therapy (PORT) was also evaluated.

PATIENTS AND METHODS

Patients. Fifty-two patients with pancreatic ductal adenocarcinoma confirmed by histopathologic diagnosis who underwent pancreatic resection at the Department of Surgery and Surgical Basic Science, Kyoto University, from January 1995 to

Presented at the 65th Annual Meeting of the Society of University Surgeons, St. Louis, Missouri, February 11-14, 2004.

Supported by a Grant-in-Aid (#15390395) from the Ministry of Education of Japan.

Reprint requests: Ryuichiro Doi, MD, Department of Surgery and Surgical Basic Science, Kyoto University, 54 Shogoinkawara-cho, Sakyo, Kyoto 606-8507, Japan.

0039-6060/$ - see front matter

doi:10.1016/j.surg.2004.05.023

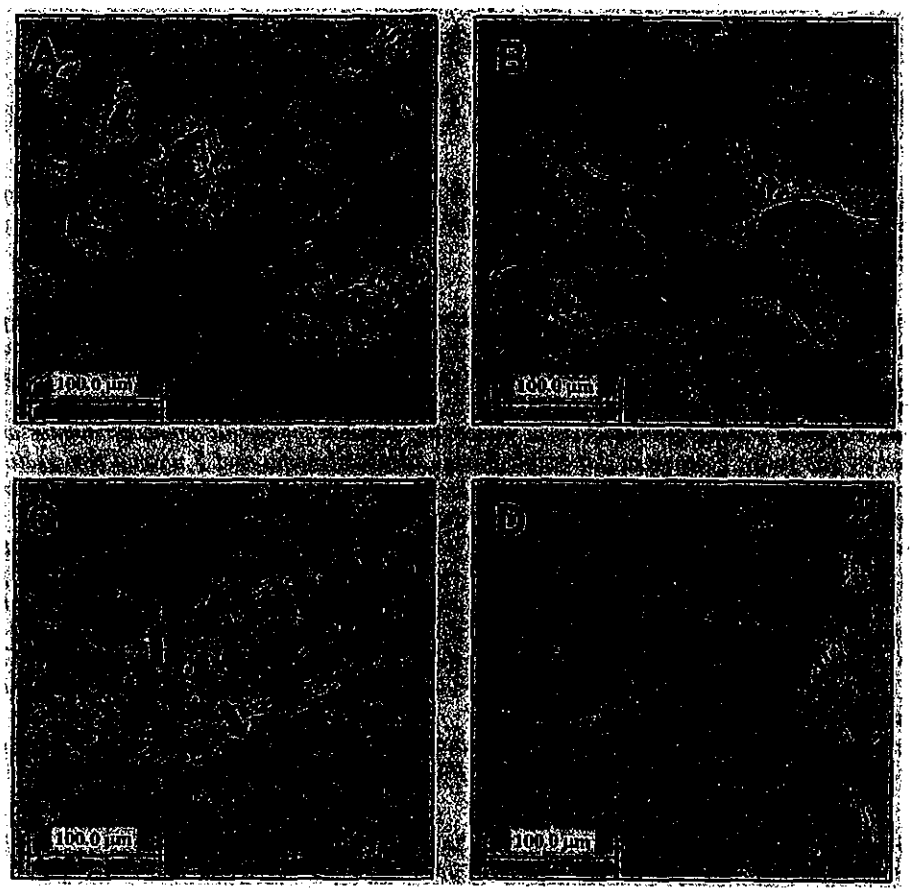

Fig 1. Survivin expression by immunohistochemistry in pancreatic cancer tissues. A-B, Typical expression of immunoreactive survivin in the cytoplasm of the pancreatic cancer cells (*brown color*). **C,** Expression of survivin in the pancreatic islet cells (*arrow*). **D,** Normal pancreatic tissues. (Original magnification ×40).

December 2001 were included in this study. The patients included 26 men and 21 women, with ages that ranged from 46 to 76 years at the time of the operation (64.7 ± 8.3 years [mean ± SD]). The follow-up data of the patients were updated in August 2003. The patients underwent margin-free curative pancreatic resection and D2 lymph node dissection. We used the clinical data from 41 patients whose outcome and context of adjuvant therapy have been confirmed. Among them, 14 patients underwent PORT. Tumor samples were collected after the patients provided informed consent, in accordance with the institutional guidelines.

Tissue samples. Tissue samples were fixed in 4% paraformaldehyde, embedded in paraffin, and cut into consecutive 4-μm-thick sections. Forty-seven pancreatic ductal adenocarcinomas and 5 normal pancreatic tissues were obtained from 52 pancreatic carcinoma patients. Paraffin sections were prepared for the immunohistochemistry.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue sections were deparaffinized in 3 changes of xylene, rehydrated in descending concentrations of ethanol, and washed 2 times for 3 minutes each with double-distilled water. Antigen retrieval was carried out by pressure cooking in 0.01 mol/L sodium citrate buffer (pH 6.0). The sections were incubated for 30 minutes at room temperature in 0.3% hydrogen peroxide in methanol to block endogenous peroxidase activity, and then were incubated for 30 minutes at room temperature with 0.01 mol/L phosphate-buffered saline solution (pH 7.4) that contained 5% normal goat serum and 1% bovine serum albumin. The sections were then immunostained for survivin by overnight incubation at 4°C with mouse monoclonal antibody Survivin Ab-1 (clone 8E2; LAB VISION, Fremont, Calif) at dilution of 1:100 for all cases. Five sections were also immunostained with the use of polyclonal antibody Surv11A (AlphaDiagnostics, San Antonio, Tex) at a dilution of 1:50 to verify whether the

Table I. Survivin expression and clinicopathologic factors

Variables	*Survivin*		P *value*
	Negative (*n* = 15)	*Positive* (*n* = 32)	
Age			.80
≥60 yo	10	24	
<60 yo	4	6	
Gender			.89
Male	8	18	
Female	7	14	
Tumor size			.79
≥4 cm	6	10	
<4 cm	9	22	
Grade			.34
1–2	10	26	
3–4	5	5	
pT			.99
1–2	4	7	
3–4	11	25	
pN			.78
Negative	7	12	
Positive	8	20	
pM			1.00
Negative	13	28	
Positive	2	4	
pTNM (UICC)			.72
Stage 1	4	3	
Stage 2	6	17	
Stage 3	3	8	
Stage 4	2	4	

yo, Years old; *UICC*, Union Internationale Contre le Cancer.

staining pattern is identical with a different antibody. To exclude the possibility of background staining by a secondary antibody, we immunostained serial sections using nonspecific mouse immunoglobulin G. Then the sections were washed 2 times for 3 minutes in phosphate-buffered saline solution. Bound antibody was detected with the use of a Envision/HRP (DAB) immunostaining kit (DakoCytomation, Kyoto, Japan). Then the slides were counterstained with hematoxylin and eosin. The expression of survivin was evaluated according to the mean percentage of positive-stained cells among cancer cells in at least 4 areas at 400-fold magnification and assigned to 1 of the following 4 categories: 0 = none; 1 = 1% to 10%; 2 = 11% to 50%; 3 = 51% to 100%.

The specimens graded into 0 and 1 were regarded as negative, and the specimens graded into 2 and 3 as positive. The expression of survivin was evaluated independently by 2 investigators (K.K. and M.W.) who had no knowledge of the patients' clinicopathologic features.

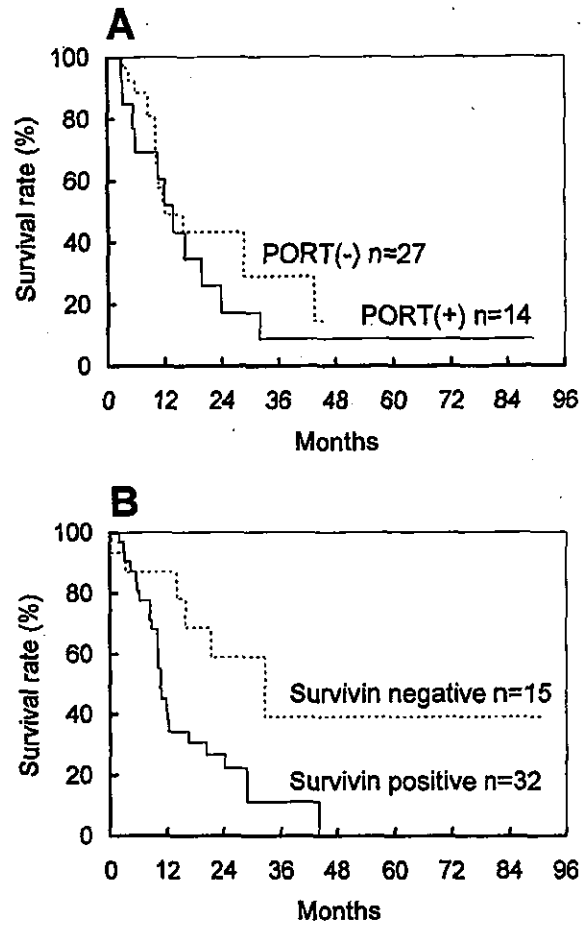

Fig 2. A, Survival curves for patients with PORT (PORT(+), n = 14) and patients without the therapy (PORT(-), n = 27). There were no significant differences between the 2 groups ($P = .49$, log-rank test). **B,** Survival curves for survivin-positive patients (n = 32) and survivin-negative patients (n = 15). Statistically significant differences was detected between the 2 groups ($P = .02$, log-rank test).

Statistical analysis. Survival time was calculated from the date of resection to the date of death or date of the latest follow-up. Survival curves were plotted according to the Kaplan-Meier method; statistical differences were analyzed by the log-rank test. The correlation between survivin expressions and various clinicopathologic characteristics was analyzed by using either the chi-square test or Student *t* test. To assess the prognostic significance of individual variables and to identify independent predictors of survival, we used Cox regression analysis and stepwise selection procedure. The statistical analyses were performed with Stat View software version 5.0 (Abacus Concepts, Berkeley, Calif) and SPSS software version 11.0 (SPSS Inc,

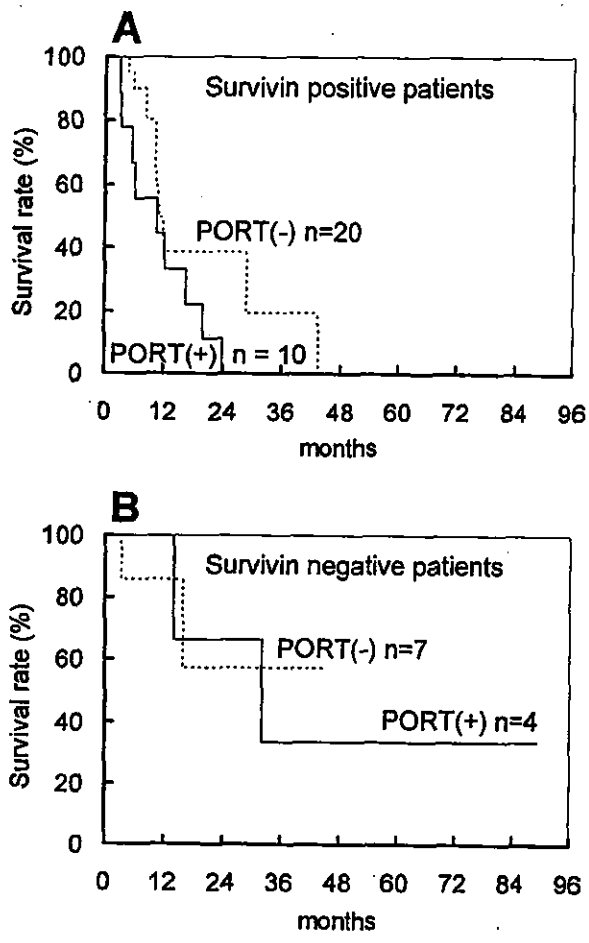

Fig 3. **A,** Survival curves for survivin-positive patients with PORT (PORT(+), n = 10) and patients without the therapy (PORT(-), n = 20). There were no significant differences between the 2 groups ($P = .12$, log-rank test). **B,** Survival curves for survivin-negative patients with PORT (PORT(+), n = 4) and patients without the therapy (PORT(-), n = 7). There were no significant differences between the 2 groups ($P = .95$, log-rank test).

Chicago, Ill). A P value less than .05 was regarded as statistically significant.

RESULTS

Survivin expression in pancreatic tissues. The expression of survivin was observed in the cytoplasm of the pancreatic cancer cells and occasionally in the nuclei of islet cells (Fig 1). Normal pancreatic exocrine tissues showed no expression of survivin. According to the criteria described in Material and Methods, 32 of 47 pancreatic cancer tissues (68%) were positive for survivin expression, and 15 were negative (32%). Normal pancreatic tissues were negative for survivin (0 of 5). Strength and localization of the survivin expression were identical to each other by 2 antibodies: clone 8E2 and SURV11A.

Survivin expression and clinicopathologic factors. Table I shows the relationship between the expression of survivin and clinicopathologic factors. There were no significant differences between the 2 groups in the parameters, such as tumor size, tumor grade (1-2 vs 3-4), pT, pN, pM, and pTNM stage (Union Internationale Contre le Cancer pTNM system).

PORT and prognosis. Figure 2, *A*, shows the survival curves for pancreatic cancer patients with and without PORT. Median survival time of patients with PORT was 11.2 months and that of patients without PORT was 10.9 months. There was no significant difference between the 2 groups ($P = .49$, log-rank test).

Survivin expression and prognosis. Figure 2, *B* shows the survival curves for patients with survivin-positive and survivin-negative pancreatic cancers. Median survival time of patients with survivin-positive cancers was 10.4 months and that of patients with survivin-negative cancers was 15.7 months. The survival rate of the survivin-negative group was significantly better than the survivin-positive group ($P = .02$, log-rank test). To evaluate the relationship between survivin expression and the effect of PORT in terms of survival of pancreatic cancer patients, we plotted Kaplan-Meier curves for patients with survivin-positive cancers or survivin-negative cancers, according to the presence of PORT, respectively (Fig 3). In survivin-positive patients, there was no significant difference in survival between PORT (+) and PORT (-) groups ($P = .12$, log-rank test). Similarly, in the survivin-negative patients, there was no significant difference in survival between PORT (+) and PORT (-) groups ($P = .95$, log-rank test).

We next performed a multivariate Cox proportional hazard model analysis with the variables of surviving expression, tumor grade (1-2 vs 3-4), PORT (+ vs -), pT (T1-2 vs T3-4), pN, and pM to determine the independent prognostic value with respect to the overall survival of pancreatic cancer patients. By univariate or multivariate analysis, the survivin expression and pM were independent variables that significantly affected overall survival (Table II).

DISCUSSION

The aim of this study was to determine whether survivin expression was predictive of sensitivity to radiation therapy and outcome of pancreatic cancer patients. We first demonstrated that pancreatic

Table II. Univariate and multivariate Cox proportional hazard model

Variables	Univariate analysis		Multivariate analysis		
	LRχ2	P *value*	*Risk ratio (95% CI)*	*LR*χ^2	P *value*
pM (negative vs positive)	6.78	.009*	0.71 (0.18-0.282)	14.1	.002*
Survivin expression (negative vs positive)	5.66	.01*	0.108 (0.028-0.417)	10.4	.01*.

LRχ2, Likelihood ratioχ^2; *CI*, confidence interval.
*Denotes significant value.

cancer patients without survivin expression had significantly longer survival rates than patients with survivin expression. Furthermore, we demonstrated that PORT might have no significant effects on postoperative survival of the pancreatic cancer patients regardless of survivin expression.

Satoh et al[14] and Sarela et al[13] have reported strong expression of survivin in pancreatic ductal carcinoma by immunohistochemistry. Satoh et al did not evaluate the correlation between survivin expression and survival of the patients. Sarela et al reported that there was no association between survival and survivin expression in pancreatic ductal carcinoma, and suggested that survivin expression might independently predict a worse outcome in specific subgroups of patients with early stage cancer. They did not evaluate pTNM stage of the patients, but 93% of the patients were graded into pT3 in their series. In contrast, patients with pT1 and pT2 were occupied 23% (11/47) in the present study. In addition, we used a monoclonal antibody for the evaluation of survivin expression, although Sarela et al used a different polyclonal antibody. The discordance in the results between Sarela et al and our study might be attributed to the differences described above. Strong expression of survivin in several human cancers is regarded an important predictor of shorter survival, chemoresistance, and radioresistance. It has been reported that survivin directly downregulates radiosensitivity of pancreatic cancer cells in vitro.[17] Therefore, we tested the effect of radiation therapy on pancreatic cancer with and without survivin expression. Contrary to expectations, in our study, PORT did not affect survival of pancreatic cancer patients regardless of the survivin expression. This result was inconsistent with our previous report in another series of patients in whom PORT showed no significant effect on survival of the patients with resectable pancreatic cancer.[20] Similarly, a large-scale clinical trial has recently shown that there was no survival benefit for adjuvant chemoradiotherapy on resectable pancreatic cancer patients.[21]

In the present study, survivin expression was detected by a monoclonal antibody, clone 8E2, in the cytoplasm but not in the nuclei of the cancer cells, and occasionally in the nuclei of islet cells. On the other hand, it has been reported that survivin was detected in both the cytoplasm and nuclei of gastric cancer cells with another survivin antibody, such as SURV11A.[22] Furthermore, nuclear survivin expression has been described as an independent variable of favorable survival in gastric[22] and breast cancers.[23]

As has been suggested by Kennedy et al,[23,24] it may be true that the monoclonal antibody clone 8E2 could not recognize nuclear survivin. Therefore, we further tested if another antibody, SURV11A, can recognize nuclear expression of survivin. However, in the serial sections of the pancreatic cancer tissues, we could not detect nuclear expression of survivin in cancer cells by either antibody. It has been reported that nuclear expression of survivin stained with polyclonal antibodies in several cancers is a good prognostic marker. In our study, survivin expression was limited to the cytoplasm in cancer cells, which may be attributed to the devastatingly unfavorable outcome in pancreatic cancer patients.

We also found survivin expression in human adult islets, although we did not study what kind of human islet cells were stained with antisurvivin antibody. Liggins et al[25] has reported that survivin expression was detected in alpha cells of human adult islets and that beta cells were developmentally regulated by the expression of survivin. Similar to other apoptosis-related molecules, survivin could be involved in the lineage of the pancreatic development.

CONCLUSION

The present results suggest that survivin protein expression in pancreatic cancer tissue is not a predictor of sensitivity to PORT but could be a useful prognostic indicator in pancreatic cancer patients.

REFERENCES

1. Altieri DC. The molecular basis and potential role of survivin in cancer diagnosis and therapy. Trends Mol Med 2001;7:542-7.
2. Li F, Ambrosini G, Chu EY, Plescia J, Tognin S, Marchisio PC, et al. Control of apoptosis and mitotic spindle checkpoint by survivin. Nature 1998;396:580-4.
3. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997;3:917-21.
4. Adida C, Berrebi D, Peuchmaur M, Reyes-Mugica M, Altieri DC. Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. Lancet 1998;351:882-3.
5. Lehner R, Lucia MS, Jarboe EA, Orlicky D, Shroyer AL, McGregor JA, et al. Immunohistochemical localization of the IAP protein survivin in bladder mucosa and transitional cell carcinoma. Appl Immunohistochem Mol Morphol 2002;10:134-8.
6. Kamihira S, Yamada Y, Hirakata Y, Tomonaga M, Sugahara K, Hayashi T, et al. Aberrant expression of caspase cascade regulatory genes in adult T-cell leukaemia: survivin is an important determinant for prognosis. Br J Haematol 2001; 114:63-9.
7. Katoh M, Wilmotte R, Belkouch MC, de Tribolet N, Pizzolato G, Dietrich PY. Survivin in brain tumors: an attractive target for immunotherapy. J Neurooncol 2003;64: 71-6.
8. Tanaka K, Iwamoto S, Gon G, Nohara T, Iwamoto M, Tanigawa N. Expression of survivin and its relationship to loss of apoptosis in breast carcinomas. Clin Cancer Res 2000;6:127-34.
9. Kawasaki H, Altieri DC, Lu CD, Toyoda M, Tenjo T, Tanigawa N. Inhibition of apoptosis by survivin predicts shorter survival rates in colorectal cancer. Cancer Res 1998; 58:5071-4.
10. Kato J, Kuwabara Y, Mitani M, Shinoda N, Sato A, Toyama T, et al. Expression of survivin in esophageal cancer: correlation with the prognosis and response to chemotherapy. Int J Cancer 2001;95:92-5.
11. Ito T, Shiraki K, Sugimoto K, Yamanaka T, Fujikawa K, Ito M, et al. Survivin promotes cell proliferation in human hepatocellular carcinoma. Hepatology 2000;31:1080-5.
12. Monzo M, Rosell R, Felip E, Astudillo J, Sanchez JJ, Maestre J, et al. A novel anti-apoptosis gene: Re-expression of survivin messenger RNA as a prognosis marker in non–small-cell lung cancers. J Clin Oncol 1999;17:2100-4.
13. Sarela AI, Verbeke CS, Ramsdale J, Davies CL, Markham AF, Guillou PJ. Expression of survivin, a novel inhibitor of apoptosis and cell cycle regulatory protein, in pancreatic adenocarcinoma. Br J Cancer 2002;86:886-92.
14. Satoh K, Kaneko K, Hirota M, Masamune A, Satoh A, Shimosegawa T. Expression of survivin is correlated with cancer cell apoptosis and is involved in the development of human pancreatic duct cell tumors. Cancer 2001;92:271-8.
15. Xing N, Qian J, Bostwick D, Bergstralh E, Young CY. Neuroendocrine cells in human prostate over-express the anti-apoptosis protein survivin. Prostate 2001;48:7-15.
16. Lu CD, Altieri DC, Tanigawa N. Expression of a novel antiapoptosis gene, survivin, correlated with tumor cell apoptosis and p53 accumulation in gastric carcinomas. Cancer Res 1998;58:1808-12.
17. Asanuma K, Kobayashi D, Furuya D, Tsuji N, Yagihashi A, Watanabe N. A role for survivin in radioresistance of pancreatic cancer cells. Jpn J Cancer Res 2002;93:1057-62.
18. Rodel C, Haas J, Groth A, Grabenbauer GG, Sauer R, Rodel F. Spontaneous and radiation-induced apoptosis in colorectal carcinoma cells with different intrinsic radiosensitivities: survivin as a radioresistance factor. Int J Radiat Oncol Biol Phys 2003;55:1341-7.
19. Pennati M, Binda M, Colella G, Folini M, Citti L, Villa R, et al. Radiosensitization of human melanoma cells by ribozyme-mediated inhibition of survivin expression. J Invest Dermatol 2003;120:648-54.
20. Nishimura Y, Hosotani R, Shibamoto Y, Kokubo M, Kanamori S, Sasai K, et al. External and intraoperative radiotherapy for resectable and unresectable pancreatic cancer: analysis of survival rates and complications. Int J Radiat Oncol Biol Phys 1997;39:39-49.
21. Neoptolemos JP, Dunn JA, Stocken DD, Almond J, Link K, Beger H, et al. Adjuvant chemoradiotherapy and chemotherapy in resectable pancreatic cancer: a randomised controlled trial. Lancet 2001;358:1576-85.
22. Okada E, Murai Y, Matsui K, Isizawa S, Cheng C, Masuda M, et al. Survivin expression in tumor cell nuclei is predictive of a favorable prognosis in gastric cancer patients. Cancer Lett 2001;163:109-16.
23. Kennedy SM, O'Driscoll L, Purcell R, Fitz-Simons N, McDermott EW, Hill AD, et al. Prognostic importance of survivin in breast cancer. Br J Cancer 2003;88:1077-83.
24. Fortugno P, Wall NR, Giodini A, O'Connor DS, Plescia J, Padgett KM, et al. Survivin exists in immunochemically distinct subcellular pools and is involved in spindle microtubule function. J Cell Sci 2002;115:575-85.
25. Liggins C, Orlicky DJ, Bloomquist LA, Gianani R. Developmentally regulated expression of survivin in human pancreatic islets. Pediatr Dev Pathol 2003;6:392-7.

Hepatic regeneration and enforced PDX-1 expression accelerate transdifferentiation in liver

Masayuki Koizumi, MD, Ryuichiro Doi, MD, Eiji Toyoda, MD, Sidhartha S. Tulachan, MD, Kazuhiro Kami, MD, Tomohiko Mori, MD, Daisuke Ito, MD, Yoshiya Kawaguchi, MD, Koji Fujimoto, MD, George K. Gittes, MD, *and* **Masayuki Imamura, MD,** *Kyoto, Japan, and Kansas City, Mo*

Background. *Pancreatic duodenal homeobox gene-1 (PDX-1) has a dual task as a key regulator in pancreatic organogenesis and in functional maintenance of β cells in adults. Recent studies have shown a close lineage relationship between the liver and the pancreas. In this study, we analyzed the plasticity of the liver by enforced expression of PDX-1 in streptozotocin (STZ)-treated mice under the condition of hepatic regeneration.*

Methods. *Replication-deficient adenoviruses were constructed by the cosmid-adenoviral DNA terminal protein complex method. Mice were treated with STZ (200 mg/kg ip), and a 40% partial hepatectomy was performed at day 0. After 24 hours, Ad-pdx-1 or Ad-lacZ 2.0×10^9 PFU/body was injected via the tail vain into nontreated (control), STZ-treated, or STZ plus partial hepatectomy (Hx)–treated ICR mice. After 7 and 14 days, expression of PDX-1 and islet hormones was examined by immunohistologic and reverse transcription-polymerase chain reaction analysis. Blood glucose concentrations were measured every 2 days. Immunoreactive insulin (IRI) of serum and liver extract was measured by ELISA.*

Results. *Most hepatocytes of Ad-pdx-1–infected mice were positive for PDX-1 expression by immunohistochemistry. In nontreated mice, very few cells expressed insulin and other hormones. In contrast, insulin and somatostatin were expressed in STZ-treated mice, and more cells were expressed in STZ plus Hx–treated mice. In addition, other β-cell markers like GLUT2 and glucokinase were observed. Hyperglycemia was improved in STZ-treated mice and STZ plus Hx–treated mice. IRI of serum and liver extract was increased in STZ-treated mice and STZ plus Hx–treated mice. The insulin positive area of the liver in STZ plus Hx–treated mice was larger than that in nontreated and STZ-treated mice.*

Conclusions. *Ectopic PDX-1 expression alone may be insufficient to induce insulin-producing cells in the liver. STZ-induced hyperglycemia plus partial hepatectomy that leads to diabetic state and hepatic regeneration may stimulate the transdifferentiation of liver cells into insulin-producing cells.* *(Surgery 2004;136:449-57.)*

From the Department of Surgery and Surgical Basic Science, Kyoto University, Kyoto, Japan; and Surgical Research, Children's Mercy Hospital, Kansas City, Mo

DIABETES MELLITUS IS A METABOLIC DISORDER characterized by hyperglycemia resulting from defects in insulin secretion and insulin function.[1] Patients affected by diabetes present various degrees of islet cell dysfunction and insulin resistance. The insufficient release of insulin by β cells is caused by either autoimmune-mediated cell destruction or failure to compensate for an increasing demand of insulin. On the other hand, extended surgical resection for advanced malignant tumor in the hepato-pancreatico-biliary region can provoke postoperative severe hyperglycemia that leads to various serious complications. Especially in pancreatectomy, a massive reduction of β cells in the pancreas causes hyperglycemia that is difficult to deal with. As an approach to restore the decrease of insulin production both in diabetes mellitus and in surgically induced hyperglycemic patients,

Presented at the 65th Annual Meeting of the Society of University Surgeons, St. Louis, Missouri, February 11-14, 2004.

Supported by a Grant-in-Aid (#15390395) from the Ministry of Education of Japan, and PRESTO, Japan Science and Technology Agency, Japan.

Reprint requests: Ryuichiro Doi, MD, Department of Surgery and Surgical Basic Science, Kyoto University, 54 Shogoinkawara-cho, Sakyo, Kyoto 606-8507, Japan.

0039-6060/$ - see front matter

doi:10.1016/j.surg.2004.05.024

surrogate β cells have been generated from embryonic stem cells[2] and somatic non-β cells such as pancreatic ductal cells,[3] exocrine cells,[4] the liver,[5,6] intestinal epithelial cells,[7,8] and bone marrow.[9] Among those surrogates for β cells, the liver could be the most promising and ideal source because it has the highest regenerative capacity among all parenchymal organs in the adult.

A number of reports have focused on the mutual plasticity between the liver and pancreas; both have a similar structural organization and share a common developmental origin. The liver is derived from the endoderm adjacent to the area from where the pancreas is derived.[10] Fibroblast growth factor and bone morphogenetic protein signals from the mesoderm influence the fate of the endoderm to compose the liver during development.[11] In contrast, lower levels of these components lead the endoderm to the ventral pancreas. The plasticity from the pancreas to the liver has been reported in regeneration of the pancreas of hamsters,[12] in copper depletion-repletion rats,[13] and in keratinocyte growth factor (KGF) transgenic mice (under the control of insulin promoter).[14] Shen et al[15] has elucidated the molecular basis of this transdifferentiation by using pancreatic exocrine cell lines with dexamethasone treatment.

On the other hand, the plasticity from the liver to the pancreas was observed in polychlorinated biphenyl–treated rats[16] and in fish liver tumors.[17] All of the converted cells were exocrine cells, and endocrine conversion was not reported. In order for endocrine cells to have emerged in the liver, genetic modification seems indispensable as was observed in vivo[5,18] and in vitro.[6] These experimental conversion models suggested that PDX-1 is an important master switch regulator for the conversion of the liver to the pancreas.[5,6,18] In pancreas development, PDX-1 is an indispensable gene: homozygous disruption of PDX-1 results in pancreatic agenesis both in the mouse and humans.[19] In the mouse, PDX-1 expression is first detected at e8.5 in endoderm cells in the dorsal gut. At e9.5, dorsal and ventral pancreatic buds are formed; PDX-1 is expressed in the pancreatic buds and in the duodenal wall between them and becomes downregulated in acinar and duct cells. In the adult, PDX-1 is expressed mainly in β and δ cells of the pancreatic islets,[20] and in gastrointestinal epithelia from the pyloric antrum to the duodenum.[21] PDX-1 regulates a number of genes involved in maintaining β-cell identity and function, including insulin, glucose transporter gene-2 (GLUT2), glucokinase and islet amyloid polypeptide; it also regulates somatostatin in δ cells.

In the present study, we investigated the adenovirus-mediated gene transfer of PDX-1 to the mouse liver and the conversion of the liver to pancreatic endocrine cells. We further analyzed the effect of a streptozotocin (STZ)-induced hyperglycemic condition and the effect of hepatectomy on an adenovirus-mediated gene transfer of PDX-1 to the mouse liver.

MATERIAL AND METHODS

Cell lines. Human embryonic kidney 293 cells (HEK293) and COS7 cells were purchased from the American Type Culture Collection (ATCC, Rockville, Md), and maintained in an appropriate medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humid atmosphere of 5% CO_2/95% air.

Recombinant adenovirus vectors. We used a replication-deficient recombinant adenovirus in which the E1A, E1B, and E3 regions of the human adenovirus type 5 (Ad5) serotype were deleted.[22] Recombinant adenovirus vectors were prepared as reported previously.[23] Briefly, the recombinant replication-defective adenoviruses were constructed by the cosmid-adenoviral DNA terminal protein complex (COS-TPC) method.[24,25] The mouse PDX-1 cDNA was excised from pBluescript 2 SK (+)-pdx-1 (a gift from Dr C.V. Wright, Vanderbilt University, Nashville, Tenn). The PDX-1–coding sequence was inserted into the pAdCAwt cassette cosmid that contains a CAG promoter (chicken β-actin promoter associated with a CMV enhancer), an artificial splice sequence, and rabbit β-globlin poly A sequence. pAdCAG-pdx-1 was constructed by subcloning, and pAdCAG-pdx-1 and adenoviral DNA-TPC were co-transfected into HEK293 cells to produce recombinant adenovirus through homologous recombination (Fig 1, *A*). Ad-pdx-1, an adenovirus that contains the PDX-1 gene directly driven by the CAG promoter, strongly expresses the PDX-1 gene in all types of infected cells. Control viruses that express *Escherichia coli*-lacZ (Ad-lacZ) were also prepared. All of the adenoviral vectors were propagated in 293 cells, purified by 2 rounds of cesium chloride density centrifugation, dialyzed, and stored at −70°C. The titer, expressed as plaque-forming units (PFU)/mL of each viral stock was determined by plaque assay with HEK293 cells. All of the vector preparations were demonstrated to be free of replication-competent adenoviruses.

Animal experiments. Male ICR mice aged 6 to 9 weeks and weighing 25 to 30 g were purchased from

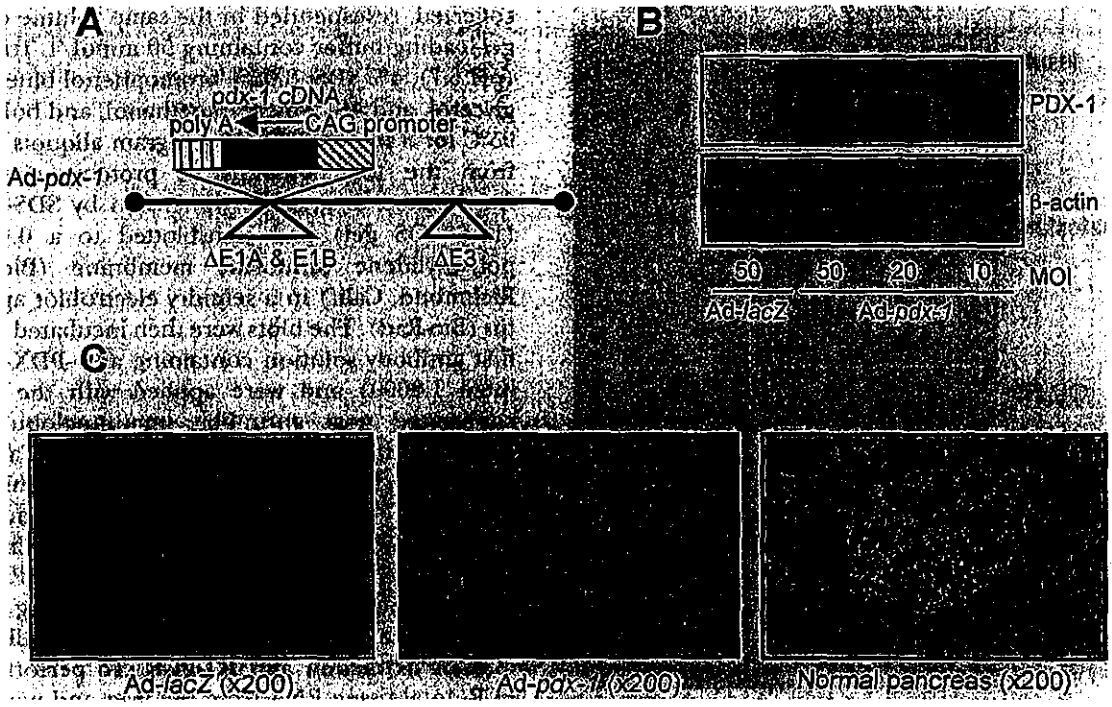

Fig 1. Preparation of recombinant adenovirus and detection of adenovirus-mediated PDX-1 expression in vitro and in vivo. **A,** Structure of recombinant adenovirus Ad-pdx-1. The E1 region of the Ad5 genome was replaced with the PDX-1 expression cassette, which consists of a cytomegalovirus enhancer and a chicken β-actin promoter (CAG promoter), PDX-1 cDNA with a rabbit β-globin polyadenylation signal (poly A). The control adenovirus Ad-lacZ was prepared accordingly. **B,** The expression of PDX-1 protein was determined by Western blot analysis in COS7 cells. The COS7 cells were infected with Ad-pdx-1 or Ad-lacZ at 10, 20, and 50 MOI. The expression of PDX-1 was detected in a MOI-dependent manner. **C,** The expression of PDX-1 was determined by immunohistochemical analysis in Ad-lacZ–infected liver (*left*), in Ad-pdx-1–infected liver (*middle*), and in normal pancreas (*right*) (original magnification ×200).

SLC (Hamamatsu, Japan). All animals were treated in accordance with the Institutional Guidelines of Kyoto University. Diabetes was induced by intraperitoneal injection of STZ at 200 mg/kg body weight. Blood samples were collected from ocular veins before and every 2 days after STZ injection or after hepatectomy; blood glucose levels were measured with the use of GT-1640 (Arkray, Kyoto, Japan). Mice were subjected to 40% partial hepatectomy under general anesthesia by using diethyl ether on the same day of STZ treatment. After 24 hours, the mice were injected with recombinant adenovirus solutions (2.0×10^9 PFU in 300 μL lactated Ringer's solution) into the tail vein. Mice were sacrificed on day 7 or 14. Tissue samples from the liver and pancreas, and blood samples were kept for the purpose of immunohistochemical analysis, reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, and ELISA, as described below.

Immunohistochemical analysis. Tissues were fixed in 4% paraformaldehyde for 4 hours and then cryoprotected overnight in 30% sucrose, embedded in TissueTek OCT compound, frozen in liquid nitrogen, and cut into 6-μm sections with the Microtome HM505E cryostat (Carl Zeiss Co, Jena, Germany). Sections for immunoperoxidase were pretreated with 0.3% hydrogen peroxide to reduce nonspecific binding to endogenous peroxidase enzymes. Primary antibodies to the following antigens were used at the indicated dilutions: insulin (guinea pig antiswine, DAKO, Carpinteria, Calif) 1:800, somatostatin (rabbit antihuman; DAKO) 1:500, glucagon (rabbit antihuman; DAKO) 1:600, GLUT2 (rabbit antihuman; DAKO) 1:500, glucokinase (donkey antirabbit) 1:500, PDX-1 (rabbit antibody against mouse PDX-1; a gift from Dr C.V. Wright, Vanderbilt University, Nashville, Tenn) 1:1000, α-amylase (rabbit antihuman; Sigma, St. Louis, Mo) 1:500. Primary antibodies were incubated at room temperature for 1 hour (insulin, somatostatin, glucagons, GLUT2, glucokinase) or at 4°C overnight (PDX-1, α-amylase). For secondary antibodies, ready-to-use HRP-conjugated antirabbit

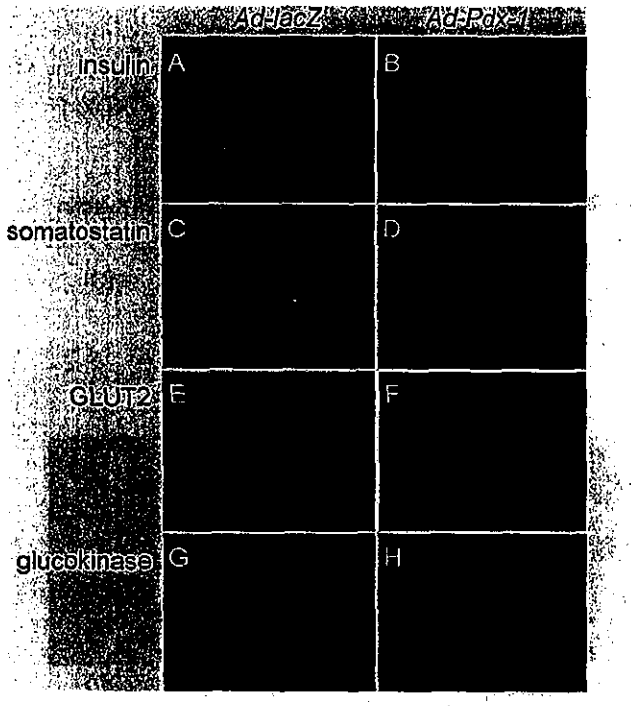

Fig 2. Expression of pancreatic endocrine markers in liver. Fluorescent immunohistochemical analyses at day 7 for insulin (**A, B**), somatostatin (**C, D**), GLUT2 (**E, F**), and glucokinase (**G, H**) were performed for serial sections of liver tissue. Panels of the left column are tissues from Ad-lacZ–infected mice (**A, C, E, G**) and panels in the right column are tissues from Ad-pdx-1–infected mice (**B, D, F, H**). (original magnification ×200) Ad-pdx-1–induced pancreatic hormones GLUT2 and glucokinase. The red color is Cy3; the green color is Yo-Pro's nuclear staining.

imunnoglobulin G (Envision Kit/HRP; DAKO) was used. Immunoperoxidase was detected by DAB kit (DAKO) for PDX-1, and Cy3 conjugated goat antirabbit or donkey antibody (Jackson ImmunoResearch Lab, West Grove, Pa) was used for insulin, somatostatin, glucagon, GLUT2, and glucokinase. For nuclear counter staining, Yo-Pro (Molecular Probes, Eugene, Ore) 1:5000 was incubated at room temperature for 10 minutes. Fluorescently labeled samples were imaged by using a fluorescent microscope (Olympus, Tokyo, Japan).

Western blot analysis. COS7 cells were infected with various multiplicities of infection (MOIs) of recombinant adenovirus; 48 hours later the cells were collected into microtubes with a cell scraper and lysed for 60 minutes in phosphorylation inhibitory RIPA buffer containing 50 mmol/L HEPES (pH 7.0), 250 mmol/L NaCl, 0.1% Nonidet P-40, 1 mmol/L PMSF, and 20 μg/mL gabexate mesilate. They were then sonicated for 20 seconds. Total extracts were centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatants were collected, resuspended in the same volume of the gel-loading buffer containing 50 mmol/L Tris-HCl (pH 6.7), 4% SDS, 0.02% bromophenol blue, 20% glycerol, and 4% 2-mercaptoethanol, and boiled at 95°C for 5 minutes. Ten-microgram aliquots taken from the total quantity of protein were size fractionated to a single dimension by SDS-PAGE (12% SDS gel) and transblotted to a 0.45-μm polyvinylidene difluoride membrane (Bio-Rad, Richmond, Calif) in a semidry electroblot apparatus (Bio-Rad). The blots were then incubated in the first antibody solution containing anti–PDX-1 (diluted 1:2000) and were applied with the HRP-conjugated goat antirabbit immunoglobulin G (Zymed Laboratories Inc, San Francisco, Calif) as a secondary antibody diluted 1:1000 for 60 minutes at room temperature. Enhanced chemiluminescence system (ECL kit; Amersham, Arlington Heights, Ill) was used for detection. Protein concentration was determined by using BCA Protein Assay Reagents (Pierce, Rockford, Ill).

RNA extraction and RT-PCR. To perform RT-PCR, total tissue RNA from the liver and pancreas was prepared using TRIZOL Reagent (Life Technologies Inc, Rockville, Md). Complementary DNA (cDNA) was prepared according to the manufacturer's instructions by random priming from 1 μg of total RNA with the use of a First-Strand cDNA Synthesis kit (Pharmacia Biotech, North Peapack, NJ). PCR was carried out with a mixture consisting of cDNA derived from 100 ng of RNA, 0.2 μmol/L each of upstream and downstream primers for the sequences of the insulin-1 gene and insulin-2 gene, 0.2 μmol/L of deoxynucleotide triphosphate at a final concentration, and 2.5 units of Taq DNA polymerase with reaction buffer (TaKaRa, Kyoto, Japan) in a final volume of 50 μL. The reaction of PCR was performed for 35 cycles in a thermal cycler (Gene Amp PCR system 2400; PE Applied Biosystems, Foster City, Calif) as follows: 60 seconds at 94°C for denaturation, 60 seconds at 58°C for annealing, and 60 seconds at 72°C for extension. β actin was used as an internal control; adult pancreas was used as a positive control. Products of amplification were separated on 1.5% agarose gel and photographed after ethidium bromide staining. Each positive result was confirmed by at least 3 repeats. Primer sequences used are listed as forward then reversed 5′ to 3′. β-actin primers 5′GGCATCGTGATGGACTCCG3′ and 5′GCTGGAAGGTGGACAGCG3′ amplify a product of 612 bp; PDX-1 primers 5′CCTTGATATCGCTGCCACCATGAACAG3′ and 5′CTGCGCTCGAGTGTAGGCAGTACG3′ amplify a product of 484 bp; insulin-1 primers 5′TAGTGACCAGCTATAATCAGAG3′ and

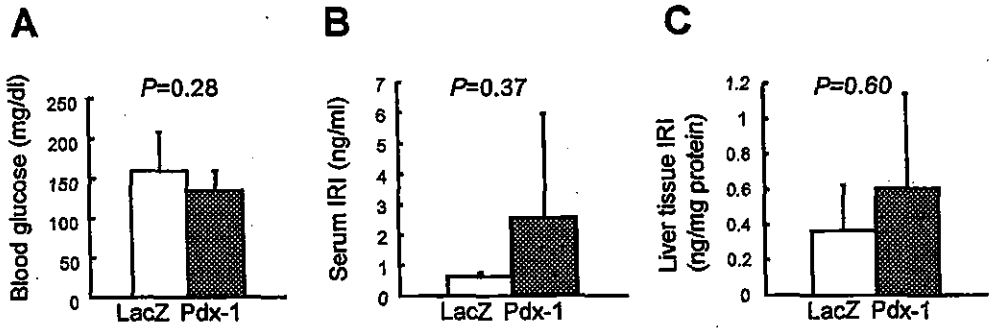

Fig 3. Effects of PDX-1 on blood glucose and IRI of serum and liver tissue extract in mice. Mice were subjected to adenovirus injection at day 0; the samples were collected at day 7. The differences between the Ad-lacZ–treated mice and Ad-pdx-1 mice were not statistically significant in blood glucose (A), serum IRI (B), and liver tissue IRI (C).

5′ACGCCAAGGTCTGAAGGTCC3′ amplify a product of 407 bp; insulin-2 primers 5′CCCTGCTGGCCCTGCTCTT3′ and 5′AGGTCTGAAGGTCACCTGCT3′ amplify a product of 213 bp.

Other procedures. We used a commercial ELISA kit (AKRIN-011; Shibayagi, Gunma, Japan) for the determination of immunoreactive insulin. The area positive for insulin staining was calculated with the use of NIH image software. Statistical analysis was preformed by Student *t* test for the comparison of insulin levels and blood glucose levels or by ANOVA for the insulin-positive area with Stat View software, version J-5, (Abacus Concepts, Berkeley, Calif).

RESULTS

Adenovirus-mediated PDX-1 expression was determined in vitro and in vivo. Replication-defective adenovirus carrying mouse PDX-1 gene was constructed as described in Material and Methods (Fig 1, *A*). The obtained recombinant adenovirus, Ad-pdx-1, was purified, and its protein expression was determined in COS 7 cells (Fig 1, *B*). PDX-1 protein was successfully determined by Western blot analysis; its expression was increased in an MOI-dependent manner.

To determine the expression of PDX-1 in vivo, we injected ICR mice with 2.0×10^9 PFU/body of recombinant adenoviruses; liver samples were collected at day 7. In Ad-lacZ–infected mice, more than 90% of the liver cells were positive for X-gal staining (data not shown). In Ad-pdx-1–infected mice, most of the hepatocytes were positive for PDX-1 expression (Fig 1, *C*).

Induction of pancreatic endocrine markers in the liver by adenoviral PDX-1 expression. To examine whether ectopic expression of PDX-1 induces pancreatic endocrine markers in the liver, we performed immunohistochemical analysis. Insulin-positive cells were not detected in the liver of Ad-lacZ–infected mice (Fig 2, *A*). In contrast, scattering insulin-positive cells were detected in the liver from Ad-pdx-1–infected mice (Fig 2, *B*). The insulin-positive cells formed a cluster, and the number of the cluster was small. In the serial sections, the insulin-positive cells were also positive for somatostatin staining (Fig 2, *D*). Furthermore, glucose transporter 2 (GLUT2) and glucokinase, which are the targets of PDX-1 and essential for insulin regulation, were examined in the serial sections (Fig 2, *F, H*). The expression of GLUT2 in Ad-pdx-1–infected mice was mainly in the cytoplasm, although normal liver cells and normal endocrine cells showed membranous expression pattern (Fig 2, *E, F*). Glucokinase was not visualized in the liver of Ad-lacZ–infected mice, but cytoplasmic expression of glucokinase was detected in the liver of Ad-pdx-1–infected mice, which was similar to that of normal endocrine cells.

To examine the function of ectopic endocrine cells in the liver, we measured the blood glucose and immunoreactive insulin (IRI) in serum and liver extract (Fig 3). Ad-pdx-1–infected mice tended to have a lower blood glucose level and higher IRI in serum and liver tissue extract when compared to Ad-lacZ–infected mice, although there was no significant difference.

Effects of PDX-1 on blood glucose and IRI of serum and liver tissue extract in STZ-treated mice. Mice treated with STZ at 200 mg/kg at day 0 showed gradual hyperglycemia. At day 1, recombinant adenoviruses were injected into the tail vein at 2.0×10^9 PFU per body. At day 15, mice were sacrificed and samples were collected to measure the IRI of serum and liver tissue extract (Fig 4, *A, B*). The IRI level of liver tissue extract was significantly different between Ad-pdx-1–infected

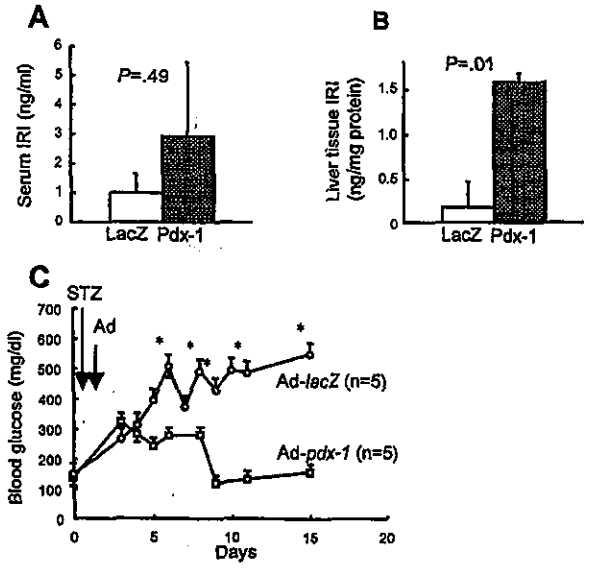

Fig 4. Effects of PDX-1 on blood glucose and IRI of serum and liver tissue extract in STZ-treated mice. Mice were treated with STZ at day 0 and received adenovirus injection at day 1. The tissue samples were collected at day 15. **A,** Serum IRI levels were not significantly different between Ad-lacZ–infected mice and Ad-pdx-1–infected mice. **B,** The IRI levels in liver tissue extract were significantly higher in Ad-pdx-1–infected mice than in Ad-lacZ–infected mice. **C,** STZ-induced hyperglysemia was reversed to within a normal range in Ad-pdx-1–infected mice. $*P < .05$ vs Ad-pdx-1 mice.

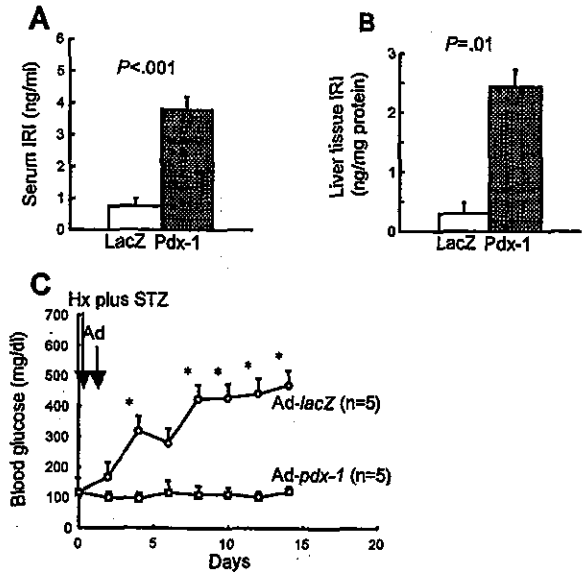

Fig 5. Effects of PDX-1 on blood glucose and IRI of serum and liver tissue extract in STZ-treated and hepatectomized mice. Mice were subjected to STZ treatment plus hepatectomy at day 0 and received adenovirus injection at day 1. The tissue samples were collected at day 14. Serum IRI levels **(A)** and liver tissue IRI levels **(B)** of Ad-pdx-1–infected mice were significantly higher than that of Ad-lacZ–infected mice. **C,** STZ-induced hyperglysemia was reversed to within a normal range in Ad-pdx-1–infected mice. $*P < .05$ vs Ad-pdx-1 mice.

mice and Ad-lacZ–infected mice. The serum IRI level of Ad-lacZ–treated mice was higher than Ad-pdx-1–treated mice, but the difference was not statistically significant. The STZ-induced hyperglycemia was reversed to within a normal range in Ad-pdx-1–treated mice until 14 days after Ad-infection (Fig 4, *C*).

Effects of PDX-1 on blood glucose and IRI of serum and liver tissue extract in STZ-treated and hepatectomized mice. To assess how hepatic regeneration affects the emergence of insulin-positive cells in the liver, a 40% partial hepatectomy was performed in ICR mice. The hepatectomized mice were simultaneously treated with STZ at 200 mg/kg. The next day, recombinant adenoviruses were administered at 2.0×10^9 PFU/body. Blood samples were obtained from the ocular vein, and the blood glucose level was measured every 2 days until day 14. Serum and liver tissues were collected at day 14, and IRI levels were measured by ELISA. The IRI levels of serum (Fig 5, *A*) and liver tissue extract (Fig 5, *B*) were significantly higher in Ad-pdx-1–infected mice than in Ad-lacZ–infected mice. Furthermore, STZ-induced hyperglycemia was reversed to within a normal range in Ad-pdx-1–infected mice (Fig 5, *C*).

Effects of PDX-1, STZ treatment, and hepatectomy on the emergence of insulin-positive cells. The insulin-positive area was not detected in the liver of Ad-lacZ–infected mice (Fig 6, *A*). As described, Ad-pdx-1 induced a small number of insulin-producing cells in nontreated mice (Fig 2, *B*). The insulin-positive area was clearly detected in Ad-pdx-1–infected mice treated with STZ (Fig 6, *B*). The insulin-positive area was more pronounced in the liver of Ad-pdx-1–infected mice treated with STZ plus hepatectomy (Fig 6, *C, D*).

The area of insulin-positive cells was quantitated by an image-analyzing software (Fig 6, *E*). By this method, an insulin-positive area was not detected in Ad-lacZ–infected mice that received any treatment. The Ad-pdx-1–infected mice treated with STZ plus hepatectomy showed a significantly wider area of insulin staining when compared to Ad-pdx-1–infected mice that did not receive STZ and hepatectomy and to Ad-pdx-1–infected mice treated with STZ.

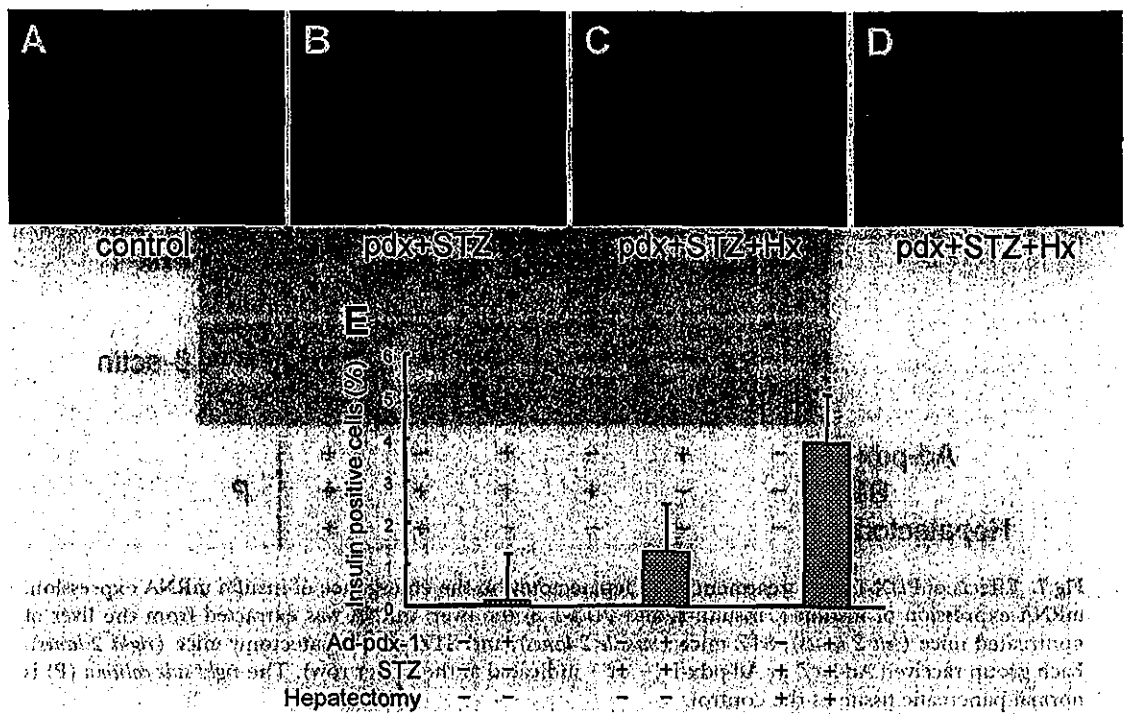

Fig 6. Effects of PDX-1, STZ treatment, and hepatectomy on the emergence of insulin-positive cells. **A,** Ad-lacZ–infected control mice; **B,** Ad-pdx-1–infected mice that received STZ treatment; **C and D,** Ad-pdx-1–infected mice that received STZ treatment plus hepatectomy (Hx). Original magnification ×200 **(A, B, C)** or ×400 **(D). E,** Estimated area of insulin-positive cells. $*P < .01$ vs Ad-pdx-1–infected mice and Ad-pdx-1–infected mice that received STZ treatment plus hepatectomy.

The mRNA expression of insulin-1 and insulin-2 were further analyzed by RT-PCR (Fig 7). Ad-pdx-1–infected mice who received STZ treatment plus hepatectomy showed both insulin-1 and insulin-2 mRNA. However, insulin mRNA was not detected in any other group of mice.

DISCUSSION

We have successfully introduced an ectopic expression of PDX-1 in the liver by adenovirus-mediated transfer of the PDX-1 gene. It is known that the adenovirus can infect a wide variety of replicating and nonreplicating cells,[26] although systemic injection of the adenovirus resulted in an efficient delivery of the target gene, especially to the liver. Enforced PDX-1 expression in the liver by recombinant adenovirus was successfully performed and verified by immunohistochemical detection of PDX-1 protein. Effects of overexpression of PDX-1 on non-endocrine cells have been investigated in vivo and in vitro. Grapin-Botton[27] induced overexpression of PDX-1 in the gut of a chick embryo by electroporation, but endocrine cytodifferentiation from the intestinal epithelia was not completed. Targeted expression of PDX-1 in exocrine cells caused no change of β-cell mass, whereas exocrine tissue showed marked dysmorphogenesis.[28] Adenovirus-mediated gene transfer of PDX-1 was first reported by Ferber et al.[5] The insulin-producing cell they found was a small proportion of the liver. They thought the origin of those insulin-producing cells were derived from hepatic stem cells, so-called oval cells. However, recently, Horb et al[6] used modified PDX-1, whose transcriptional activity was enforced by VP-16, and showed that the conversion of the liver to the pancreas occurred directly from mature hepatocytes in vitro and in vivo. However, it is not elucidated what kind of cell in the liver was responsible for the generation of insulin-producing cells in vivo. For mild hepatic damages like partial hepatectomy, mature hepatocytes are reported to be responsible for the regenerative process. From our data, we speculate that the increment of hepatic insulin-producing cells in STZ plus Hx group over STZ group could be attributed to transdifferentiation of mature hepatocytes.

In our experiment, administration of the recombinant adenoviruses to nontreated mice

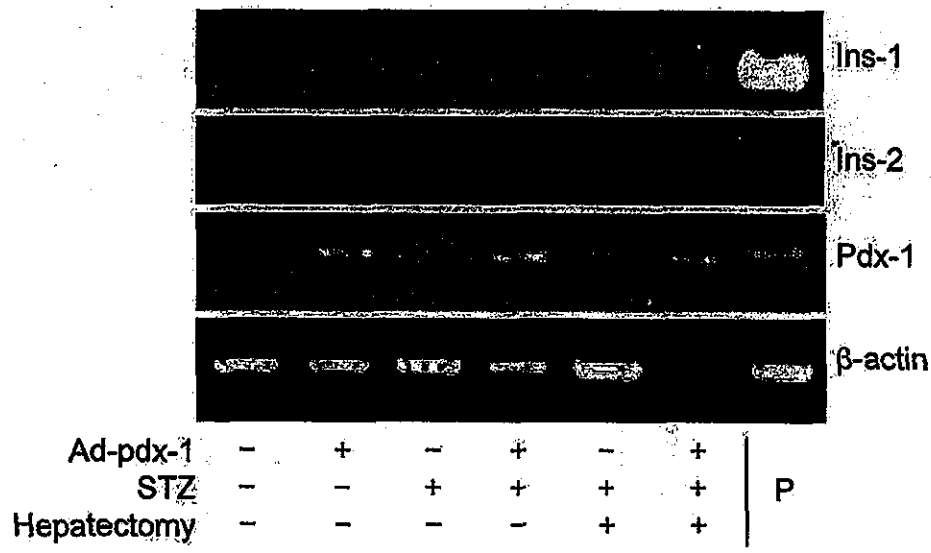

Fig 7. Effects of PDX-1, STZ treatment, and hepatectomy on the emergence of insulin mRNA expression. mRNA expression of insulin-1, insulin-2, and PDX-1 in the liver: mRNA was extracted from the liver of nontreated mice (*left 2 lanes*), STZ mice (*middle 2 lanes*), and STZ plus hepatectomy mice (*right 2 lanes*). Each group received Ad-lacZ or Ad-pdx-1 (− or + indicated at the upper row). The *right side column* (P) is normal pancreatic tissue as the control.

induced a small subpopulation of insulin-positive cells in the liver. The insulin-positive area was observed as a cluster-like structure of cells. Immunoreactive somatostatin was positive for the identical cells to the insulin-positive cells in the serial sections. On the other hand, glucagon and pancreatic polypeptide were not detected in those cells (data not shown). These results suggest that the cluster-like structure with insulin- and somatostatin-positive cells was not identical to the pancreatic islet that expresses in nature other endocrine hormones such as glucagon at the periphery. Previous studies using an adenovirus vector that carried the PDX-1 gene have reported that both endocrine and exocrine cells were introduced in the liver.[29,30] However, in our experiment, the expression of exocrine markers such as amylase was not detected by either immunohistochemical analysis or RT-PCR (data not shown).

Although we verified an ectopic expression of insulin by immunohistochemical analysis, it should be more important whether the ectopic insulin functions as a regulatory peptide. To clarify the function of insulin-producing cells in the liver, we examined the expression of GLUT2 and glucokinase. The expression of GLUT2 in the normal liver showed a membranous pattern that was altered to a cytoplasmic pattern by the Ad-pdx-1 infection. Similarly, the expression of glucokinase of the liver from Ad-pdx-1–infected mice showed a cytoplasmic pattern, which was not visualized in the liver from Ad-lacZ–infected mice. GLUT2 is important for glucose sensing by β cells and stands as a downstream target for PDX-1. Glucokinase is also one of the targets of PDX-1 and is essential for insulin regulation. Therefore, alteration of the expression pattern may be attributed to the introduction of enforced PDX-1 expression. In addition, the normalization of STZ-induced hyperglycemia by PDX-1 indicates that the ectopic insulin was biologically active in vivo and that the insulin-producing cells in the liver might have a glucose-sensing ability similar to that of pancreatic β cells.

In Ad-pdx-1–infected mice who did not receive STZ treatment or hepatectomy, almost all the liver cells showed PDX-1 expression. However, the number of insulin-positive cells was very small, indicating that Ad-pdx-1 infection alone was not sufficient to drive insulin production. Ad-pdx-1–infected mice who did receive STZ treatment showed an increase in insulin-positive cells. The difference in blood glucose levels between nontreated control mice and STZ mice was in accordance with the difference of the area of insulin-positive cells. Furthermore, STZ treatment plus hepatectomy further expanded the area of insulin-positive cells. Taken together, the results suggest that STZ treatment and hepatectomy would act as an additional but effective factor that stimulates insulin production. Though detailed molecular mechanism remains to be elucidated, it is conceivable that a systemic hyperglycemic

condition or regenerative condition of the liver induced by hepatectomy could promote the endocrine transdifferentiation of the liver cells by enforced PDX-1 expression.

CONCLUSION

Ectopic PDX-1 expression alone may be insufficient to induce insulin-producing cells in the liver. STZ-induced hyperglycemia and partial hepatectomy that leads to hepatic regeneration might stimulate the transdifferentiation of liver cells into insulin-producing cells.

REFERENCES

1. Atkinson MA, Maclaren NK. The pathogenesis of insulin-dependent diabetes mellitus. N Engl J Med 1994;331:1428-36.
2. Lumelsky N, Blondel O, Laeng P, Velasco I, Ravin R, McKay R. Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets. Science 2001;292: 1389-94.
3. Ramiya VK, Maraist M, Arfors KE, Schatz DA, Peck AB, Cornelius JG. Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells. Nat Med 2000;6:278-82.
4. Mashima H, Shibata H, Mine T, Kojima I. Formation of insulin-producing cells from pancreatic acinar AR42J cells by hepatocyte growth factor. Endocrinology 1996;137: 3969-76.
5. Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, et al. Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. Nat Med 2000;6: 568-72.
6. Horb ME, Shen CN, Tosh D, Slack JM. Experimental conversion of liver to pancreas. Curr Biol 2003;13:105-15.
7. Kojima H, Nakamura T, Fujita Y, Kishi A, Fujimiya M, Yamada S, et al. Combined expression of pancreatic duodenal homeobox 1 and islet factor 1 induces immature enterocytes to produce insulin. Diabetes 2002;51:1398-408.
8. Yoshida S, Kajimoto Y, Yasuda T, Watada H, Fujitani Y, Kosaka H, et al. PDX-1 induces differentiation of intestinal epithelioid IEC-6 into insulin-producing cells. Diabetes 2002;51:2505-13.
9. Ianus A, Holz GG, Theise ND, Hussain MA. In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J Clin Invest 2003;111:843-50.
10. Wells JM, Melton DA. Vertebrate endoderm development. Annu Rev Cell Dev Biol 1999;15:393-410.
11. Zaret KS. Hepatocyte differentiation: from the endoderm and beyond. Curr Opin Genet Dev 2001;11:568-74.
12. Scarpelli DG, Rao MS. Differentiation of regenerating pancreatic cells into hepatocyte-like cells. Proc Natl Acad Sci U S A 1981;78:2577-81.
13. Rao MS, Subbarao V, Reddy JK. Induction of hepatocytes in the pancreas of copper-depleted rats following copper repletion. Cell Differ 1986;18:109-17.
14. Krakowski ML, Kritzik MR, Jones EM, Krahl T, Lee J, Arnush M, et al. Pancreatic expression of keratinocyte growth factor leads to differentiation of islet hepatocytes and proliferation of duct cells. Am J Pathol 1999;154:683-91.
15. Shen CN, Slack JM, Tosh D. Molecular basis of transdifferentiation of pancreas to liver. Nat Cell Biol 2000;2: 879-87.
16. Rao MS, Bendayan M, Kimbrough RD, Reddy JK. Characterization of pancreatic-type tissue in the liver of rat induced by polychlorinated biphenyls. J Histochem Cytochem 1986;34:197-201.
17. Lee BC, Hendricks JD, Bailey GS. Metaplastic pancreatic cells in liver tumors induced by diethylnitrosamine. Exp Mol Pathol 1989;50:104-13.
18. Miyatsuka T, Kaneto H, Kajimoto Y, Hirota S, Arakawa Y, Fujitani Y, et al. Ectopically expressed PDX-1 in liver initiates endocrine and exocrine pancreas differentiation but causes dysmorphogenesis. Biochem Biophys Res Commun 2003;310:1017-25.
19. Jonsson J, Carlsson L, Edlund T, Edlund H. Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 1994;371:606-9.
20. McKinnon CM, Docherty K. Pancreatic duodenal homeobox-1, PDX-1, a major regulator of beta cell identity and function. Diabetologia 2001;44:1203-14.
21. Guz Y, Montminy MR, Stein R, Leonard J, Gamer LW, Wright CV, et al. Expression of murine STF-1, a putative insulin gene transcription factor, in beta cells of pancreas, duodenal epithelium and pancreatic exocrine and endocrine progenitors during ontogeny. Development 1995;121:11-8.
22. Ishii A, Hagiwara Y, Saito Y, Yamamoto K, Yuasa K, Sato Y, et al. Effective adenovirus-mediated gene expression in adult murine skeletal muscle. Muscle Nerve 1999;22:592-9.
23. Koizumi M, Doi R, Toyoda E, Masui T, Tulachan SS, Kawaguchi Y, et al. Increased PDX-1 expression is associated with outcome in patients with pancreatic cancer. Surgery 2003;134:260-6.
24. Saito I, Oya Y, Yamamoto K, Yuasa T, Shimojo H. Construction of nondefective adenovirus type 5 bearing a 2.8-kilobase hepatitis B virus DNA near the right end of its genome. J Virol 1985;54:711-9.
25. Miyake S, Makimura M, Kanegae Y, Harada S, Sato Y, Takamori K, et al. Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. Proc Natl Acad Sci U S A 1996;93:1320-4.
26. Kanegae Y, Lee G, Sato Y, Tanaka M, Nakai M, Sakaki T, et al. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. Nucleic Acids Res 1995;23:3816-21.
27. Grapin-Botton A, Majithia AR, Melton DA. Key events of pancreas formation are triggered in gut endoderm by ectopic expression of pancreatic regulatory genes. Genes Dev 2001;15:444-54.
28. Heller RS, Stoffers DA, Bock T, Svenstrup K, Jensen J, Horn T, et al. Improved glucose tolerance and acinar dysmorphogenesis by targeted expression of transcription factor PDX-1 to the exocrine pancreas. Diabetes 2001;50:1553-61.
29. Kojima H, Fujimiya M, Matsumura K, Younan P, Imaeda H, Maeda M, et al. NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. Nat Med 2003;9:596-603.
30. Ber I, Shternhall K, Perl S, Ohanuna Z, Goldberg I, Barshack I, et al. Functional, persistent, and extended liver to pancreas transdifferentiation. J Biol Chem 2003;278: 31950-7.

Chronic exposure of transforming growth factor beta 1 confers a more aggressive tumor phenotype through downregulation of $p21^{WAF1/CIP1}$ in conditionally immortalized pancreatic epithelial cells

Daisuke Ito, MD, Koji Fujimoto, MD, Ryuichiro Doi, MD, Masayuki Koizumi, MD, Eiji Toyoda, MD, Tomohiko Mori, MD, Kazuhiro Kami, MD, Yoshiya Kawaguchi, MD, Robert Whitehead, PhD, *and* **Masayuki Imamura, MD, FACS,** *Kyoto, Japan, and Nashville, Tenn*

Background. *Recent studies have demonstrated that transforming growth factor beta 1 (TGF-β1) expression is markedly enhanced in invasive ductal pancreatic adenocarcinomas, although the precise role of TGF-β1 in pancreatic carcinogenesis remains unclear. We analyzed TGF-β1 expression in pancreatic intraepithelial neoplasias (PanINs) and the effects of chronic TGF-β1 exposure on conditionally immortalized pancreatic epithelial (IMPE) cells.*

Methods. *Sixty-one PanIN lesions were immunohistochemically stained with a polyclonal rabbit antibody against human TGF-β1. Growth-inhibitory effects of short-term exposure to TGF-β1 were examined in IMPE cells. IMPE cells resistant to TGF-β1 (IMPE-Tr cells) were generated by continuous exposure to 1 ng/mL of TGF-β1 for more than 50 days. Phenotypic alterations of IMPE-Tr cells were examined by soft agar and Matrigel assay and Western blot analysis. IMPE and IMPE-Tr cells were injected subcutaneously into nude mice for an in vivo tumorigenicity assay.*

Results. *Forty-six percent of PanINs (28/61) were positive for TGF-β1 expression, whereas all the epithelia of normal pancreatic ducts were negative. TGF-β1 treatment showed the marked growth-inhibitory effects (>75%) in IMPE cells, whereas its effects were not observed in IMPE-Tr cells. IMPE-Tr cells were more spindle shaped compared with IMPE cells. In soft agar and Matrigel, formations of many colonies were observed in IMPE-Tr cells, but not in IMPE cells. Interestingly, the expression of $p21^{WAF1/CIP1}$ was induced by short-term exposure to TGF-β1 in IMPE cells, whereas the induction was decreased in IMPE-Tr cells. All of the IMPE-Tr cell-injected mice (5/5) had subcutaneous tumors, although no tumor was found in the IMPE cell-injected mice.*

Conclusions. *TGF-β1 expression in PanINs and neoplastic transformation of IMPE cells by long-term exposure to TGF-β1 suggest that TGF-β1 may act as a tumor promoter in the early stage of pancreatic carcinogenesis. (Surgery 2004;136:364-74.)*

From the Department of Surgery and Surgical Basic Science, Kyoto University, Kyoto, Japan; and the Departments of Medicine and Cell Biology, Vanderbilt University Medical Center, Nashville, Tenn

Presented at the 65th Annual Meeting of the Society of University Surgeons, St. Louis, Missouri, February 11-14, 2004.

Supported by a grant (no.14370386) to K.F. from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, and funded in part by NIH grant 5P30 DK58404-02 to the Vanderbilt Digestive Disease Research Center.

Reprint requests: Koji Fujimoto, MD, Department of Surgery and Surgical Basic Science, Kyoto University, 54 Shogoin-kawaracho, Sakyo, Kyoto 606-8507, Japan.

0039-6060/$ - see front matter

doi:10.1016/j.surg.2004.05.012

TRANSFORMING GROWTH FACTOR BETA 1 (TGF-β1) is a pleiotropic polypeptide growth factor that belongs to the TGF-β superfamily of structurally related peptides that regulate various physiologic functions, including cell growth, differentiation, plasticity and migration.[1] TGF-β was first reported as a stimulator of phenotypic transformation of rat kidney fibroblasts, but later it has been more frequently reported as an inhibitor of proliferation in a broad range of cell types including epithelial cells.[1] The role of TGF-β in tumorigenesis is somewhat paradoxical. Previous studies have