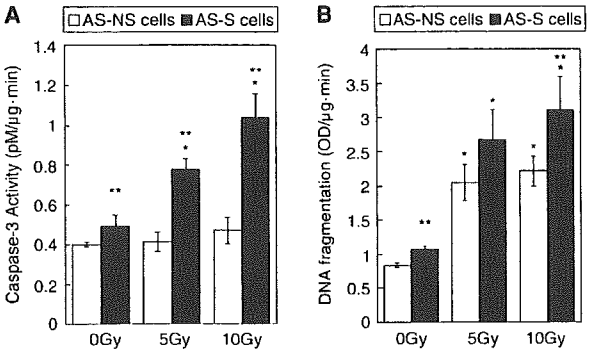

Fig 5. Effect of siRNA on radiation-induced caspase-3 activity and DNA fragmentation. **A,** Basal and radiation-induced caspase-3 activity in AS-S cells and AS-NS cells. Caspase-3 activity was expressed as picomoles of substrate cleaved per minute and per microgram of protein in nonirradiated cells (0 Gy) and at 48 hours after irradiation with 5 or 10 Gy. In AS-NS cells, caspase-3 activity was not increased by radiation, whereas a significant increase in caspase-3 activity was induced by radiation in AS-S cells. $*P < .05$ versus 0 Gy; $**P < .05$ vs AS-NS cells. **B,** Basal and radiation-induced DNA fragmentation in AS-S cells and AS-NS cells. Apoptotic DNA fragmentation was expressed as the absorbance per minute and per microgram of protein in nonirradiated cells (0 Gy) and at 48 hours after irradiation with 5 or 10 Gy. DNA fragmentation was increased by radiation in both AS-S and AS-NS cells, but DNA fragmentation was significantly greater in AS-S cells than in AS-NS cells at 10 Gy. $*P < .05$ vs 0 Gy; $**P < .05$ vs AS-NS cells. The experiment was performed in triplicate; data are the mean ± SD. *AS-NS,* AsPC-1 cells treated with nonsilencing scramble siRNA; *AS-S cells,* AsPC-1 cells treated with silencing survivin siRNA.

performed with survivin siRNA and radiation.[23] However, it is not known whether downregulation of survivin expression and decreased radioresistance can be achieved by using siRNA in the case of pancreatic cancer.

Previous studies evaluated survivin expression at only the mRNA and protein levels. In the current study, we examined the transcriptional activity of the survivin promoter as well as the level of mRNA expression. We also examined the relationship between survivin expression and the radioresistance of pancreatic cancer cells. Finally, we evaluated the effect of survivin siRNA on the radioresistance of pancreatic cancer cells.

We showed that transcriptional activity of the survivin promoter was associated with the level of expression of survivin mRNA transcripts in the cell lines, indicating that survivin gene expression by human pancreatic cancer cells may be regulated at the transcriptional level by the 397-bp sequence

Table II. Relative surviving mRNA expression, caspase-3 activity, and apoptotic index in AS-S cells*

	0 GY	*5 Gy*	*10 Gy*
Survivin mRNA expression	0.38 ± 0.05†	0.30 ± 0.06†	0.26 ± 0.03†‡
Caspase-3 activity	1.23 ± 0.13†	1.88 ± 0.12†‡	2.21 ± 0.24†‡
Apoptotic index	1.27 ± 0.06†	1.31 ± 0.21	1.40 ± 0.21†

mRNA, Messenger RNA; *AS-S cells,* AsPC-1 cells treated with silencing survivin siRNA.
*Survivin expression, caspase-3 activity, and apoptotic index in AS-S cells are expressed as relative values to those in AS-NS cells after various doses of radiation.
†$P < .05$ vs AS-NS cells.
‡$P < .05$ vs 0 Gy.

upstream of the translation initiation site. The level of survivin expression was associated with the degree of resistance to radiation. Both survivin promoter activity and mRNA expression were increased by irradiation, and the increment of transcriptional activity was associated strongly with the extent of resistance to irradiation. These results suggest that both the basal expression and radiation-induced increment in transcriptional activity of the survivin promoter and survivin mRNA are important determinants of the radioresistance of pancreatic cancer.

We selected AsPC-1 as a radiation-resistant cell line and further examined the mechanisms involved. AsPC-1 cells responded to irradiation with an increase in caspase-3 activity and DNA fragmentation (indicators of apoptosis), suggesting that the radiation-evoked death signal pathway is intact in this cell line. However, the cells were extremely resistant to radiation, probably because of high expression of survivin. When we used survivin siRNA to target AsPC-1 cells, treated cells showed a significant decline of radioresistance, an increase in caspase-3 activity, and an increased apoptotic index after exposure to 10 Gy, compared with AsPC-1 cells targeted by scramble siRNA. These results suggest that suppression of survivin expression by siRNA may activate signaling via caspase-3 and eventually influence tumor cell apoptosis after exposure to radiation. Our results also suggest that regulation of survivin expression at the transcriptional level by the survivin promoter may be involved directly in the apoptotic response of human pancreatic cancer cell lines after exposure to radiation.

We have previously reported that 68% of human pancreatic cancers are survivin-positive and that normal pancreatic exocrine tissues are survivin-negative.[6] We also have reported that patients with

survivin-positive pancreatic cancer show a significantly shorter survival time than those with survivin-negative cancer.[6] Therefore, inhibition of survivin mRNA by survivin-specific siRNA may be a reasonable approach when combined with radiation therapy for unresectable or recurrent pancreatic cancer. However, more-efficient introduction of siRNA into target cells will be needed because low efficiency of transduction is one of the weaknesses of this type of gene therapy.

CONCLUSION

Survivin may play an important role as a radioresistance factor. Downregulation of survivin by siRNA diminishes the radioresistance of pancreatic cancer cells. Radiation therapy combined with inhibition of survivin may be useful for the treatment of pancreatic cancer.

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. Ambrosini G, Adida C, Altieri DC. A novel anti-apoptosis gene, survivin, expressed in cancer and lymphoma. Nat Med 1997;3:917-21.
3. Adida C, Berrebi D, Peuchmaur M, Reyes-Mugica M, Altieri DC. Anti-apoptosis gene, survivin, and prognosis of neuroblastoma. Lancet 1998;351:882-3.
4. 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.
5. 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.
6. Kami K, Doi R, Koizumi M, et al. Survivin expression is a prognostic marker in pancreatic cancer patients. Surgery 2004;136:443-8.
7. 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.
8. Pennati M, Binda M, Colella G, et al. Radiosensitization of human melanoma cells by ribozyme-mediated inhibition of survivin expression. J Invest Dermatol 2003;120: 648-54.
9. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 1998;391: 806-11.
10. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001;411: 494-8.
11. Bertrand JR, Pottier M, Vekris A, Opolon P, Maksimenko A, Malvy C. Comparison of antisense oligonucleotides and siRNAs in cell culture and in vivo. Biochem Biophys Res Commun 2002;296:1000-4.
12. Aoki Y, Cioca DP, Oidaira H, Kamiya J, Kiyosawa K. RNA interference may be more potent than antisense RNA in human cancer cell lines. Clin Exp Pharmacol Physiol 2003; 30:96-102.
13. Carvalho A, Carmena M, Sambade C, Earnshaw WC, Wheatley SP. Survivin is required for stable checkpoint activation in taxol-treated HeLa cells. J Cell Sci 2003;116: 2987-98.
14. Gelmini S, Orlando C, Sestini R, et al. Quantitative polymerase chain reaction-based homogeneous assay with fluorogenic probes to measure c-erbB-2 oncogene amplification. Clin Chem 1997;43:752-8.
15. Masui T, Hosotani R, Tsuji S, et al. Expression of METH-1 and METH-2 in pancreatic cancer. Clin Cancer Res 2001; 7:3437-43.
16. Tajiri T, Tanaka S, Shono K, et al. Quick quantitative analysis of gene dosages associated with prognosis in neuroblastoma. Cancer Lett 2001;166:89-94.
17. Asanuma K, Moriai R, Yajima T, et al. Survivin as a radioresistance factor in pancreatic cancer. Jpn J Cancer Res 2000;91:1204-9.
18. Coma S, Noe V, Lavarino C, et al. Use of siRNAs and antisense oligonucleotides against survivin RNA to inhibit steps leading to tumor angiogenesis. Oligonucleotides 2004;14:100-13.
19. Tsuruma T, Hata F, Torigoe T, et al. Phase I clinical study of anti-apoptosis protein, survivin-derived peptide vaccine therapy for patients with advanced or recurrent colorectal cancer. J Transl Med 2004;2:19.
20. Ling X, Li F. Silencing of antiapoptotic survivin gene by multiple approaches of RNA interference technology. Biotechniques 2004;36:450-4, 6-60.
21. Ning S, Fuessel S, Kotzsch M, et al. siRNA-mediated downregulation of survivin inhibits bladder cancer cell growth. Int J Oncol 2004;25:1065-71.
22. Uchida H, Tanaka T, Sasaki K, et al. Adenovirus-mediated transfer of siRNA against survivin induced apoptosis and attenuated tumor cell growth in vitro and in vivo. Mol Ther 2004;10:162-71.
23. Kappler M, Taubert H, Bartel F, et al. Radiosensitization, after a combined treatment of survivin siRNA and irradiation, is correlated with the activation of caspases 3 and 7 in a wt-p53 sarcoma cell line, but not in a mt-p53 sarcoma cell line. Oncol Rep 2005;13:167-72.

Pancreatic epithelial cells can be converted into insulin-producing cells by GLP-1 in conjunction with virus-mediated gene transfer of *pdx-1*

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

Background. *Glucagonlike peptide-1 (GLP-1) stimulates insulin secretion and proliferation by islet cells in vitro and in vivo, associated with an activation of pancreatic duodenal homeobox gene-1* (pdx-1) *function. The effect of GLP-1 on the conditionally immortalized pancreatic epithelial cells (IMPE cells) is not clear when they are treated in conjunction with the adenovirus-mediated gene transfer of* pdx-1.
Methods. *IMPE cells were established from the pancreas of H-2K^b-tsA58 transgenic mice. IMPE cells were maintained at 33°C with 10 U/mL interferon (IFN)-γ and the experiments were performed at 39°C without IFN-γ. IMPE cells were infected with 20 multiplicities of Ad-*pdx-1 *or control Ad-*lacZ *at 39°C without IFN-γ and were incubated with various concentrations of GLP-1. After 48 hours, immunofluorescence and reverse transcriptase-polymerase chain reaction for insulin and* pdx-1 *expression were examined. Immunoreactive insulin in the cell lysate and supernatant was also analyzed. The glucose concentration in the culture medium was changed to test the insulin secretory responsiveness of the IMPE cells.*
Results. *The treatment with GLP-1 in conjunction with Ad-*pdx-1 *induced insulin production by IMPE cells, but the treatment with either GLP-1 or Ad-*pdx-1 *alone failed to induce insulin production. Insulin production and secretion were increased by GLP-1 and by glucose in a dose-dependent manner. In addition, the insulin-producing IMPE cells acquired a rapid insulin secretory responsiveness to the changes of extracellular glucose concentration.*
Conclusions. *GLP-1 and* pdx-1 *work together to induce insulin-producing cells from IMPE cells, which bear unique characteristics of pancreatic ductal cells. The results suggest that GLP-1 may be another important determiner of pancreatic endocrine differentiation as is* pdx-1. *(Surgery 2005;138:125-33.)*

From the Department of Surgery and Surgical Basic Science,[a] Kyoto University, Kyoto, and the Department of Surgical Research,[b] Children's Mercy Hospital, Kansas City

MANY EFFORTS have been made to establish pancreatic epithelial cell lines that can be used to investigate carcinogenesis of the pancreas and endocrine differentiation of the pancreas. It has been difficult to maintain pancreatic epithelial cells in a long-term in vitro culture condition by either direct isolation or by immortalization with viral oncogenes. Little success has been achieved so far; however, in the early 1990s, the development of a transgenic mouse harboring a temperature-sensitive mutant of simian virus 40 large tumor antigen (tsA58) under the control of interferon (IFN)-γ–inducible promoter (*H-2K^b*) has allowed us to culture conditionally immortalized cell lines including intestine,[1] liver,[2] and vascular endothelial cells.[3,4] These cell lines can proliferate indefinitely under permissive conditions (33°C with IFN-γ) but cease proliferation under nonpermissive

Presented at the 66th Annual Meeting of the Society of University Surgeons, Nashville, Tennessee, February 9-12, 2005.
Supported by Grants-in-Aid (#17390364 and #07659409) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.
Reprint requests: Ryuichiro Doi, MD, Department of Surgery and Surgical Basic Science, Kyoto University, 54 Shogoinkawara-cho, Sakyo, Kyoto 606-8507, Japan. E-mail: doir@kuhp.kyoto-u.ac.jp.
0039-6060/$ - see front matter

doi:10.1016/j.surg.2005.06.008

conditions (39°C without IFN-γ). In other words, the cells under nonpermissive conditions seem to behave like normal cells, and the cells under permissive conditions can passage indefinitely.

We previously established the character of the conditionally immortalized pancreatic epithelial cell lines from this transgenic mouse, which were designated as IMPE cells (IMortalized Pancreatic Epithelial cells).[5] IMPE cells showed epithelial markers such as E-cadherin and β-catenin, and a specific duct marker cytokeratin-19, indicating that IMPE cells are derived from pancreatic duct cells. Adenovirus-mediated gene transfer of an oncogenic ras gene into IMPE cells showed anchorage-independent growth in soft agar. In addition, long-term exposure of tumor growth factor β1 to IMPE cells conferred a tumor phenotype thorough p21downregulation.[6] These results suggest that IMPE cells are a useful model of pancreatic duct cells to study the process involved in pancreatic carcinogenesis and development.

For regenerative medicine, restoring insulin-producing β cells to treat diabetes mellitus is a major challenge. Diabetes mellitus is a metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion and insulin function. 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. Recently, it has been demonstrated that neogenesis of β cells in the islets is derived from the replication of existing β cells by using conditional lineage tracing study.[7] However, as an approach to restoring the decrease of insulin production in diabetes mellitus, replacement of β cells have been generated from embryonic stem cells[8] and somatic non–β cells such as hepatocytes,[9] intestinal epithelial cells,[10] and bone marrow.[11] In pancreas development, pancreatic duodenal homeobox gene-1 (*pdx-1*) is an indispensable gene (ie, homozygous disruption of *pdx-1* results in pancreatic agenesis).[12] *Pdx-1* regulates a number of factors involved in maintaining β cell identity and function, including insulin, glucose transporter gene-2, glucokinase, and islet amyloid polypeptide.

Glucagonlike peptide 1 (GLP-1) is a peptide hormone that has both incretin and insulinotrophic actions.[13] GLP-1 is a proglucagon-derived peptide hormone that is synthesized and secreted from the enteroendocrine L cells in the distal ileum and the colon. Oral meal intake stimulates L cells to synthesize proglucagon; then, proglucagon is processed to produce glicentin or oxyntomodulin and GLP-1. GLP-1 is then released from L cells and stimulates β cell of the pancreas to release and produce insulin, downregulates glucagon in α cells, and reduces gastric motility. Recent studies have demonstrated that GLP-1 increased insulin secretion in vitro[14] and in vivo,[15] and is associated with the nuclear translocation and production of *pdx-1*, and proliferation of islet cells.[16,17]

In the previous study, we demonstrated that IMPE cells have the characteristics of pancreatic duct cells and lack endocrine markers and *pdx-1* expression. In the current study, we investigated the effects of GLP-1 in conjunction with the adenovirus-mediated gene transfer of *pdx-1* in IMPE cells (ie, whether or not IMPE cells could be altered to produce insulin and to function like insulin-producing cells).

MATERIAL AND METHODS

Cell lines. IMPE cell lines were generated from the pancreas of *H-2K*^{b}-tsA58 transgenic mouse as reported previously.[5] The cells were maintained in RPMI medium supplemented with 5% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin either at 33°C with 10 U/mL IFN-γ (for maintenance) or 39°C without IFN-γ (for experiments) in a humid atmosphere of 5% CO_2/95% air; the medium was replaced as needed.

Recombinant adenovirus vectors. We used a replication-deficient recombinant adenovirus in which the E1A, E1B, and E3 regions of human adenovirus type 5 (Ad5) serotype were deleted. Virus vectors were prepared as reported previously.[18] Briefly, the recombinant replication-defective adenoviruses were constructed by the cosmid-adenoviral DNA terminal protein complex (COS-TPC) method. The mouse *pdx-1* complementary DNA (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 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 cotransfected into HEK293 cells to produce recombinant adenovirus through homologous recombination. 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

Table I. The characteristics of pancreatic epithelial cell lines derived from the pancreas of *H-2Kb*-tsA58 transgenic mouse

		Exocrine marker				*Endocrine marker*				*Epithelial marker*	
		Amylase	*CK8*	*CK18*	*CK19*	*Insulin*	*Glugagon*	*Somatostatin*	*Pdx-1*	*E-Cadherin*	*β-Catenin*
33°C w/ IFN-γ	RT-PCR	+	+	+	+	−	nt	nt	−	nt	nt
	IF	+	+	+	nt	−	−	−	−	+	+
39°C w/o IFN-γ	RT-PCR	−	+	+	+	−	nt	nt	−	nt	nt
	IF	−	+	+	nt	−	−	−	−	+	+

IFN-γ, Interferon γ; *RT-PCR*, reverse transcriptase-polymerase chain reaction; *nt*, not tested; *IF*, immunocytofluorescence.

−70°C. The titer of each viral stock, expressed as plaque-forming units (PFU) per milliliter, was determined by a plaque assay with HEK293 cells. All of the vector preparations were demonstrated to be free of replication-competent adenoviruses.

Infection of adenoviruses, treatment of GLP-1, and measurement of insulin. Experiments for IMPE cells were performed under the condition of 39°C without IFN-γ. IMPE cells grown to 100% confluence were infected with Ad-*lacZ* at various multiplicities of infection (MOI), and the efficiency of the gene expression was tested by X-gal staining at 48 hours. Induction of *pdx-1* protein by Ad-*pdx-1* was evaluated by immunofluorescence of *pdx-1*.

The following experiments were performed in IMPE cells grown to 80% confluence at 20 MOI of recombinant adenoviruses. After 24 hours of adenoviral infection, the cell layer with serum-free medium was washed, replaced with serum-free medium, and treated with GLP-1 (7-36 amide) (Sigma-Aldrich, St. Louis, Mo) at various concentrations (10, 20, 50, 100, and 200 nmol/L) for 48 hours in the presence of fixed concentration of glucose (12 mmol/L).

Next, glucose-dependent insulin secretion was evaluated at various concentrations of GLP-1 (0, 20, and 100 nmol/L). Immunoreactive insulin (IRI) levels released into the medium and in the cell lysate were measured by enzyme-linked immunosorbent assay (ELISA) kit (AKRIN-011; Shibayagi, Gunma, Japan) according to the manufacturer's instructions. Total insulin accumulation in the culture medium was then normalized by total cellular protein content in each individual well. To determine total cellular content of insulin, we collected the cells into microtubes with a cell scraper and lysed them 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*g* for 10 minutes at 4°C. The supernatants were collected for insulin ELISA. Protein concentration was determined by using BCA Protein Assay Reagents (Pierce, Rockford, Ill).

Immunocytochemical analysis. Cells grown on sterile glass coverslips were fixed with 4% paraformaldehyde dissolved in phosphate-buffered saline (PBS) for 10 minutes at room temperature and permealized in PBS with 2% Triton X-100 for 5 minutes. Primary antibodies were diluted in PBS containing 0.2% bovine serum albumin and incubated for 1 hour at room temperature. Primary antibodies to the following antigens were used at the indicated dilutions: insulin (guinea pig antiswine; DAKO, Carpinteria, Calif) 1:800; *pdx-1* (rabbit antibody against mouse *pdx-1*, a gift from Dr C V Wright, Vanderbilt University, Nashville, Tenn) 1:1000. Blocking was performed in PBS containing 2% of normal goat serum for 30 minutes at room temperature. Then the cells were washed 3 times with PBS with 2% Triton X-100 and incubated for 1 hour at room temperature with the secondary antibody, Cy-3–conjugated goat antirabbit IgG antibody (Jackson Immuno Research Laboratories, West Grove, Pa), mounted in Vectashield (Vector Laboratories, Burlingame, Calif), and viewed under an fluorescence microscope (Axioskop 2 plus; Carl Zeiss Co, Jena, Germany).

RNA extraction and reverse-transcriptase-polymerase chain reaction. Total cellular RNA was prepared from IMPE cells with the use of TRIZOL Reagent (Life Technologies Inc, Rockville, Md) and cDNA 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 polymerase chain reaction (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

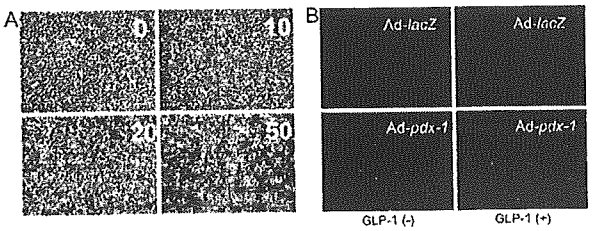

Fig 1. The efficiency of adenovirus-mediated gene transfer was determined. **A,** X-gal staining in IMPE cells. X-gal staining in IMPE cells with Ad-*lacZ* was dose-dependent. At 20 MOI, more than 80% of the cells were positive for X-gal staining. (Original magnification: ×40.) **B,** Expression of *pdx-1* protein in IMPE cells. *Pdx-1* protein expression was not detected in IMPE cells with Ad-*lacZ*. *Pdx-1* protein expression was detected in IMPE cells with Ad-*pdx-1* irrespective of GLP-1 treatment. (Original magnification: ×100.) *GLP-1,* Glugagonlike peptide-1. (Color figures available in online version of the article.)

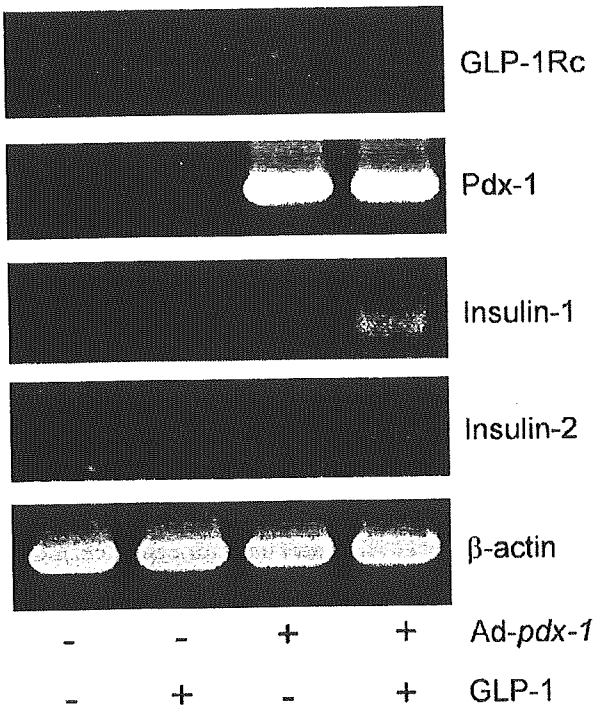

Fig 2. Expression of mRNAs of GLP-1 receptor, *pdx-1,* insulin-1 and insulin-2 in IMPE cells. IMPE cells were infected with Ad-*lacZ* (*left 2 lanes*) or Ad-*pdx-1* (*right 2 lanes*) at 20 MOI and treated with or without GLP-1 at 20 nmol/L. The GLP-1 receptor gene was constitutively transcribed. *Pdx-1* mRNA was observed only in IMPE cells that were infected with Ad-*pdx-1*. Insulin mRNA was not detected in IMPE cells either with Ad-*pdx-1* or with GLP-1. Concomitant treatment of GLP-1 and Ad-*pdx-1*successfully induced the expression of mRNA for both insulin-1 and insulin-2. *GLP-1,* Glugagonlike peptide-1.

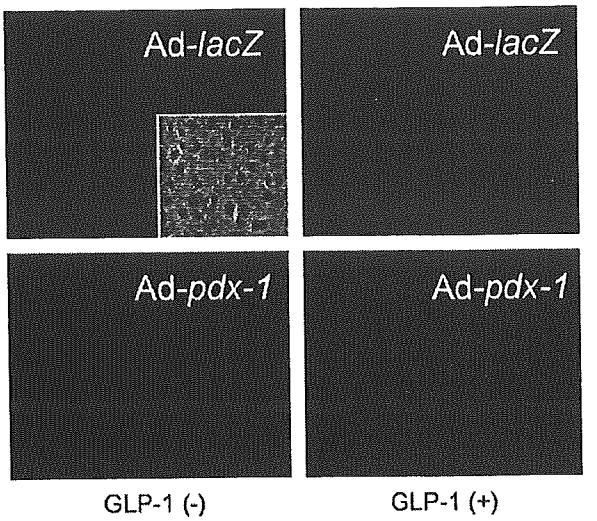

Fig 3. Immunocytochemistry for insulin in IMPE cells. IMPE cells were infected with Ad-*lacZ* (*upper 2 panels*) or Ad-*pdx-1* (*lower 2 panels*) at 20 MOI and treated with or without GLP-1 at 20 nmol/L. Fluorescent immunocytochemistry shows IMPE cells treated with Ad-*pdx*-1 and GLP-1 were positive for insulin (*right lower panel*). The inset in *left upper panel* is a phase-contrast image of IMPE cells for these experiments. (Original magnification ×100.) *GLP-1,* Glugagonlike peptide-1. (Color figures available in online version of the article.)

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. 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. The primer sequences used are listed as forward then reverse 5′ to 3′. β-Actin primers 5′-GGCATCGTGATGGACTCCG-3′ and 5′-GCTGGAAGGTGGACAGCG-3′ amplify a product of 612 bp, *pdx-1* primers 5′-CCTTGATATCGCTGCCACCATGAACAG-3′ and 5′-CTGCGCTCGAGTGTAGGCAGTACG-3′ amplify a product of 484 bp, insulin-1 primers 5′-TAGTGACCAGCTATAATCAGAG-3′ and 5′-ACGCCAAGGTCTGAAGGTCC-3′ amplify a product of 407 bp, insulin-2 primers 5′-CCCTGCTGGCCCTGCTCTT-3′ and 5′-AGGTCTGAAGGTCACCTGCT-3′ amplify a product of 213 bp, GLP-1 receptor primers 5′-CCTGAACCTGTTTGCATCCTTC-3′ and 5′-CTGGAAGGAAGTGAAGGAG-3′ amplify a product of 643 bp.

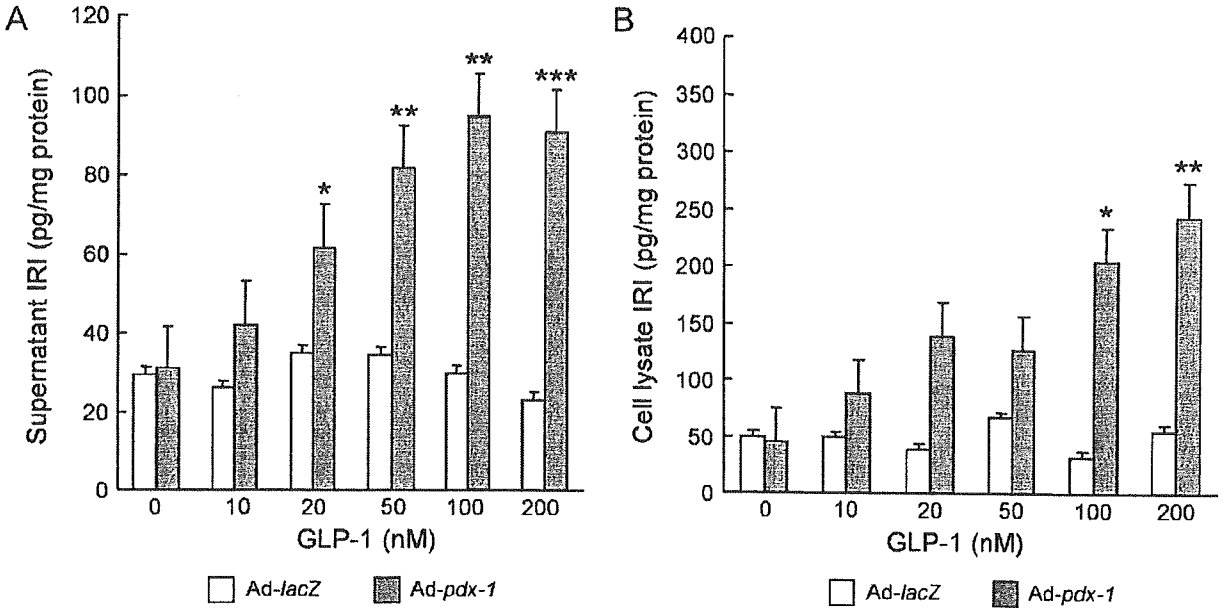

Fig 4. Effects of GLP-1 on insulin secretion and production by IMPE cells. IMPE cells were infected with Ad-*pdx-1* or Ad-*lacZ* at 20 MOI and treated with various concentrations of GLP-1 in serum-free medium at 39°C without IFN-γ. After the incubation of GLP-1 for 48 hours, the supernatant (**A**) and cell lysate (**B**) were collected separately, and the immunoreactive insulin was determined. Both the supernatant (**A**) and cell lysate (**B**) of IMPE cells with Ad-*pdx-1* showed a dose-dependent increase in insulin concentration by GLP-1, whereas those of IMPE cells with Ad-*lacZ* did not show any increase in insulin concentration. Each experiment was repeated at least 3 times; the data are expressed as the means ± SE. Statistical analysis was evaluated by analysis of variance. $*P < .05$; $**P < .001$; $***P < .0001$. *IRI,* Immunoreactive insulin; *GLP-1,* glugagonlike peptide-1.

Statistical analysis. The data are expressed as means ± SE. Statistical analysis was preformed by analysis of variance with Stat View software (version J-5; Abacus Concepts, Berkeley, Calif).

RESULTS

Adenovirus-mediated gene transfer of *pdx-1* in IMPE cells. The characteristics of the IMPE cells are summarized in Table I. Briefly, IMPE cells under nonpermissive condition (39°C without IFN-γ) demonstrate the character of pancreatic duct cells, whereas IMPE cells under permissive condition (33°C with IFN-γ) tend to demonstrate the character of pancreatic exocrine cells. In either condition, endocrine markers such as glucagon, insulin, and somatostatin were negative, and, more importantly, *pdx-1* is negative in either condition.

The ability of Ad-*lacZ* (control recombinant adenovirus) to infect cells and cause gene expression was analyzed at various MOIs (Fig 1, *A*). X-gal staining revealed that the gene induction of Ad-*lacZ* in IMPE cells occurred in a dose-dependent manner. At 20 MOI, more than 80% of the cells were positive for X-gal staining.

The effect of GLP-1 treatment on the induction of *pdx-1* protein in IMPE cells by adenovirus-mediated *pdx-1* gene transfer was tested by immunocytochemistry (Fig 1, *B*). IMPE cells were infected with Ad-*pdx-1* or Ad-*lacZ* at 20 MOI for 24 hours, and the culture medium was replaced with serum-free medium with or without 20 nmol/L GLP-1. After incubation for an additional 48 hours, IMPE cells were subjected to immunocytochemistry for *pdx-1* protein. Enforced expression of *pdx-1* protein was successfully observed irrespective of GLP-1 treatment.

Induction of pancreatic endocrine markers by GLP-1 in conjunction with adenovirus-mediated *pdx-1* gene transfer. IMPE cells were infected with recombinant adenoviruses at 20 MOI and treated with or without 20 nmol/L GLP-1, and subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. The expressions of mRNAs of GLP-1 receptor, z, insulin-1, insulin-2, and β-actin were evaluated (Fig 2).

The GLP-1 receptor gene was transcribed constitutively. *Pdx-1* mRNA was observed only in IMPE cells which were infected with Ad-*pdx-1*. Insulin mRNA was not detected in IMPE cells either with

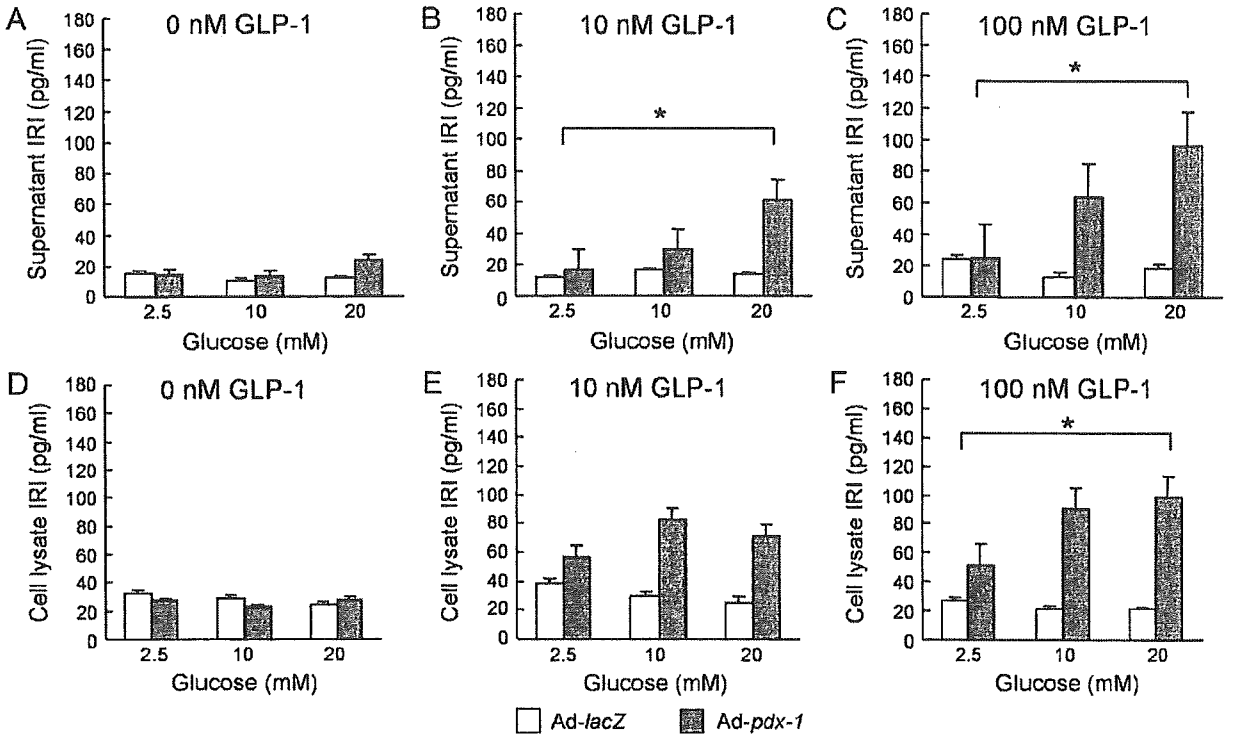

Fig 5. Effects of glucose on insulin secretion and production by IMPE cells. IMPE cells infected with Ad-*pdx-1* or Ad-*lacZ* (20 MOI) were treated with various concentrations of glucose (2.5, 10, and 20 mmol/L) and GLP-1 (0, 10, and 100 nmol/L) in serum-free medium at 39°C without IFN-γ. Without GLP-1 in the culture medium, insulin was not produced and released at any concentration of glucose (**A** and **D**). When IMPE cells were incubated with 10 nmol/L of GLP-1, IMPE cells with Ad-*pdx-1* showed a significant increase of insulin in the supernatant by glucose in a dose-dependent manner (**B** and **E**). When IMPE cells were incubated in 100 nmol/L of GLP-1, IMPE cells with Ad-*pdx-1* showed a significant increase in insulin in both the supernatant and the cell lysate in a dose-dependent manner (**C** and **F**). Each experiment was repeated at least 3 times; the data are expressed as the means ± SE. Statistical analysis was evaluated by analysis of variance. $*P < .05$. *IRI,* Immunoreactive insulin; *GLP-1,* glugagonlike peptide-1.

Ad-*pdx-1* or with GLP-1. Concomitant treatment of GLP-1 and Ad-*pdx-1* successfully induced the expression of mRNA for both insulin-1 and insulin-2 in IMPE cells (Fig 2).

Immunocytochemistry for insulin in IMPE cells was compatible to the results of mRNA detection. IMPE cells infected with Ad-*lacZ* were negative for insulin by immunocytofluorescence irrespective of GLP-1 treatment. IMPE cell infected with Ad-*pdx-1* and without GLP-1 also showed negative expression of insulin. Finally, IMPE cells treated with Ad-*pdx*-1 and GLP-1 were positive for insulin (Fig 3).

Dose-dependent insulin production by GLP-1 and glucose. To analyze GLP-1–dependent secretion and production of insulin, IMPE cells were infected with Ad-*pdx-1* or Ad-*lacZ* at 20 MOI and treated with various concentrations of GLP-1 in serum-free medium at 39°C without IFN-γ. After the incubation of GLP-1 for 48 hours, the cells and supernatants were collected separately to determine the concentration of immunoreactive insulin (IRI; Fig 4). Both the cell lysate and supernatant of IMPE cells with Ad-*pdx-1* showed a dose-dependent increase of insulin by GLP-1, whereas those of IMPE cells with Ad-*lacZ* did not show any increase in insulin concentration.

To analyze glucose-dependent secretion and production of insulin, IMPE cells were incubated with various concentrations of glucose. IMPE cells infected with Ad-*pdx-1* or Ad-*lacZ* (20 MOI) were treated with various concentrations of glucose (2.5, 10, and 20 mmol/L) and GLP-1 (0, 10, and 100 nmol/L) in serum-free medium at 39°C without IFN-γ. After 48-hour incubation, the cell lysate and supernatant were collected separately for insulin ELISA (Fig 5). Without GLP-1 in the culture medium, insulin was not produced and released at any concentration of glucose. When IMPE cells were incubated with 10 nmol/L of GLP-1, IMPE cells with Ad-*pdx-1* showed a significant

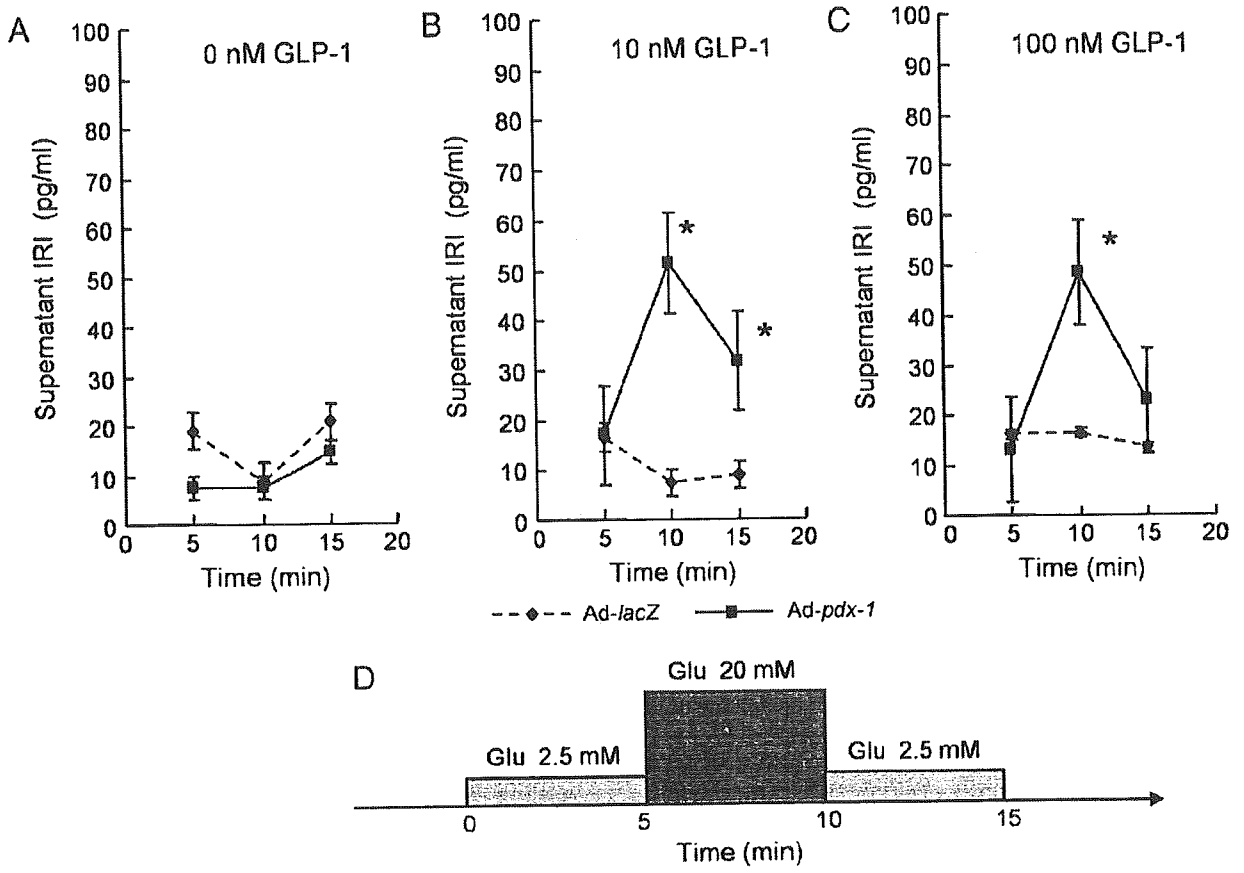

Fig 6. Responsiveness of IMPE cells to the change of extracellular glucose concentration. **D,** The cells treated with GLP-1 and Ad-*pdx-1* were incubated at 5-minute intervals in medium at a low concentration of glucose (2.5 mmol/L), at a high concentration of glucose (20 mmol/L), and then again at a low concentration of glucose (2.5 mmol/L). **A,** IMPE cells treated without GLP-1 were not stimulated by a high-glucose medium even when they were infected with Ad-*pdx-1*. **B** and **C,** IMPE cells treated with GLP-1 (10 and 100 nmol/L) and Ad-*pdx-1* showed a 4.7- and 3.0-fold increase in insulin secretion, respectively, as indicated by high concentrations of glucose. Each experiment was repeated at least 3 times; the data are expressed as the means ± SE. Statistical analysis was evaluated by analysis of variance. $*P < .05$. *IRI,* Immunoreactive insulin; *GLP-1,* glugagonlike peptide-1.

increase of insulin in supernatant by glucose in a dose-dependent manner. When IMPE cells were incubated in 100 nmol/L of GLP-1, IMPE cells with Ad-*pdx-1* showed a significant dose-dependent increase in insulin in the cell lysate and supernatant.

Responsiveness of IMPE cells to the change of extracellular glucose concentration. To investigate whether a change in extracellular glucose concentration affects insulin secretion by IMPE cells, the cells treated with GLP-1 and Ad-*pdx-1* were incubated for 5-minute intervals in medium at a low concentration of glucose (2.5 mmol/L), at a high concentration of glucose (20 mmol/L), and then at a low concentration of glucose (2.5 mmol) again. Media were collected every 5 minutes, and insulin was measured by ELISA (Fig 6). IMPE cells treated without GLP-1 were not stimulated by the high-glucose medium even when they were infected with Ad-*pdx-1*. IMPE cells treated with GLP-1 (10 and 100 nmol/L) and Ad-*pdx-1* showed a 4.7- and 3.0-fold increase in insulin secretion, respectively, as indicated by high concentrations of glucose.

DISCUSSION

In the present study, we investigated whether IMPE cells from a ductal cell origin could be differentiated into insulin-producing endocrine cells by undergoing treatment with GLP-1 and enforced *pdx-1* expression. We successfully obtained insulin-producing cells by concomitant

treatment with GLP-1 and *pdx-1*. The effects of GLP-1 on pancreatic differentiation have been reported in several pancreatic cell lines such as AR42J,[19] ARIP,[20] Panc-1,[20] and Capan-1.[21] These cell lines were treated with GLP-1 or exndin-4, a GLP-1 receptor agonist; the, insulin production by these cells was reported. From these studies, *pdx-1* seemed to be indispensable for endocrine differentiation and upregulated by GLP-1. In contrast to these established cell lines, IMPE cells are not transformed cells. Soft agar assay showed an absence of anchorage-independent growth under permissive and nonpermissive conditions. In an in vivo transplantation assay, IMPE cells showed lack of tumor formation in nude mice.[6] Although IMPE cells can be cultured under permissive condition (33°C with IFN-γ), all experiments in the current study, including the treatment of adenoviruses and GLP-1, were performed under nonpermissive condition (39°C without IFN-γ), which is the normal body temperature of mice. Therefore, this is the first study to report that nontransformed ductal cells can be altered to insulin-producing cells.

We showed that, in IMPE cells, mRNA of GLP-1 receptor is constitutively expressed. Immunohistochemistry also showed that *pdx-1* protein was successfully introduced in IMPE cells by *pdx-1* gene infection. However, induction of *pdx-1* alone could not accomplish insulin production by IMPE cells. Even when IMPE cells were infected with a high titer of Ad-*pdx-1* (at 50 MOI), *pdx-1* alone could not induce insulin production at mRNA and protein levels. GLP-1 secreted from intestinal L cells in response to oral intake of glucose stimulates β cells to secrete insulin by phosphorylating *pdx-1* protein and translocating *pdx-1* from the cytoplasm to the nucleus.[16] In addition, *pdx-1*–lacking Panc-1 cells could be differentiated to insulin-producing cells only when the cells were treated with GLP-1 and stably transfected with *pdx-1*.[20] We showed that GLP-1 and Ad-*pdx-1* could alter IMPE cells to produce insulin mRNA and insulin protein (Figs 2 and 3). Either GLP-1 or Ad-*pdx-1* alone could not induce mRNA or protein expression of insulin by IMPE cells. Again, *pdx-1* is a master regulator gene for pancreas organogenesis and β cell identity although enforced expression of *pdx-1* alone could not promote insulin production in IMPE cell. Taken together, these findings show it is conceivable that GLP-1 is essential for these cells to produce insulin.

To investigate the function of insulin-producing cells derived from IMPE cells, we tested the effect of different concentrations of GLP-1. As shown in Figure 4, insulin levels in both the supernatant and the cell lysate were increased significantly by GLP-1 in a dose-dependent manner. In the same way, when the effect of different concentrations of glucose was tested, insulin levels in both the supernatant and the cell lysate were increased significantly by glucose in a dose-dependent manner (Fig 5). In addition, we demonstrated that the IMPE-derived insulin-producing cells gained the quick responsiveness to the change of extracellular glucose concentration (Fig 6). It is well-known that glucose stimulates insulin production and secretion in normal β cells via the activation of *pdx-1*.[22] Our data suggest that IMPE-derived insulin-producing cells become differentiated to respond to glucose as the normal insulin-producing β cells do. We noted that other endocrine markers, such as glucagon and somatostatin, and exocrine marker amylase were not detected in IMPE cells even when the cells were treated with GLP-1 and Ad-*pdx-1*. Further study is needed to clarify the mechanism by which GLP-1 and *pdx-1* selectively activate insulin gene.

In the present study, we demonstrated that GLP-1 and *pdx-1* work together to induce insulin-producing cells from novel, conditionally immortalized pancreatic epithelial cells; the insulin-producing cells basically bear unique characteristics of pancreatic duct cells. The results suggest that GLP-1 is another important factor for pancreatic endocrine differentiation as is *pdx-1*.

REFERENCES

1. Whitehead RH, VanEeden PE, Noble MD, Ataliotis P, Jat PS. Establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2Kb-tsA58 transgenic mice. Proc Natl Acad Sci U S A 1993;90: 587-91.
2. Allen KJ, Reyes R, Demmler K, Mercer JF, Williamson R, Whitehead RH. Conditionally immortalized mouse hepatocytes for use in liver gene therapy. J Gastroenterol Hepatol 2000;15:1325-32.
3. Langley RR, Ramirez KM, Tsan RZ, Van Arsdall M, Nilsson MB, Fidler IJ. Tissue-specific microvascular endothelial cell lines from H-2K(b)-tsA58 mice for studies of angiogenesis and metastasis. Cancer Res 2003;63:2971-6.
4. Ehler E, Jat PS, Noble MD, Citi S, Draeger A. Vascular smooth muscle cells of H-2Kb-tsA58 transgenic mice. Characterization of cell lines with distinct properties. Circulation 1995;92:3289-96.
5. Koizumi M, Ito D, Fujimoto K, et al. Conditional transformation of mouse pancreatic epithelial cells: an in vitro model for analysis of genetic events in pancreatocarcinogenesis. Biochem Biophys Res Commun 2004;319:612-21.
6. Ito D, Fujimoto K, Doi R, et al. 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. Surgery 2004;136:364-74.
7. Dor Y, Brown J, Martinez OI, Melton DA. Adult pancreatic beta-cells are formed by self-duplication rather than stem-cell differentiation. Nature 2004;429:41-6.
8. 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.
9. Horb ME, Shen CN, Tosh D, Slack JM. Experimental conversion of liver to pancreas. Curr Biol 2003;13:105-15.
10. Yoshida S, Kajimoto Y, Yasuda T, et al. PDX-1 induces differentiation of intestinal epithelioid IEC-6 into insulin-producing cells. Diabetes 2002;51:2505-13.
11. 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.
12. Jonsson J, Carlsson L, Edlund T, Edlund H. Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 1994;371:606-9.
13. Drucker DJ. Minireview: the glucagon-like peptides. Endocrinology 2001;142:521-7.
14. Wang X, Cahill CM, Pineyro MA, Zhou J, Doyle ME, Egan JM. Glucagon-like peptide-1 regulates the beta cell transcription factor, PDX-1, in insulinoma cells. Endocrinology 1999;140:4904-7.
15. Stoffers DA, Kieffer TJ, Hussain MA, et al. Insulinotropic glucagon-like peptide 1 agonists stimulate expression of homeodomain protein IDX-1 and increase islet size in mouse pancreas. Diabetes 2000;49:741-8.
16. Wang X, Zhou J, Doyle ME, Egan JM. Glucagon-like peptide-1 causes pancreatic duodenal homeobox-1 protein translocation from the cytoplasm to the nucleus of pancreatic beta-cells by a cyclic adenosine monophosphate/protein kinase A-dependent mechanism. Endocrinology 2001;142:1820-7.
17. Buteau J, Roduit R, Susini S, Prentki M. Glucagon-like peptide-1 promotes DNA synthesis, activates phosphatidylinositol 3-kinase and increases transcription factor pancreatic and duodenal homeobox gene 1 (PDX-1) DNA binding activity in beta (INS-1)-cells. Diabetologia 1999;42:856-64.
18. Koizumi M, Doi R, Toyoda E, et al. Increased PDX-1 expression is associated with outcome in patients with pancreatic cancer. Surgery 2003;134:260-6.
19. Zhou J, Wang X, Pineyro MA, Egan JM. Glucagon-like peptide 1 and exendin-4 convert pancreatic AR42J cells into glucagon- and insulin-producing cells. Diabetes 1999;48:2358-66.
20. Hui H, Wright C, Perfetti R. Glucagon-like peptide 1 induces differentiation of islet duodenal homeobox-1-positive pancreatic ductal cells into insulin-secreting cells. Diabetes 2001;50:785-96.
21. Zhou J, Pineyro MA, Wang X, Doyle ME, Egan JM. Exendin-4 differentiation of a human pancreatic duct cell line into endocrine cells: involvement of PDX-1 and HNF3beta transcription factors. J Cell Physiol 2002;192:304-14.
22. MacDonald PE, El-Kholy W, Riedel MJ, Salapatek AM, Light PE, Wheeler MB. The multiple actions of GLP-1 on the process of glucose-stimulated insulin secretion. Diabetes 2002;51(Suppl 3):S434-42.

Expression and prognostic value of tuberous sclerosis complex 2 gene product tuberin in human pancreatic cancer

Kozo Kataoka, Koji Fujimoto, MD, PhD, Daisuke Ito, MD, Masayuki Koizumi, MD, Eiji Toyoda, MD, Tomohiko Mori, MD, Kazuhiro Kami, MD, *and* **Ryuichiro Doi, MD, PhD,** *Kyoto, Japan*

Background. *Tuberous sclerosis complex (TSC) is an autosomal dominant disorder caused by mutation of either of 2 tumor suppressor genes, TSC1 or TSC2, which encode hamartin and tuberin, respectively. Several studies have shown that tuberin functions independently of hamartin and inhibits signaling pathways via the mammalian target of rapamycin, a critical regulator of cell proliferation. Recent studies have revealed that the signaling pathways regulating the mammalian target of rapamycin such as Akt and S6K1 are frequently activated in pancreatic cancer. We hypothesized that tuberin might be involved in the proliferation and survival of pancreatic cancer cells.*

Methods. *We immunohistochemically examined the expression of tuberin in 42 pancreatic cancerous and noncancerous pancreatic tissue specimens using an antituberin antibody. The correlations between tuberin expression and various clinicopathologic features, including survival, were evaluated. Reverse transcriptase-polymerase chain reaction was performed to evaluate the level of tuberin expression in paired samples of pancreatic cancer and noncancerous tissue.*

Results. *Twenty-four of the 42 pancreatic cancer samples (57%) were negative for tuberin expression. The patients with tuberin-negative tumors had a significantly higher incidence of pT3 or pT4 disease (primary tumor extent by the TNM classification) than those with tuberin-positive tumors (P = .024). Female patients had a significantly higher incidence of tuberin-positive tumors than male patients (P = .014). The survival rate of the tuberin-positive group tended to be better than that of the tuberin-negative group, but there was no significant difference (P = .4). Expression of TSC2 in cancer tissue was lower than in the corresponding noncancerous tissue for 7 of the 9 samples examined.*

Conclusions. *This study demonstrates that reduced expression of tuberin might be involved in the progression of pancreatic cancer. Accordingly, tuberin may provide a new therapeutic target in patients with this type of cancer. (Surgery 2005;138:450-5.)*

From the Department of Surgery and Basic Surgical Science, Kyoto University

TUBEROUS SCLEROSIS COMPLEX (TSC) is an autosomal dominant disorder characterized by development of benign tumors called hamartomas in the brain, heart, kidneys, and skin.[1] Genetic studies have mapped this disorder to the *TSC1* and *TSC2* genes, which encode hamartin and tuberin, respectively.[2] TSC patients have a mutant *TSC1* or *TSC2* gene in each of their somatic cells, and it has been demonstrated that these are tumor suppressor genes that function by inhibiting cell growth and proliferation.[3,4] Several studies have shown that the *TSC2* gene product tuberin has functions independent from those of the *TSC1* gene product hamartin,[5] and it is known to play a critical role in the regulation of cell cycle progression from G0/G1 phase to S phase.[6]

Pancreatic cancer is the fifth leading cause of cancer-related death in most Western countries.[7] It is difficult to detect this disease at an early stage, and the cancer shows resistance to almost all available chemotherapy and radiation regimens; therefore, the prognosis remains dismal with a 5-year survival rate of less than 10%.[8] In recent years, considerable insight into the genetic basis of this disease has been obtained, and studies have often

Presented at the 66th Annual Meeting of the Society of University Surgeons, Nashville, Tennessee, February 9-12, 2005.

Supported by a grant from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (K.F.).

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

0039-6060/$ - see front matter

doi:10.1016/j.surg.2005.06.028

45

detected alterations in tumor suppressor genes, such as $p16^{INK4A}$, p53, and SMAD4, as well as various oncogenes (e.g., Ki-ras), controlling critical steps of the cell cycle, genetic stability, and growth regulation.[9]

Genetic studies performed in Drosophila and biochemical studies on mammalian cells have demonstrated that hamartin and tuberin act downstream of phosphatidylinositol 3-kinase (PI3K) and Akt, but upstream of the mammalian target of rapamycin (mTOR). mTOR is regulated by mitogens, nutrients, and the cellular energy level, and it is known to modulate cell growth and proliferation through regulation of at least 2 downstream targets, which are p70 ribosomal S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1).[10] Recent studies have demonstrated that the mTOR-S6K1–signaling pathway is activated constitutively in pancreatic cancer and appears to be necessary for cell cycle progression and growth of tumor cells.[11] More recently, Schlieman et al[12] have shown that Akt is frequently activated in pancreatic cancer and that phosphorylation of Akt is associated with tumor grade, an important prognostic factor. In fact, phosphorylation of Akt decreases the ability of TSC2 to inhibit phosphorylation of S6K1 and 4E-BP1, which are substrates for mTOR.[13] We have found that signaling pathways regulating mTOR, such as Akt and S6K1, are frequently activated in pancreatic cancer, and that an mTOR inhibitor (the rapamycin derivative CCI-779) inhibits tumor growth in xenograft models as a single agent or combined with gemcitabine.[14] Moreover, recent studies have shown that rapamycin induces tumor vessel thrombosis related to the death of endothelial cells and decreases tumor angiogenesis, resulting in inhibition of tumor growth.[15,16]

These observations led us to hypothesize that the expression of tuberin might be modulated in human pancreatic cancer, and that this tumor suppressor gene product might be involved in the proliferation and survival of pancreatic cancer cells. To verify this hypothesis, we examined tuberin expression in pancreatic cancer tissues and assessed the correlation between various clinicopathologic features (including survival) and tuberin expression. We found that tuberin expression was negative in 24 of 42 pancreatic cancers (57%), and negative expression was correlated with a higher pT category (the extent of the primary tumor according to the TNM classification) and with male gender of the patient. Moreover, the survival rate of the patients without tuberin expression tended to be worse than that of the patients with positive expression, although there was no significant difference ($P = .4$).

MATERIAL AND METHODS

Patients and tissue specimens. Formalin-fixed, paraffin-embedded tissues were obtained from the Department of Surgery and Basic Surgical Science at Kyoto University (Kyoto, Japan) between July 1994 and January 2002. Specimens were harvested from 42 patients with invasive pancreatic ductal adenocarcinoma (PDA) and were collected after obtaining informed consent in accordance with institutional guidelines. The average age at surgery was 62.8 years (range, 45-75 years). We enrolled only patients who had survived at least 60 days after surgery to exclude any bias related to perioperative mortality. Follow-up data were updated on November 30, 2004. Tissue samples were fixed with 10% formaldehyde in phosphate-buffered saline (PBS), embedded in paraffin, and cut into serial sections 4-μm thick. Pancreatic cancer was staged according to the Putnam (Union Internationale Contra Cancrum [UICC]) system. A total of 42 tumor samples were subjected to tuberin immunohistochemistry, while cancer tissue and adjacent noncancerous tissue obtained from 9 patients were used for reverse transcriptase-polymerase chain reaction (RT-PCR). The 9 samples for the RT-PCR were from 42 tissue samples used for immunohistochemistry, which were frozen at −80°C at the time of surgery. Since, in the experiment using pancreatic cancer tissue, it is important to confirm the presence of cancer cells on the tissue slides, we carefully confirmed the presence of cancer cells with a hematoxylin-eosin slide of the serial section when immunohistochemistry for tuberin was performed.

Determination of tuberin. Tissue sections were deparaffinized, preheated in 10 mmol/L citrate buffer (pH 6.0) in a microwave oven for 1 minute and in an autoclave for 9 minutes, and pretreated with 1% hydrogen peroxide solution for 20 minutes. Blocking was performed with 5% normal goat serum (NGS) in PBS. Then, the sections were incubated overnight at 4°C with a polyclonal rabbit antituberin antibody (C20, Santa Cruz, Calif) diluted 1:500 in 5% NGS/PBS, and then for 45 minutes with a 1:250 dilution of biotinylated goat antirabbit IgG in PBS. Sections subsequently were incubated with avidin and biotin–peroxidase complex (DakoCytomation, Kyoto, Japan). Reaction products were visualized with the use of DAB, followed by counterstaining with hematoxylin.

Evaluation of immunostaining. Expression of tuberin was classified into 3 grades: 0, undetectable;

1, weak staining; 2, strong staining. Five normal pancreatic tissue specimens were also stained with antituberin antibody at the same time. We found that normal duct cells and islet cells showed strong tuberin expression. Therefore, those cells were used as an internal control for tuberin staining, being defined as grade 2 (strong staining). The proportion of positive cancer cells also was classified into 3 grades: 0 = none; 1 = 1% to 49%; and 2 = 50% to 100%. Then the staining intensity score was multiplied by the score for positive cells to obtain the overall score. Tissues with a score of ≥ 2 were regarded as positive, and tissues with a score of ≤ 1 were defined as negative. Tuberin expression was evaluated independently by 2 investigators (K.K. and D.I.) who had no knowledge of the patients' clinicopathologic features.

RNA preparation and semiquantitative RT-PCR analysis. Using TRIZOL Reagent (Life Technologies, Rockville, Md), we extracted total cellular RNA from cancer tissue and adjacent noncancerous tissue. Complementary DNA (cDNA) was synthesized by the random primer method from 1 μg of total RNA using a first-strand cDNA synthesis kit (Pharmacia Biotech, North Peapack, NJ) according to the manufacturer's instructions. PCR was carried out with a mixture of cDNA (from 100 ng of RNA), 2 μmol/L each of the sense and antisense primers, 0.2 μmol/L of deoxynucleotide triphosphate, and 2.5 U of *Taq* DNA polymerase in a reaction buffer (TaKaRa, Kyoto, Japan) with a final volume of 50 μL. PCR was performed in a thermal cycler (Gene Amp PCR system 2400; PE Applied Biosystems, Foster City, Calif). The following oligonucleotide primers were used to amplify *TSC2* and β-actin:

TSC2-(sense) 5′-TGCTCATCAACAGGCAGTTC-3′,
(anti-sense) 5′-GCCACATCCCTTTCTTCCA-3′
β-actin-(sense) 5′-ATGGATGACGATATCGCT-3′,
(anti-sense) 5′-ATGAGGTAGTCTGTCAGGT-3′

The size of the product was 204 bp for *TSC2* and 569 bp for β-actin, respectively. For PCR, initial denaturation was done at 94°C for 10 minutes, followed by 37 cycles of 55°C for 1 minute, 72°C for 1 minute, and 94°C for 30 seconds, with final extension at 72°C for 10 minutes. Then the PCR products were separated on 2.5% agarose gel (*TSC2*) or 1.5% agarose gel (β-actin).

Assessment of expression of tuberin was performed by densitometric analysis. Band areas were analyzed by scanning densitometry (ATTO, Osaka, Japan) and each value converted to a percentage of the total densitometric value. Normalization of the data was performed by using the reference gene β-actin as an internal control.

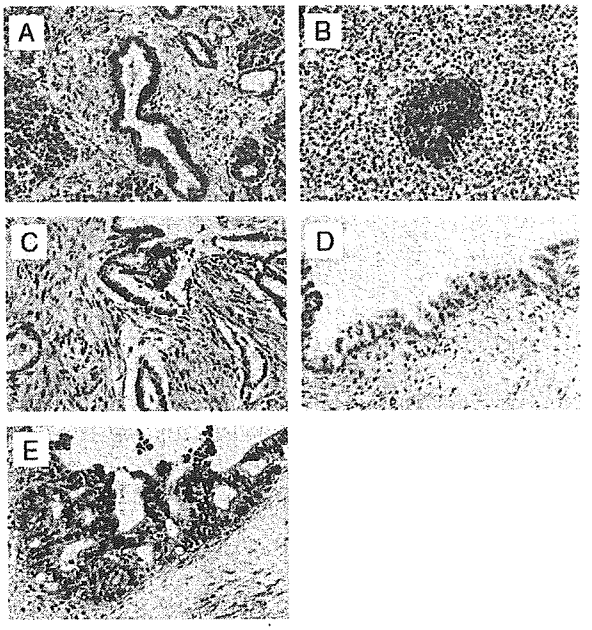

Fig 1. Immunohistochemical analysis of tuberin expression in normal pancreas tissues and PDAs. **A,** Strong staining was detected in normal ducts (*small arrow*). Some acinar cells were also stained moderately. (Original magnification: ×400.) **B,** Strong staining was detected n the cytoplasm of islet cells. (Original magnification: ×400.) **C, D,** Typical photomicrograph of PDAs regarded as negative (**C,** no staining, **D,** weak staining) are shown. (Original magnification: ×400.) **E,** A typical photomicrograph of PDA regarded as positive is shown. Cytosolic staining of tuberin was seen. The nuclei were counterstained with Mayer's hematoxylin. (Original magnification: ×400.)

Statistical analysis. The correlations between tuberin immunoreactivity and clinicopathologic variables were analyzed by chi-square test or Fisher exact test. Survival was calculated by the Kaplan-Meier method, and differences between each group were evaluated with the log-rank test. Statistical significance was defined as $P < .05$.

RESULTS

Immunohistochemical tuberin expression in pancreatic cancer. In normal pancreatic tissue specimens, tuberin was strongly positive in the cytoplasm of islet cells and duct cells (Fig 1, *A* and *B*). Some normal acinar cells also showed moderate tuberin reactivity. In pancreatic cancer tissues, tuberin staining was identified primarily in cytoplasm of cancer cells. Twenty-four of the 42 pancreatic cancers (57%) were judged to have negligible or

Table. Correlation between tuberin expression and clinicopathological features

		Tuberin expression		
Variables	*Category*	*Negative (n = 24)*	*Positive (n = 18)*	P *value*
Age	<65y	10	8	0.69
	≥65y	14	10	
Gender	Male	16	5	**0.014***
	Female	8	13	
pT	1 or 2	4	9	**0.024***
	3 or 4	20	9	
pN	Negative	6	8	0.16
	Positive	18	10	
pM	Negative	20	15	0.67
	Positive	4	3	
UICC	Stage I or II	7	8	0.25
	Stage III or IV	17	10	
Tumor size	<3.5 cm	12	12	0.22
	≥3.5 cm	12	6	
Grade	1 or 2	20	15	0.67
	3 or 4	4	3	

*$P < 0.05$.

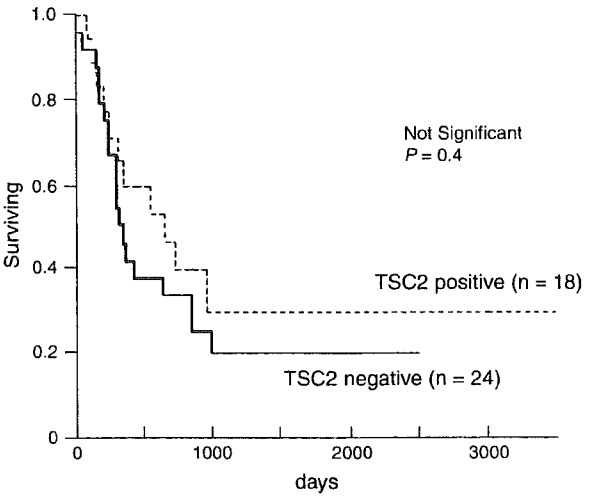

Fig 2. Kaplan-Meier analysis of the overall survival of patients with PDAs according to tuberin expression. Patients with negative tuberin expression tended to be a worse prognosis than those with positive expression, but there was no statistical significance (log-rank test, $P = .4$).

weak expression of tuberin (Fig 1, *C* and *D*), while moderate to marked immunoreactivity was found in 18 pancreatic cancer tissues (43%) (Fig 1, *E*). In our procedure, samples with a low staining score for tuberin (≤1) were classified as negative (n = 24), and those with a high staining score (≥2) were classified as positive (n = 18).

Correlation between tuberin expression and clinicopathologic features. The relationship between tuberin expression and various clinicopathologic features was analyzed. As shown in the Table, gender and pT category (primary tumor extent in the TNM classification) were correlated significantly with tuberin expression. We found that female patients had a significantly higher incidence (13/21, 61.9%) of tuberin expression in their tumors than male patients (5/21, 23.8%) ($P = .014$). Moreover, patients without tuberin expression had a significantly higher incidence (20/24, 83.3%) of pT3 or pT4 disease (UICC classification) than those (9/18, 50%) who were tuberin-positive ($P = .024$). There were no significant relationships between tuberin expression and any of the other clinicopathologic features.

Kaplan-Meier survival analysis. Survival curves were plotted according to the Kaplan-Meier method for 18 patients with tuberin expression and 24 patients without its expression (Fig 2). The survival rate of the tubulin-negative group tended to be worse than that of the tubulin-positive group, but there was no significant difference ($P = .4$, log-rank test).

***TSC2* expression in pancreatic cancer tissue and noncancerous tissue.** Total cellular RNA was extracted from cancer tissue and the adjacent noncancerous tissue of the same patients. Semiquantitative RT-PCR was performed to evaluate *TSC2* expression in 9 pairs of samples, and densitometry was performed (ATTO, Osaka, Japan) (Fig 3, *A*). Cancer tissue showed lower *TSC2* expression than noncancerous tissue in 7 of the 9 patients, with a marked decrease of <50%, compared with their noncancerous tissue (Fig 3, *B*).

DISCUSSION

Tuberin (TSC2) is a tumor suppressor gene product. It acts as a critical regulator of cell growth and differentiation, and thus is involved in tumor suppression,[6,17] with functions that are independent of those of the *TSC1* gene product hamartin.[5] The present study demonstrated negative tuberin expression in 24 of 42 (57%) pancreatic cancers, and lack of expression was correlated with a higher pT category and male gender. In addition, survival rate of the tuberin-negative group tended to be worse than that of the tuberin-positive group.

First, an immunohistochemical study of tuberin was performed to determine the incidence of its expression and to assess its prognostic value in 42 patients with PDA. To our knowledge, this is the first study of tuberin expression in PDA tissues. We found that tuberin was expressed strongly by normal duct cells and islet cells (Fig 1). Since tuberin

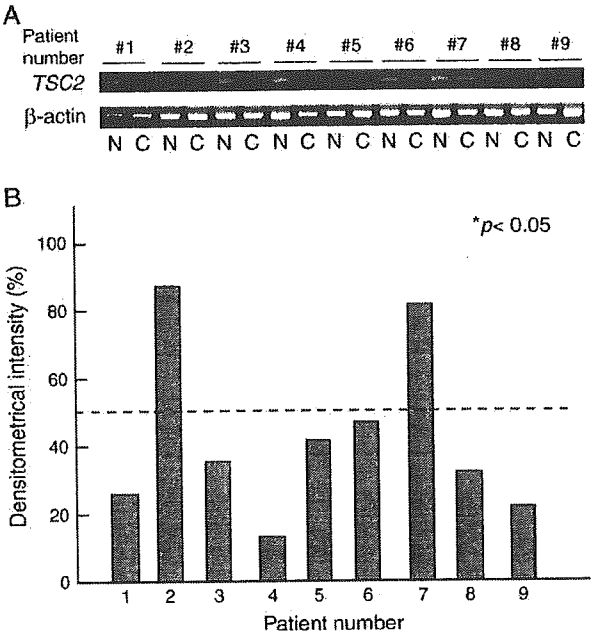

Fig 3. A, Semiquantitative RT-PCR of *TSC2* in pancreatic cancer and noncancerous tissues. **B,** Expression level of *TSC2*. Expression level of *TSC2* was evaluated by a densitometry (ATTO, Osaka, Japan). Results are expressed as per cent of the tuberin/β-actin ratio of cancer versus noncancerous tissues. Similar results were obtained in 3 independent experiments.

is known to be a tumor suppressor gene product, this expression profile in normal tissues seems reasonable. Recently, Francalanci et al[18] reported a case of an epileptic and mentally retarded child with TSC who developed a malignant islet cell tumor of the pancreas. They observed immunostaining for tuberin in normal pancreatic islet cells, but not in tumor cells, since both TSC2 alleles had been knocked out by a germline mutation. In the present study, tuberin expression was reduced markedly in 57% (24/42) of our patients with PDA. Moreover, RT-PCR revealed that 7 of 9 cancer tissue samples showed lower tuberin expression than noncancerous tissues (Fig 3). These findings might indicate the involvement of tuberin in the progression of pancreatic exocrine and endocrine tumors.

Next, we assessed whether tuberin expression was a useful marker for predicting the prognosis. Statistical analysis of the relationship between tuberin and clinicopathologic features revealed that gender and pT category were correlated significantly with tuberin expression (Table). Female patients had a significantly higher incidence (13/21, 61.9%) of tuberin-positive tumors than male patients (5/21, 23.8%) (P = .014). Several studies have shown already that females have a survival advantage over males among PDA patients.[19,20] Since patients with tuberin-positive tumors tended to have a better prognosis than those with negative tumors in our series, the significant correlation between gender and tubein expression seems to be reasonable. With respect to pT category, the patients with tuberin-negative tumors had a significantly higher pT category (pT3 or pT4) than those with tuberin-positive tumors (P = .024) in this series. This finding means that tumors without tuberin expression might show more aggressive local invasion.

Deregulation of pathways that control cell proliferation is a hallmark of cancer, but the exact requirements for oncogenic transformation of specific signaling pathways remain a major question. TSC is a tumor syndrome caused by mutation in *TSC1* or *TSC2* genes; however, TSC tumorigenesis is not always accompanied by loss of heterozygosity. Recent studies have shown that extracellular signal-regulated kinase (Erk) has been activated in TSC lesions lacking TSC1 or TSC2, and that Erk may play a critical role in TSC progression through post-translational inactivation of TSC2.[21] Interestingly, Ma et al[21] have revealed that the Ras/MAPK pathway exists upstream of the TSC complex and that Erk may modulate mTOR signaling and contribute to disease progression through phosphorylation and inactivation of TSC2. In the case of pancreatic cancer, activating ras mutations are thought to be the first genetic changes, and loss of SMAD4 expression occurs later in neoplastic progression.[22] We also have recently shown that both activation of ras and inactivation of p53 are necessary for acquisition of in vitro tumorigenicity by normal pancreatic epithelial cells.[23] Intriguingly, recent studies have revealed a molecular association between the activated Ras/MAPK or TGF-β/SMAD signaling pathways and tuberin.[24,25] Roux et al[24] reported that activated ras can induce phosphorylation of tuberin and thus cause its inactivation, resulting in increased mTOR signaling. Furthermore, other investigators recently have demonstrated that tuberin strongly augments the TGF-β1–signaling pathway, including activation of SMAD.[25] Although the reason why expression of tuberin was reduced markedly in more than half of the tumors in our series remains unclear, it might be associated with activating mutations of ras or inactivation of SMAD4 in pancreatic cancer. It might be interesting to investigate the association of tuberin with tumor suppressor genes such as $p16^{INK4A}$, p53, and SMAD4 as well as ras oncogene to clarify the mechanisms of regulating tuberin expression in PDA.

CONCLUSION

This study demonstrated that tuberin expression was negative in 24 of 42 pancreatic cancers (57%), and negative expression was correlated with a higher pT category and male gender of the patient. Moreover, the survival rate of the tuberin-negative group tended to be worse than that of the tuberin-positive group. This association between the *TSC2* gene product tuberin and tumor extent (pT) may suggest new therapeutic targets for the treatment of pancreatic cancer.

REFERENCES

1. Cheadle J, Reeve MP, Sampson JR, et al. Molecular genetic advances in tuberous sclerosis. Hum Genet 2000;107: 97-114.
2. Young J, Povey S. The genetic basis of tuberous sclerosis. Mol Med Today 1998;4:313-9.
3. Ito N, Rubin GM. gigas, a Drosophila homolog of tuberous sclerosis gene product-2, regulates the cell cycle. Cell 1999; 96:529-39.
4. Tapon N, Ito N, Dickson BJ, et al. The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. Cell 2001;105:345-55.
5. Miloloza A, Kubista M, Rosner M, et al. Evidence for separable functions of tuberous sclerosis gene products in mammalian cell cycle regulation. J Neuropathol Exp Neurol 2002;61:154-63.
6. Soucek T, Pusch O, Wienecke R, et al. Role of the tuberous sclerosis gene-2 product in cell cycle control. Loss of the tuberous sclerosis gene-2 induces quiescent cells to enter S phase. J Biol Chem 1997;272:29301-8.
7. Greenlee R, Hill-Harmon MB, Murray T, et al. Cancer statistics, 2001. CA Cancer J Clin 2001;51:15-36.
8. Rosewicz S, Wiedenmann B. Pancreatic carcinoma. Lancet 1997;349:485-9.
9. Bardeesy N, Depinho R. Pancreatic cancer biology and genetics. Nat. Rev. Cancer 2002;2:897-909.
10. Richardson C, Schalm S, Blenis J. PI3-kinase and TOR: PIKTORing cell growth. Semin Cell Dev Biol 2004;15: 147-59.
11. Shah SA, Potter MW, Ricciardi R, et al. FRAP-p70s6K signaling is required for pancreatic cancer cell proliferation. J Surg Res 2001;97:123-30.
12. Schlieman MG, Fahy BN, Ramsamooj R, et al. Incidence, mechanism and prognostic value of activated AKT in pancreas cancer. Br J Cancer 2003;89:2110-5.
13. Dan HC, Sun M, Yang L, et al. Phosphatidylinositol 3-kinase/Akt pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberin. J Biol Chem 2002;277(38):35364-70.
14. Fujimoto K, Ito D, Doi R, et al. Antitumor activity of the mTOR inhibitor, CCI-779 in pancreatic cancer preclinical models as single agent and in combination chemotherapy with gemcitabine. HPB 2004;6:11.
15. Bruns C, Koehl GE, Guba M, et al. Rapamycin-induced endothelial cell death and tumor vessel thrombosis potentiate cytotoxic therapy against pancreatic cancer. Clin Cancer Res 2004;10:2109-19.
16. Stephan S, Datta K, Wang E, et al. Effect of rapamycin alone and in combination with antiangiogenesis therapy in an orthotopic model of human pancreatic cancer. Clin Cancer Res 2004;10:6993-7000.
17. Soucek T, Holzl G, Bernaschek G, et al. A role of the tuberous sclerosis gene-2 product during neuronal differentiation. Oncogene 1998;16:2197-204.
18. Francalanci P, Diomedi-Camassei F, Purificato C, et al. Malignant pancreatic endocrine tumor in a child with tuberous sclerosis. Am J Surg Pathol 2003;27:1386-9.
19. Chang KJ, Parasher G, Christie C, et al. Risk of pancreatic adenocarcinoma. Cancer 2004.
20. Andren-Sandberg A, Ihse I. Factors influencing survival after total pancreatectomy in patients with pancreatic cancer. Ann Surg 1983;198:605-10.
21. Ma L, Chen Z, Erdjument-Bromage H, et al. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell 2005;121:179-93.
22. Wilentz RE, Iacobuzio-Donahue CA, Argani P, et al. Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. Cancer Res 2000;60:2002-6.
23. Koizumi M, Ito D, Fujimoto K, et al. Conditional transformation of mouse pancreatic epithelial cells: an in vitro model for analysis of genetic events in pancreatocarcinogenesis. Biochem Biophys Res Commun 2004;319:612-21.
24. Roux PP, Ballif BA, Anjum R, et al. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc Natl Acad Sci U S A 2004;101:13489-94.
25. Birchenall-Roberts MC, Fu T, Bang O, et al. Tuberous sclerosis complex 2 gene product interacts with human SMAD proteins. J Biol Chem 2004;279:25605-13.

Available online at www.sciencedirect.com
SCIENCE DIRECT
ELSEVIER
Biochemical Pharmacology 70 (2005) 1756–1763
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Expression profiles of various transporters for oligopeptides, amino acids and organic ions along the human digestive tract

Tomohiro Terada [a], Yutaka Shimada [b], Xiaoyue Pan [a], Koshiro Kishimoto [a], Takaki Sakurai [c], Ryuichiro Doi [b], Hisashi Onodera [b], Toshiya Katsura [a], Masayuki Imamura [b], Ken-ichi Inui [a,*]

[a] *Department of Pharmacy, Kyoto University Hospital, Sakyo-ku, Kyoto 606-8507, Japan*
[b] *Surgery and Surgical Basic Science, Graduate School of Medicine, Kyoto University, Japan*
[c] *Laboratory of Anatomic Pathology, Kyoto University Hospital, Japan*

Received 31 August 2005; accepted 28 September 2005

Abstract

Various transporters such as H^+/peptide cotransporter PEPT1 are expressed in the intestine, and play important physiological and pharmacological roles in the body. Present study was performed to examine the expression profile of 20 kinds of transporters (PEPT1 and 2, P-glycoprotein, amino acid transporters and organic ion transporters) along the human digestive tract, especially focusing on PEPT1. Using normal mucosal specimens, real-time polymerase chain reactions were carried out. Immunoblot analyses were also performed for PEPT1 expression. PEPT1 mRNA was highly expressed in the small intestine (duodenum > jejunum > ileum) compared to other tissues, and some patients showed a significant level of expression in the stomach. The expressional pattern of PEPT1 in the stomach and histological diagnosis indicated that gastric PEPT1 originated from the intestinal metaplasia. The amino acid transporters showed unique mRNA expression levels and distributions in the digestive tract. For example, the expression levels of B^0AT1, a Na^+-dependent and chloride-independent neutral amino acid transporter, were increased from the duodenum to ileum, which pattern is completely inverted to that for PEPT1. There is little expression of organic ion transporters except for organic cation/carnitine transporter OCTN2. In conclusion, PEPT1 was abundantly expressed in the small intestine, and the reciprocal expression of PEPT1 and B^0AT1 may serve for the efficient absorption of protein digestive products.

Keywords: Protein absorption; Digestive tract; Transporter; PEPT1; B^0AT1; OCTN2; Organic ion transporter

1. Introduction

Cellular uptake of small peptides (di- and tri-peptides) is mediated by a H^+/peptide cotransporter at the brush-border membranes of intestinal absorptive epithelial cells [1,2]. Cloning studies have clarified the molecular nature of the intestinal H^+/peptide cotransporter (PEPT1) in various species [2,3]. Another H^+/peptide cotransporter, human PEPT2 is expressed mainly in the kidney, but not in the small intestine, and has a higher affinity for most substrates [3]. As PEPT1 has broad substrate specificity, the intestinal absorption of several pharmacologically active drugs, such as oral β-lactam antibiotics and the anti-viral agent valacyclovir are mediated by this transporter, and therefore, PEPT1 also plays important roles as a drug transporter [3].

In addition to PEPT1, various amino acid transport systems, such as L, y^+, y^+L, $b^{0,+}$, A, ASC, B^0, $B^{0,+}$ and X_{AG}^- are expressed in the small intestinal epithelial cells. These systems have been classified by their ion-dependence and substrate specificity [4], and the molecular nature of each system has been identified [5,6]. For example, system B^0, which is a Na^+-dependent and chloride-independent transporter, is responsible for the uptake of most neutral amino acids at the brush-border membranes of intestinal epithelial cells [7,8]. Two independent groups have recently isolated and characterized the transporter cDNA corresponding to system B^0 (B^0AT1), and demonstrated that a mutation of B^0AT1 results in the Hartnup disorder [9,10].

* Corresponding author. Tel.: +81 75 751 3577; fax: +81 75 751 4207.
E-mail address: inui@kuhp.kyoto-u.ac.jp (K.-i. Inui).

0006-2952/$ – see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2005.09.027

Although the number of peptide transporters is smaller than that of amino acid transporters, numerous investigations have shown that the peptide transporters play a major role in the completion of the final step of protein digestion in the human intestine [1]. These findings led us to examine the expression levels of each transporter along the human digestive tract. Thus, in the present study, using normal portions of mucosal samples from cancer patients treated surgically, mRNA expression levels of PEPT1 and seven amino acid transporters were qualified by real-time PCR techniques. Furthermore, to investigate the pharmacokinetic aspects of PEPT1, we compared mRNA expression level of PEPT1 with those of other drug transporters, i.e., organic ion transporters. We previously examined the expression profiles of organic ion transporters in the kidney [11], but there is little information about their expression in the human gut.

2. Material and methods

2.1. Patients and tissue sampling

The mucosal samples from normal tissues along the digestive tract and normal pancreatic tissue were obtained from cancer patients during surgery at the First Department of Surgery, Kyoto University Hospital. Normal mucosal samples were resected at the site most distant from the affected portions. Table 1 shows the number, sex and age of patients for each tissue sample. No patients underwent preoperative chemotherapy and/or radiation therapy. The samples were frozen in liquid nitrogen and stored at −80 °C until RNA extraction and membrane preparation. This study was conducted in accordance with the Declaration of Helsinki and its amendments, and was approved by the Ethics Committee of Kyoto University (G-39). Written informed consent was obtained from all patients for surgery and use of their resected samples.

2.2. Isolation of total RNA

Total cellular RNA was isolated from the tissue samples using a MagNA Pure LC RNA isolation Kit II (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions, and the concentrations of total cellular RNA were measured by spectrophotometry. Isolated total RNA was reverse-transcribed and the reaction mixtures were used for real-time PCR.

Table 1
The number, sex and age of patients for each tissue sample

	n	Male	Female	Age (years)
Esophagus	16	13	3	59.3 ± 1.6
Stomach	31	20	11	64.4 ± 2.3
Duodenum	13	7	6	63.8 ± 2.2
Jejunum	15	13	2	66.6 ± 3.7
Ileum	10	4	6	65.3 ± 5.7
Colon	15	8	7	71.6 ± 3.1
Rectum	15	11	4	64.6 ± 3.0
Pancreas	9	3	6	61.8 ± 2.8

2.3. Real-time PCR

Primers and probes for PEPT1 and 2, seven amino acid transporters and urate transproter (URAT1) were designed using the Primer Express® software program (Table 2). Those for multidrug resistance 1 (MDR1) [12] and organic ion transporters [11] were previously designed. Oligonucleotide probes were labelled with a fluorogenic dye, 6 carboxyfluorescein (Fam) and quenched with 6 carboxy-tetramethylrhodamine (Tamra) (Table 2). Real-time PCR was performed in an ABI PRISM 7700 (Applied Biosystems, Foster, CA) and quantification by use of standard plasmid DNA was performed as described previously [11]. For each reaction, the assay was carried out in duplicate. Glyceroladehyde 3-phosphate denydrogenase (GAPDH) mRNA was also measured as an internal control with GAPDH control reagent (Applied Biosystems).

2.4. Antibodies

Rabbit anti-human PEPT1 antibody was prepared previously, and the specificity was already confirmed [13]. Mouse anti-Na^+/K^+-ATPase monoclonal antibody was purchased from Upstate Biotechnology (Lake Placid, NY). For immunohistochemistry, we used anti-CD10 antibody (Novocastra Laboratories Ltd., Newcastle upon Tyne, UK).

2.5. Western blot analysis

Crude plasma membranes were prepared from tissue samples taken along the digestive tract as described previously [11]. Western blot analyses using affinity-purified anti-PEPT1 antibody [13] and mouse monoclonal Na^+/K^+-ATPase antibody [11] were carried out based on our previous reports.

2.6. Immunohistochemistry

Paraffin-embeded sections were stained for CD10 antibody. The sections were deparaffinized, and antigens were retrieved by autoclave in 10 mM EDTA buffer (pH 8.0) for 15 min. Non-specific antigen was blocked by 10% normal horse serum for 30 min at room temperature. Anti-CD10 antibody (×50) in PBS containing 1% bovine serum albumin was applied and sections were incubated at 37 °C for 32 min. Slides were washed six times in PBS, and then incubated with biotinylated horse anti-mouse antibody for 40 min at room temperature. The slides were then counterstained with hematoxylin.

Table 2
Primer sets and probes used for real-time PCR

	Sequence	Nucleotide numbers	Accession number
PEPT1			NM_005073
Forward primer	attgtgtcgctctccattgtctac	306–329	
Reverse primer	atgacctcacagaccacaaccat	389–367	
TaqMan probe	ttggacaagcagtcacctcagtaagctcca	334–363	
PEPT2			NM_021082
Forward primer	ttaaacaaggccccagagactct	2382–2404	
Reverse primer	cccacttagttctggacctgctt	2463–2445	
TaqMan probe	tgccccaaccagtcttcaggaggaag	2410–2439	
B^0AT1			XM_291120
Forward primer	gtgtggacaggttcaataaggacat	1646–1670	
Reverse primer	ccacgtgacttgccagaagat	1719–1699	
TaqMan probe	tcatgatcggccacaagcccaa	1676–1697	
ASCT2			U53347
Forward primer	gcgagaaatatcttcccttcca	1152–1202	
Reverse primer	gttccggtgatattcctctcttca	1266–1243	
TaqMan probe	tgtcagcagcctttcgctcatactctacca	1209–1238	
$b^{0,+}AT$			AF141289
Forward primer	ggcctgacgattctaggactca	1344–1365	
Reverse primer	ggagccagaaccaaaaacaca	1468–1448	
TaqMan probe	atcaaggtgcccgtagtcattcccgt	1404–1429	
LAT1			AF104032
Forward primer	ggaagacacccgtggagtgt	1421–1440	
Reverse primer	acaggacggtcgtggagaag	1552–1533	
TaqMan probe	tggaaaaacaagcccaagtggctcc	1498–1522	
LAT2			AF171669
Forward primer	tgaggagcttgttgatccctaca	988–1010	
Reverse primer	gcgacattggcaaagacataca	1080–1059	
TaqMan probe	tcccagagccatcttcatctccatcc	1018–1043	
y^+LAT1			AF092032
Forward primer	gatccatgttgagcggttcac	1336–1356	
Reverse primer	ccacgcacaagtagatcaatgc	1412–1391	
TaqMan probe	ccagtgccttctctgctcttcaatggtatc	1358–1387	
ATA2			AF259799
Forward primer	gacagcagcagctacagttcca	66–87	
Reverse primer	cataatggcttttcagagcagctt	144–121	
TaqMan probe	agcgacttcaactactcctaccccaccaag	90–119	
URAT1			AB071863
Forward primer	agctcttggaccccaatgc	437–455	
Reverse primer	cttcagagcgtgagagtcacaca	560–582	
TaqMan probe	cgcagcatcttcacctccacaatcgt	517–542	

Primers and probes used for real-time PCR. All primers and probes were designed using Primer Express software from Applied Biosystems. Primers were tested using RT-PCR and only those yielding a single band of the expected size were used for subsequent real-time PCR experiments.

3. Results

3.1. Quantification of PEPT1 and PEPT2 mRNA in tissues along the digestive tract and pancreas

First, to verify the conditions and experimental techniques, MDR1 mRNA expression levels were measured (Fig. 1 right). We previously found that the level of MDR1 mRNA in the jejunum from living-donor liver transplantation patients was 0.41 ± 1.19 amol/μg of total RNA (mean ± S.E., $n = 48$) using a competitive PCR-based method, and that the level of MDR1 mRNA correlated well with protein level [14]. In the present study, the MDR1 mRNA level in the jejunum was 2.73 ± 0.51 amol/μg of total RNA (mean ± S.E., $n = 16$). This value was similar to previously reported ones [14]. Furthermore, MDR1 mRNA levels gradually increased from the duodenum to ileum, well consistent with previous findings on the distribution of MDR1 mRNA [15] and P-glycoprotein [16] in the human small intestine (Fig. 1 right). The expression of MDR1 mRNA in the colon and rectum (Fig. 1 right) also corresponded to a previous report [17]. All these findings suggested that the conditions and experimental techniques were reliable.

We then examined the mRNA expression of PEPT1 and PEPT2 along the digestive tract (esophagus, stomach,