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Conditional transformation of mouse pancreatic epithelial cells: an in vitro model for analysis of genetic events in pancreatocarcinogenesis

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Abstract

Pancreatic ductal adenocarcinomas arise through the accumulation of certain genetic alterations including ras, p16, p53, and DPC4. We found that activation of ras and inactivation of p53 could cooperatively induce in vitro tumorigenicity in conditionally immortalized pancreatic epithelial (IMPE) cells. IMPE cells were established from transgenic mice bearing a temperature-sensitive mutant SV40 Large T (LT) antigen. IMPE cells grew continuously under permissive conditions (33 °C with interferon-γ), but rapidly suffered growth arrest under non-permissive conditions (39 °C without interferon-γ). The cells showed strong expression of E-cadherin and β-catenin as epithelial markers, and cytokeratin 19, a specific ductal cell marker. Cell proliferation under permissive conditions was associated with down-regulation of p21 expression through inactivation of p53 after overexpression of LT antigen. Intriguingly, the shift from the permissive to non-permissive culture conditions caused G2/M arrest of IMPE cells. Although the cells did not form colonies when cultured in soft agar without activation of ras, cells with ras activation via an adenovirus vector formed colonies under permissive conditions. These findings suggest that activation of ras and inactivation of p53 can cooperatively induce anchorage-independent growth of IMPE cells. This cell line might be useful for studying the processes involved in pancreatocarcinogenesis.

The incidence of pancreatic ductal adenocarcinoma (PDA) is increasing in Western countries and Japan. The overall 5-year survival rate is <5%, which is dismal, and emphasizes the need for early diagnosis and/or identification of susceptible patient populations [1]. In recent years, considerable insight into the genetic basis of this disease has been obtained, and studies have shown that alterations often occur in tumor-suppressor genes controlling critical steps of cell cycle regulation, genetic stability, and growth regulation, such as $p16^{INK4A}$, p53, and deleted in pancreatic carcinoma (DPC4), as well as various oncogenes (e.g., Ki-ras) [2,3]. More recently, several investigations based on surgically resected specimens of PDA have detected these genetic changes even in precancerous lesions such as pancreatic intraepithelial neoplasia (PanIN) [4–6]. Although PDA appears to develop via a multistep process, it is not clear whether all of these genetic alterations are required for tumorigenesis or whether there is specific cooperation between subsets of the mutant gene products that lead to cancer. An in vitro model based on normal pancreatic epithelial cells that can be manipulated genetically is needed to examine the overlapping actions of these molecules, but pancreatic epithelial cells have proved difficult to culture. Numerous attempts have been made to achieve long-term culture of these cells by either direct isolation or by immortalization with viral oncogenes, but there has been little success [7,8]. In the early 1990s, the development of transgenic mice bearing a temperature-sensitive mutant SV40 Large T (LT) antigen under the control of an

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interferon (IFN)-γ inducible promoter (*H-2K*^{b}) has allowed us to culture conditionally immortalized cell lines such as intestinal epithelial cells and vascular endothelial cells [9–11]. These cell lines can proliferate indefinitely under permissive culture conditions (33 °C with IFN-γ), but cease proliferation under non-permissive conditions (39 °C without IFN-γ). In other words, the cells cultured under non-permissive conditions seem to behave like normal cells.

The aims of the present study were to examine the overlapping actions of molecules known to be altered in PDA, using normal pancreatic epithelial cells. Accordingly, we planned two sets of experiments. The initial experiments involved the establishment and characterization of conditionally immortalized pancreatic epithelial cells from *H-2K*^{b}-*ts*A58 transgenic mice. Then the second sets of experiments were performed to clarify how activation of ras and inactivation of p53 modulated the phenotype of normal pancreatic epithelial cells by using these novel immortalized cells. We found that both activation of ras and inactivation of p53 are necessary for acquisition of in vitro tumorigenicity by these pancreatic epithelial cells.

Materials and methods

Generation of immortalized pancreatic epithelial cells and primary culture conditions. Three male mice, homozygous for a temperature-sensitive SV40 LT antigen (ImmortoMice; CBA/caX C57Bl/10 hybrid; Charles River Laboratories), were killed and the whole pancreas was harvested. The pancreas was placed into a 100-mm plate containing William's E medium (Life Technologies, Gaithersburg, MD) and cut into small (1 mm) fragments. The fragments were collected into 50-ml polypropylene centrifuge tubes containing 1 mg/ml of collagenase type I and dispase (Sigma Chemical, St. Louis, MO), and then immersed in a 37 °C water bath for 1 h. Next, the tubes were centrifuged at 500*g* for 5 min. The supernatants were discarded, and the tissue fragments were resuspended in William's medium with 5% fetal bovine serum (FBS). The suspensions were plated on 24-well plates (IWAKI, Tokyo, Japan) that had been coated with rat-tail collagen. William's E medium with 5% FBS was supplemented with 5 U/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 U/ml IFN-γ (Gibco-BRL). The IFN-γ was added to enhance expression of the MHC *H-2K*^{b} class I promoter, which regulates the level of LT antigen protein in ImmortoMouse-derived cells [12]. Primary culture was maintained in a 5% humidified CO_2 atmosphere at 33 °C, and the medium was replaced as needed.

Antibodies. Mouse anti-SV40 LT Ag antibody (clone PAb419) was obtained from Oncogene (CA, USA). Mouse anti-cdc2 (clone C12720), anti-p27^{kipl} (clone K25020), anti-E-cadherin (clone 36), and β-catenin (clone 14) antibodies were purchased from Transduction Laboratories (Lexington, KY). Rat anti-Troma-1 (CK8) and Troma-2 (CK18) antibodies were purchased from Hybridoma Bank (Iowa, USA). Goat anti-p15^{INK4b} (clone M-20) and anti-vimentin (clone S-20), as well as mouse anti-p16^{INK4a} (clone M-156), anti-p21^{waf1} (clone C-19), anti-CDK2 (clone M2), anti-cyclin A (clone C-19), anti-cyclin B1 (clone GNS), anti-cyclin D1 (clone R-124), and anti-cyclin E (clone M-20) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-p53 (clone 9282) antibody was purchased from Cell Signaling (Beverly, MA). Mouse anti-pan-ras antibody (clone Ab-3) was purchased from Calbiochem (La Jolla, CA), while mouse anti-β-actin (A-5441) and anti-amylase antibodies were purchased from Sigma (St. Louis, MO). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgG secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and Cy3-conjugated goat anti-mouse or FITC-conjugated goat anti-rabbit IgG was from Jackson ImmunoResearch Laboratories (West Grove, PA).

Cell proliferation assay. Cells (1×10^4) were plated in 24-well tissue culture dishes and grown under four different sets of culture conditions (33 °C with IFN-γ, 33 °C without IFN-γ, 39 °C with IFN-γ, and 39 °C without IFN-γ) for the indicated time. Then the number of cells was determined with an electronic Coulter counter (Coulter, Hialeah, FL).

Immunofluorescence. Cells grown on sterile glass coverslips were fixed with 3.7% paraformaldehyde dissolved in phosphate-buffered saline (PBS) for 10 min at room temperature (RT), and then permeabilized in PBS with 2% Triton X-100 (PBST) for 5 min. Primary antibodies were diluted in PBS containing 0.2% bovine serum albumin and incubation was done for 1 h at RT. Blocking was performed in PBS containing 2% normal goat serum for 30 min at RT. Then the cells were washed three times with PBST and incubated for 1 h at RT with the secondary antibodies. The cells were washed another three times in PBST, mounted in Vectashield (Vector Laboratories, Burlingame, CA), and viewed under an Axioplan2 fluorescence microscope (Carl Zeiss, Thornwood, NY).

Electron microscopy. Cultured cells were fixed with 2% glutaraldehyde in 0.2 M phosphate buffer (pH 7.2) for 2 h at RT and postfixed with 1% osmium tetroxide for 1.5 h at 4 °C. After dehydration in ethanol and propylene oxide, cells were embedded in Epon according to standard techniques. Ultrathin sections were cut and stained with uranyl acetate and lead citrate for observation under an electron microscope.

RT-PCR analysis. Total cellular RNA was extracted using TRIZOL Reagent (Life Technologies, Rockville, MD) and cDNA was synthesized by random priming 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 (derived from 100 ng of RNA), 0.2 μM each of the sense and antisense primers, 0.2 μM of deoxynucleotide triphosphate, and 2.5 U *Taq* DNA polymerase in 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, CA). The following oligonucleotide primers were used to amplify amylase, CK8, CK18, CK19, p21$^{WAF1/CIP1}$, and β-actin: amylase—(sense) caggcaatcctgcaggaacaa, (anti-sense) cacttgcggat aactgtgcca; mouse CK8—(sense) agtctcagatctcagacacg, (anti-sense) ccataggatgaac tcagtcc; mouse CK18—(sense) ggacctcagcaagatcatggc, (anti-serise) ccacgatcttacgggtagttg; mouse CK19—(sense) gtcctacagat tgacaatgc, (anti-sense) cacgctctggatctgtgacag; p21—(sense) agcctgaag actgtgatggg, (anti-sense) aaagttccaccgttctcgg; and β-actin—(sense) atgg atgacgat atcgct, (anti-sense) atgaggtagtctgtcaggt. The size of the products was 483 (amylase), 577 (CK8), 515 (CK18), 570 (CK19), 228 (p21), and 569 (β-actin) bp, respectively. For PCR, initial denaturation was done at 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 56 °C for 1 min, and 72 °C for 1 min, with final extension at 72 °C for 5 min. Then the PCR products were separated on 1.5% agarose gel.

Western blot analysis. Western blotting was performed as described previously [13]. Briefly, cells were lysed for 30 min in radioimmunoprecipitation assay buffer (1× PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM sodium orthovanadate). Equal amounts of protein were loaded onto SDS–polyacrylamide gels and the proteins were transferred to Immobilon-P membranes (Millipore, Bedford, MA). Then the membranes were probed with the indicated antibodies, developed using the Enhanced Chemiluminescence System (Amersham, Arlington Heights, IL), and exposed to XAR5 film (Kodak, Rochester, NY).

Flow cytometric analysis. Both adherent and non-adherent cells were harvested, washed once with PBS, and fixed in ice-cold ethanol with vigorous mixing. Next, the cells were pelleted, washed once with PBS, and resuspended at 5×10^5/ml in 1 ml PBS containing 25 μg/ml propidium iodide and 0.1 mg/ml RNase, followed by incubation for 30 min in the dark at 30–37 °C. Flow cytometric analysis was performed on a FACScan Flow cytometer (Becton–Dickinson, San Jose, CA, USA) and data from 10,000 cells were collected for cell cycle analysis.

Adenoviral vector. We used a replication-deficient recombinant adenovirus with deletion of the E1A and E1B and E3 regions of human adenovirus type 5. A recombinant adenovirus carrying CA Ha-rasV12 (Ad-rasV12) was kindly provided by Dr. L. Parada (University of Texas, Southwestern Medical Center, Dallas, TX, USA) [14]. A control adenovirus that expressed *Escherichia coli*-lacZ (Ad-lacZ) was also prepared. These 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 a plaque assay with 293 cells. All of the vector preparations were demonstrated to be free of replication-competent adenovirus. The ability of the recombinant adenovirus to infect cells and cause gene expression at various multiplicities of infection (m.o.i.) was assessed by X-gal staining. IMPE cells were infected with Ad-rasV12 or Ad-lacZ at 20 m.o.i. for 2 h. Then the vector was removed and the infected cells were subjected to a soft agar assay.

Soft agar assay. Cells (5×10^4) were suspended in 0.8% agarose (Difco, Detroit, MI) in RPMI 1640 supplemented with 10% FBS and plated onto 6-well plates (Nunc, Naperville, IL) coated with 1% agar in RPMI 1640 with 10% FBS. Cultures were incubated at 33 or 39 °C and colony formation was assessed after 2 weeks by phase-contrast microscopy.

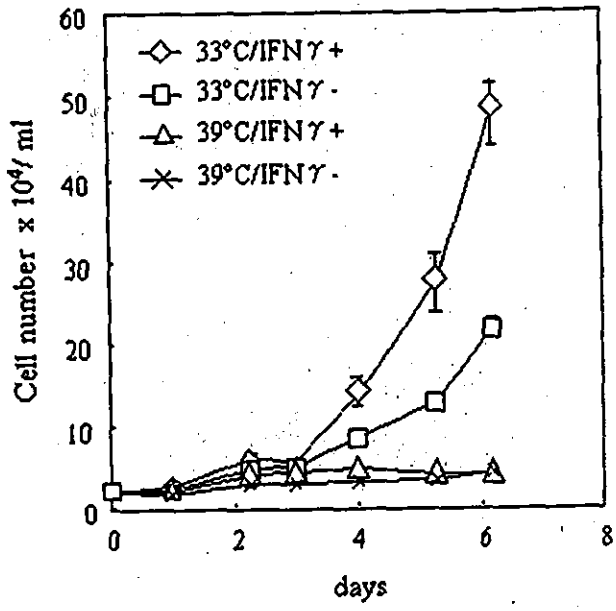

Fig. 1. Cell growth assay. IMPE cells (1×10^4) were seeded into 12-well plates and incubated under four different sets of culture conditions (33 °C with IFN-γ, 33 °C without IFN-γ, 39 °C with IFN-γ, and 39 °C without IFN-γ) for the indicated time. The number of cells was determined with an electronic Coulter counter. The experiments were repeated three times. Data represent means ± SEM of triplicate inserts from one of three representative experiments.

Results

Cell growth and morphology

A temperature-sensitive mutant of SV40 LT antigen (*ts*A58) confers temperature-dependent conformational changes on LT antigen that permit cell growth at the permissive temperature (33 °C) and stop growth at a non-permissive temperature (39 °C) [12]. When cultured at the permissive temperature, pancreatic cells attached to the collagen-coated surface of the plate and spread out to form small islands of epithelial cells. Over the first 7 days, the majority of the island cells died until only a few cells remained at the center of each island. These cells continued to proliferate until confluent monolayers were formed. The established pancreatic cells were designated as immortalized pancreatic epithelial (IMPE) cells.

Growth of IMPE cells was examined under four different sets of conditions. As shown in Fig. 1, IMPE cells cultured under absolutely permissive conditions (33 °C with IFN-γ) grew continuously, but rapidly showed growth arrest under non-permissive conditions (39 °C without IFN-γ). IMPE cells cultured under relatively permissive conditions (33 °C without IFN-γ) showed moderate growth compared with the cells under absolutely permissive conditions. These data showed that the temperature-sensitive SV40 LT antigen (*ts*A58) mutant functioned very well in this cell line.

The light microscopic appearance of IMPE cells was influenced by the culture conditions. The cells displayed a more epithelial-like appearance when cultured under permissive conditions (33 °C with IFN-γ, Fig. 2A) than under non-permissive conditions (39 °C without IFN-γ, Fig. 2B). At 39 °C, cells gradually became sparse and adopted a spindle-like morphology. Examination of the cells by electron microscopy revealed a high degree of polarity of the intracellular organelles, existence of tight junctions, and a lack of zymogen granules (Figs. 2C and D). Very few microvilli were seen. A high degree of polarity and tight junctions are key features of epithelial cells, while zymogen granules are a distinctive marker of pancreatic acinar cells.

Expression of large T-antigen by IMPE cells

We examined whether the expression of LT antigen was regulated by culture conditions. IMPE cells were cultured for 4 days under permissive or non-permissive conditions, and Western blotting and immunofluorescence were performed. Western blot analysis revealed that SV40 LT Ag was strongly expressed by the cells under permissive culture conditions, whereas expression was markedly down-regulated under non-permissive conditions (Fig. 3A). Immunofluorescence also

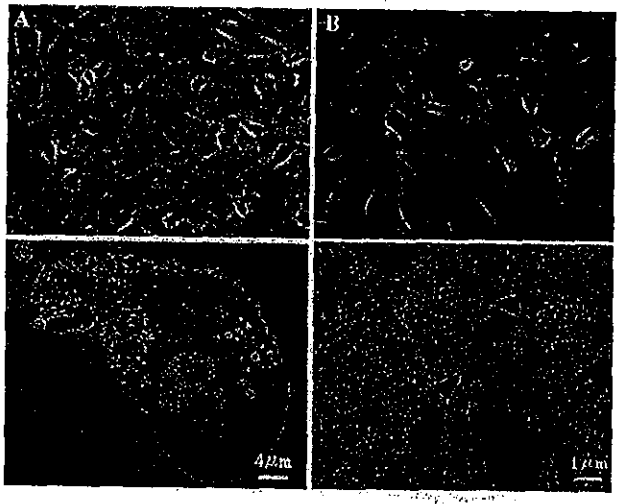

Fig. 2. Microscopic images of IMPE cells. The IMPE cells were seeded and maintained under permissive or non-permissive culture conditions for 4 days. Cell morphology was observed by light (A,B) or electron microscopy (C,D). (A) The cells grew rapidly and showed cobblestone appearance under permissive culture conditions (original magnification 200×). (B) The cells became sparse and displayed spindle-like shape under non-permissive conditions (original magnification 200×). (C) Electron microscopic image revealed a high degree of polarity in their intracellular organization and the lack of zymogen granules. Bar: 4 μm. (D) Tight junctions (arrowheads) were detected. Bar: 1 μm.

demonstrated that LT antigen was expressed in the nucleus of every cell under permissive conditions (Fig. 3B).

Expression of epithelial, exocrine, and endocrine markers by IMPE cells

Next, we investigated the characteristics of IMPE cells. In order to determine whether these cells had originated from epithelial or mesenchymal cells of the pancreas, expression of E-cadherin, β-catenin, and vimentin was examined by Western blot analysis. E-cadherin and β-catenin (epithelial markers) showed almost equal expression by IMPE cells under both permissive and non-permissive culture conditions. In contrast, the expression of vimentin (a mesenchymal marker) was not detected under both conditions (Fig. 4A). These data suggested that IMPE cells had originated from pancreatic epithelial cells.

Pancreatic epithelial cells consist of acinar, ductal, and endocrine cells. To clarify the origin of IMPE cells, amylase, CK8, CK18, CK19, insulin, and pdx-1 mRNA expression was examined by RT-PCR and immunofluorescence. RT-PCR revealed that amylase (an acinar cell marker) was weakly expressed only under permissive conditions, but not under non-permissive conditions. CK8 and 18 (acinar and ductal cell markers) were expressed under both conditions (Fig. 4B). Expression of mRNA for CK19, a specific ductal cell marker, was also observed under both culture conditions. Endocrine markers, including insulin and pdx-1, were not detected by RT-PCR under both sets of conditions (data not shown). Similarly, immunofluorescence showed weak expression of amylase only under permissive conditions. Troma-1 (CK8) and Troma-2 (CK18) expression was found under both conditions (Fig. 4C). These results indicated that IMPE cells were mainly derived from ductal or acinar cells, but not from pancreatic endocrine cells.

Flow cytometric analysis of IMPE cells

As described above, IMPE cells ceased proliferation under non-permissive conditions. In order to determine whether cells cultured under non-permissive conditions underwent apoptosis or growth arrest, flow cytometric analysis of propidium iodide-stained cells was performed and representative results are shown in Fig. 5. There was a significant increase in the population of cells in G2/M phase from 18% under permissive culture conditions to 44% under non-permissive conditions. This increase was mirrored by a decrease in the percentage of cells in G1 and S phases. Since an increase of the sub-G1 cell population was detected under non-permissive conditions, we tried to detect apoptotic cells by immunocytofluorescence of single-stranded DNA (ss-DNA) [15]. However, ss-DNA positive cells were not seen under both conditions (data not shown). These results indicated that the cessation of IMPE cell growth under non-permissive conditions was induced by G2/M arrest, but not by G1 arrest or the induction of apoptosis.

Expression of cell cycle-related molecules

SV40 LT antigen immortalizes some cells by binding to the tumor-suppressor proteins p53 and retinoblastoma protein (pRb), and blocking their activity. To examine how expression of cell cycle-associated proteins was modified by SV40 LT antigen in IMPE cells, Western blot analysis was performed. As expected, p53 protein was expressed under non-permissive culture conditions, whereas it was not detected under permissive conditions (Fig. 6A). These results suggested that the antigen bound and inactivated p53 under permissive conditions, and that p53 expression could be regulated by altering the culture temperature in this system. We also tried to examine the pRb status in this system by Western blot analysis using a mouse monoclonal antibody against pRb (clone: 3H9, Santa Cruz Biotechnology). However, we failed to detect the expression of pRb protein under non-permissive culture conditions. The Rb family is composed of at least three proteins, pRb, p107, and p130, which bind to the E2F family of transcription factors, and these proteins are thought to have overlapping functions in different stages of the cell cycle.

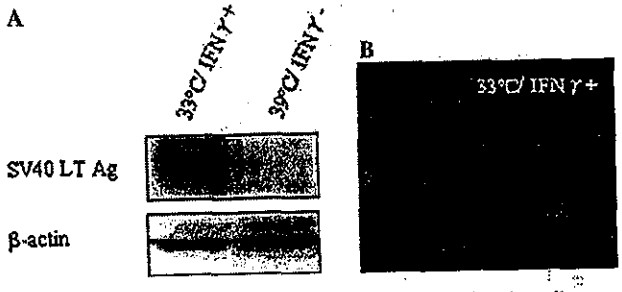

Fig. 3. Expression of SV40 LT antigen by IMPE cells. The cells were cultured under permissive or non-permissive conditions for 2 days. (A) The protein was extracted from the cultured cells. Western blot analysis showed the strong expression of SV40 LT antigen under permissive conditions, but not under non-permissive conditions. (B) Immunofluorescence study also showed the expression of the antigen in the nucleus of every cell under permissive conditions.

Although it remains unclear whether or not the status of pRb protein family could be regulated by the switch of culture conditions in this system, it might be dependent on the status of pRb, p107, and p130–E2F complexes. $p21^{WAF1/CIP1}$, which is a cyclin-dependent inhibitor (CDK) and one of the molecules downstream of p53, was expressed under non-permissive conditions, but its expression was severely attenuated under permissive conditions. As shown in Fig. 6B, p21 mRNA was only expressed under non-permissive culture conditions, indicating that p21 expression showed transcriptional regulation in this system. Other CDK inhibitors (p15, p16, and p27), CDKs (cdc2 and CDK2), and cyclins (A, B, D1, and E) showed almost equal expression under both conditions (Fig. 6). These findings demonstrated that strong expression of SV40 LT antigen under permissive conditions suppressed the expression of p53 and p21 protein, so that cells could continue to grow through by avoiding G2/M arrest.

Anchorage-independent growth

Finally, we investigated whether normal pancreatic epithelial cells could acquire anchorage-independent growth by specific cooperation between molecules

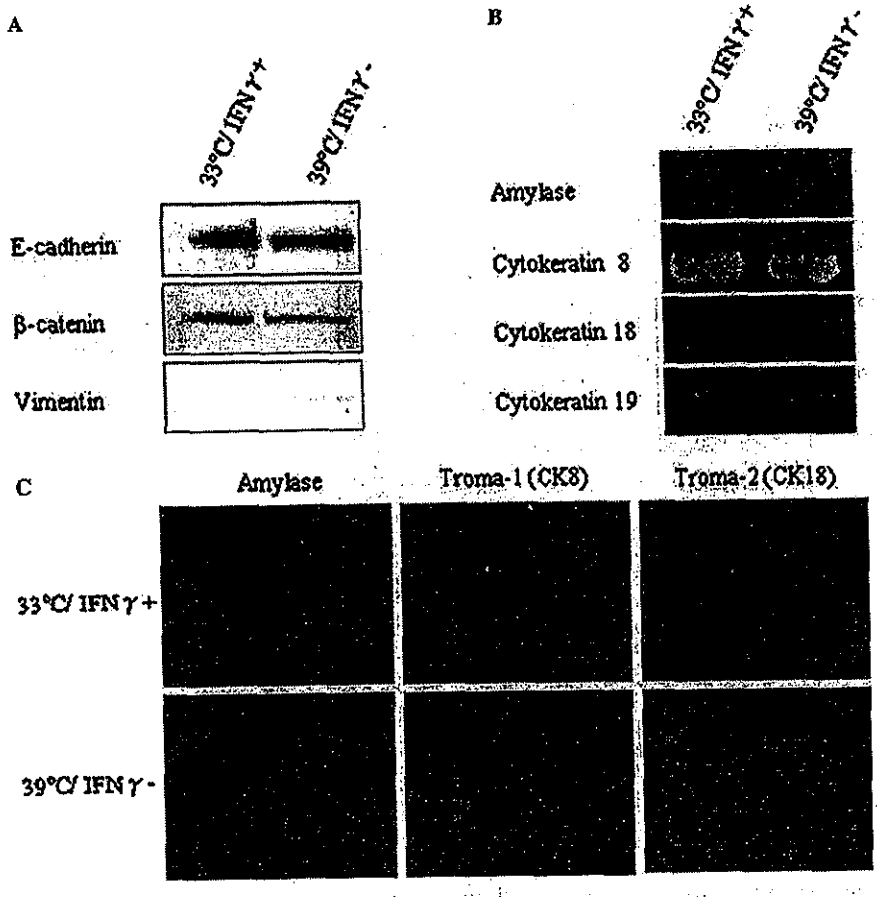

Fig. 4. Expression of epithelial, exocrine, and endocrine markers by IMPE cells. (A) Western blot analysis showed that epithelial markers such as E-cadherin and β-catenin were positive for both conditions. (B) RT-PCR analysis revealed that amylase was faintly expressed only under permissive conditions. CK8, CK18, and CK19 were also observed at both conditions. (C) Similarly, immunofluorescence study showed faint expression of amylase only under permissive conditions. Expression of Troma-1 (CK8) and Troma-2 (CK18) was observed at both conditions.

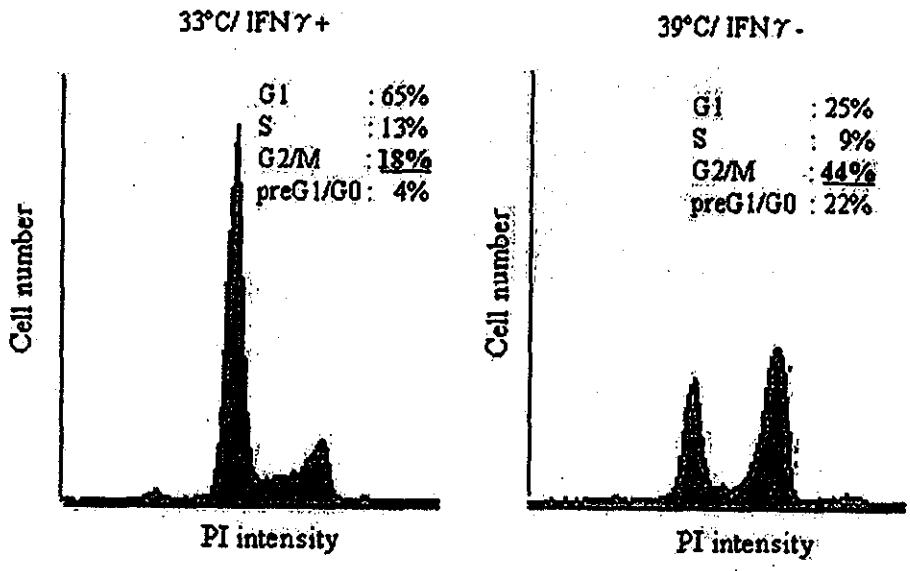

Fig. 5. Cell cycle analysis in IMPE cells. The cells were cultured under permissive or non-permissive conditions for 3 days. The data from 10,000 cells were collected for cell cycle analysis. There was a significant increase in the cells of G2/M phase from 18% under permissive conditions to 44% under non-permissive conditions. This increase was mirrored by a decrease in the proportion of cells in the G1 and S phases.

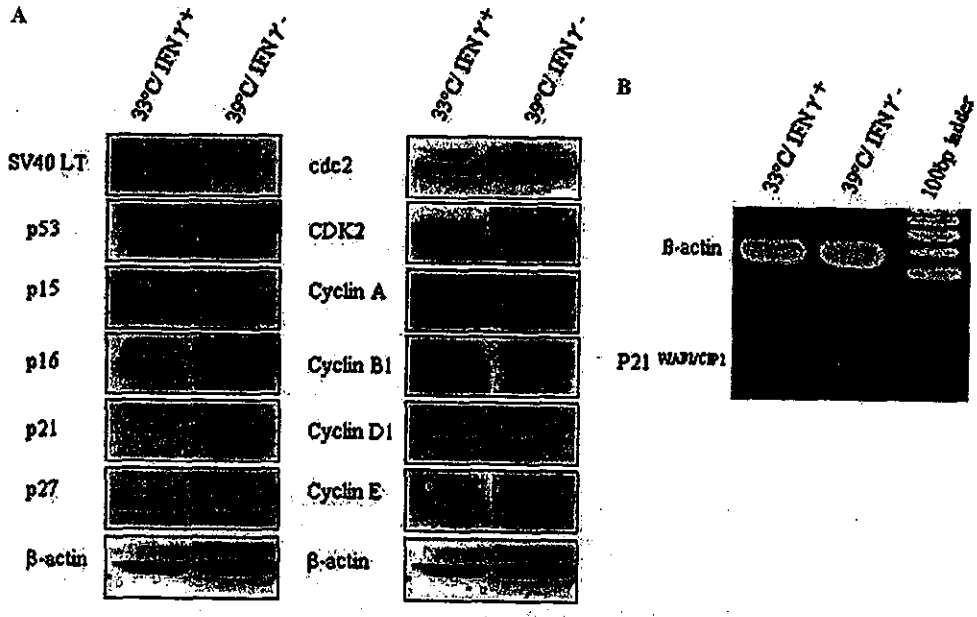

Fig. 6. Expression of cell cycle-related molecules. The IMPE cells were cultured under permissive or non-permissive conditions for 2 days. (A) Western blot analysis showed the expression of active p53 and $p21^{WAF1/CIP1}$ proteins under non-permissive conditions, whereas they were not detected under permissive conditions. Expression level of other proteins was almost equal. (B) RT-PCR analysis showed the expression of p21 mRNA only under non-permissive conditions.

known to be altered in pancreatic cancer, since it was speculated that IMPE cells could mimic normal pancreatic epithelial cells when cultured under non-permissive conditions. In particular, we were interested in assessing the combined effect of ras activation and loss of p53 function. As described above, growth under different culture conditions allowed us to examine the effects of various combinations of genetic alterations (ras activation with p53 inactivation) on the phenotype of IMPE cells. Ras was chosen as a target molecule because Ki-ras mutation occurs in more than 90% of invasive PDA and is thought to play an important role in pancreatic carcinogenesis. Although Ha-ras and Ki-ras isoforms are preferentially mutated in different types of

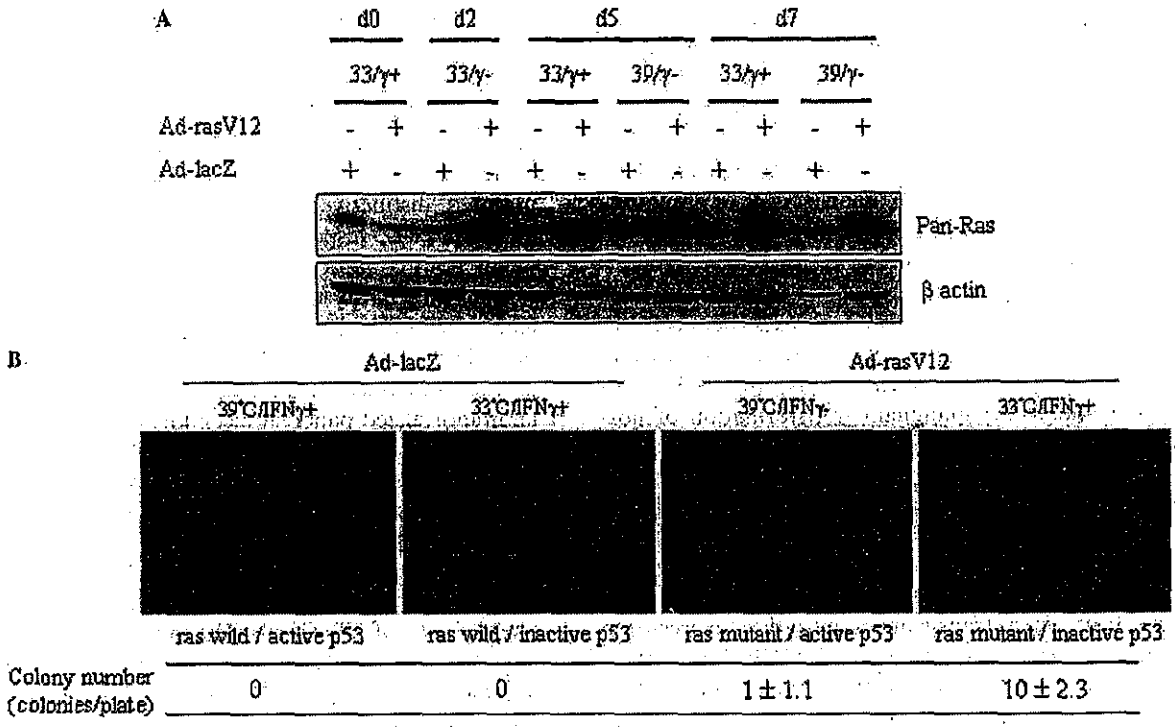

Fig. 7. Adenoviral infection of Ha-rasV12 and anchorage-independent growth in IMPE cells. (A) IMPE cells were infected with Ad-rasV12 or Ad-lacZ at 20 m.o.i. for 2 h. The infected cells were cultured under either permissive or non-permissive conditions for the indicated time, and proteins were extracted and probed with pan-ras and β-actin antibodies. (B) The cells were suspended in soft agar and cultured in a 5% humidified CO_2 atmosphere under either permissive or non-permissive conditions. Colony formation was observed on day 14. Soft agar assay was repeated three times using the different IMPE cells with or without adenoviral infection of Ha-rasV12 under permissive or non-permissive conditions. A representative colony formation composed of more than 10 cells is shown under each culture condition (original magnification 200×). The number of colonies is described as the mean ± SEM of one of three representative experiments below each photograph. Overexpression of ras under permissive conditions (ras mutant/inactive p53) led to the anchorage-independent growth as measured by soft agar assay whereas the cells without overexpression of ras showed no colony-forming activity at both conditions.

cancers, their products are similar in structure and function, and it has been thought that the oncoproteins encoded by mutated Ha-ras and Ki-ras generally impinge on identical transcriptional targets. Therefore, in the present study, we infected cells with constitutive active Ha-ras using an adenoviral Ha-rasV12 construct to examine the effects of cellular transformation by ras activation, although most of the human pancreatic cancers harbor Ki-ras mutations. To confirm whether or not mutated Ki-ras gene has the similar effects as mutated Ha-ras gene for transformation in IMPE cells, we are now trying to establish the IMPE cells infected with a retrovirus encoding human Ki-ras (G12V) in our laboratory. As shown in Fig. 7A, Ad-rasV12 was successfully used to infect IMPE cells, and strong expression of Ras protein was observed for at least 7 days after infection. Ha-ras-expressing IMPE cells proliferated more rapidly than control cells infected with Ad-lacZ under both conditions (data not shown). In order to examine anchorage-independent growth, the soft agar assay was performed under four different sets of culture conditions: (1) non-permissive conditions with Ad-lacZ infection (ras wild/active p53), (2) permissive conditions with Ad-lacZ infection (ras wild/inactive p53), (3) non-permissive conditions with Ad-rasV12 infection (ras mutant/active p53), and (4) permissive conditions with Ad-rasV12 infection (ras mutant/inactive p53). The cells did not show any colony-forming activity under both conditions without Ad-ras infection, whereas cells with Ad-ras infection were capable of anchorage-independent growth when cultured under permissive conditions, indicating that these cells had been transformed (Fig. 7B).

Discussion

In the present study, we showed that activation of ras combined with inactivation of p53 could induce in vitro tumorigenicity in conditionally immortalized pancreatic epithelial (IMPE) cells, whereas either genetic alteration alone was not sufficient for malignant transformation.

Pancreatic ductal adenocarcinoma (PDA) is thought to arise from the pancreatic ducts on the basis of histological and immunohistochemical relationships to duct cells [3]. Consistent with a ductal origin, premalignant lesions, known as pancreatic intraepithelial

neoplasias (PanINs), are found in close proximity to invasive PDA [5,16]. However, the cell-of-origin question is complicated by the developmental plasticity of the pancreas that allows transdifferentiation between cell lineages [3]. For example, acinar cells have been shown to undergo metaplastic conversion to duct-like cells [17]. Other studies have suggested that islet cells or a putative pancreatic stem cell population may also give rise to pancreatic adenocarcinoma [18]. At present, it remains unclear whether this tumor arises from any one of these differentiated cell types or from pancreatic stem cells. In addition, it also remains unclear how oncogenes and tumor-suppressor genes such as ras, p16, p53, and DPC4 modulate the malignant transformation of normal pancreatic epithelial cells.

In the present study, we established conditionally immortalized normal pancreatic epithelial (IMPE) cells from *H-2K*^{b}-*ts*A58 transgenic mice (ImmortoMouse) to examine the combined actions of molecules mentioned above on normal pancreatic epithelial cells. The ImmortoMouse is a transgenic mouse that expresses a temperature-sensitive mutation of SV40 LT antigen (*ts*A58 mutant). The protein produced by this mutant gene is rapidly degraded at 39 °C (normal mouse body temperature), but is active at 33 °C. So far, numerous immortalized cell lines have been established from the ImmortoMouse, including intestinal epithelial cells, colon epithelial cells, gastric epithelial cells, hepatocytes, vascular smooth muscle cells, and pancreatic epithelial cells [9,19–21]. As with these previously established cell lines, IMPE cells proliferated indefinitely under permissive culture conditions (33 °C with IFN-γ), but proliferation ceased under non-permissive conditions (39 °C without IFN-γ) (Fig. 1). We confirmed that SV40 LT antigen was strongly expressed by IMPE cells incubated under permissive conditions, whereas the expression was markedly down-regulated under non-permissive conditions (Fig. 3). Although the weak signal of SV40 LT Ag was still detected under non-permissive conditions and this might mean that the expression could not be completely blocked under the conditions, it appears to be important that p53 and p21$^{WAF1/CIP1}$ were clearly expressed, in spite of the weak expression of LT Ag, under non-permissive conditions (Fig. 6A). Phase-contrast microscopy revealed that IMPE monolayers showed a cobblestone-like appearance, which is one of the characteristics of epithelial cells (Fig. 2). The epithelial phenotype of IMPE cells was also confirmed by Western blot analysis using antibodies for E-cadherin and β-catenin (Fig. 4A). In order to examine the origin of IMPE cells, markers for pancreatic acinar, ductal, and endocrine cells were tested. CK8 and 18 (acinar and ductal cell markers) were expressed under both sets of culture conditions. The expression of mRNA for CK19, a specific ductal cell marker, was also observed under both conditions (Fig. 4B). Intriguingly, the expression of mRNA for amylase, a specific acinar cell marker, was only detected under permissive conditions. Markers specific for endocrine cells, such as insulin and pdx-1, could not be detected by RT-PCR under either set of conditions (data not shown). Electron microscopy revealed that neither zymogen granules nor secretary granules, distinctive markers for pancreatic acinar cells and islet cells, respectively, were observed in the cells under both conditions. It is well known that epithelial cells show polarity of their intracellular organelles and feature tight junctions. IMPE cells showed a high degree of polarity and had tight junctions on their surface (Fig. 2). Previously, Blouin et al. [21] have also reported an immortalized pancreatic cell line (IMPAN cells) that was derived from the *H-2K*^{b}-*ts*A58 transgenic mice. They demonstrated that IMPAN cells displayed the characteristics of acinar cells, including strong expression of amylase and zymogen granules, at the permissive temperature of 33 °C, although the expression of markers of the cellular origin at the non-permissive temperature of 39 °C and the phenotypic alterations including cell proliferation under different culture conditions were not reported. In contrast to IMPAN cells, our IMPE cells mainly showed the characteristics of duct cells, although there is a possibility that their origin was from pancreatic pluripotent stem cells, the existence of which is still controversial [22].

To further investigate the characteristics of IMPE cells, cell cycle analysis was performed using flow cytometry. We speculated that loss of SV40 LT antigen expression led to G1 arrest or apoptosis through the expression of wild-type p53 and retinoblastoma protein under non-permissive conditions. Contrary to our expectation, the shift from permissive temperature (33 °C) to non-permissive temperature (39 °C) induced G2/M arrest in IMPE cells. In addition, strong expression of p53 and p21$^{WAF1/CIP1}$ protein, which is one of the transcriptional target molecules of p53, was observed after inactivation of SV40 LT antigen under non-permissive culture conditions. Although it is well known that p21$^{WAF1/CIP1}$ can induce arrest at the G1/S transition in many types of cells, recent studies have demonstrated that induction of p21$^{WAF1/CIP1}$ expression might also participate in the G2 checkpoints [23,24]. Chang et al. [24] reported that genistein arrests hepatoma cells at G2/M phase through the induction of p21$^{WAF1/CIP1}$ expression.

Activating Ki-ras mutations are thought to be the first genetic changes detected during progression towards PDA. The p53 tumor-suppressor gene shows mutation, generally by missense alterations of the DNA-binding domain, in more than 50% of these cancers and p53 mutations arise in later-stage PanINs that have acquired features of dysplasia, reflecting the role of active p53 in preventing malignant progression [3]. Our data suggested that IMPE cells had most of the characteristics of normal

pancreatic epithelial cells under non-permissive culture conditions. The shift from non-permissive to permissive conditions conferred anchorage-dependent growth on the cells by escape from G2/M arrest with inactivation of wild-type p53. We hypothesized that activation of ras and inactivation of p53 cooperated to induce tumorigenicity of IMPE cells. In order to examine whether or not these cells could acquire anchorage-independent growth (in vitro tumorigenicity) after introduction of a mutant ras gene, we performed three-dimensional culture of adenoviral constitutively active Ha-rasV12 mutant cells. In the soft agar assay, only Ha-ras-expressing IMPE cells cultured under permissive conditions (ras mutant/inactive p53) formed colonies, whereas neither activation of ras nor inactivation of p53 alone allowed colony formation under both sets of culture conditions (Fig. 7B). These data suggest that inactivation of p53 could immortalize IMPE cells, but was not sufficient for transformation, and that cooperation between ras activation and p53 inactivation was needed to induce in vitro tumorigenicity of IMPE cells. Similarly, recent studies have demonstrated that ras mutation and p53 inactivation cooperate to induce malignant transformation [8,25]. Azzoli et al. [25] reported that loss of p53 function and v-H-ras cooperated in the transformation of mouse keratinocyte cells, which were stably transfected with temperature-sensitive p53 and constitutively active v-H-ras. Consistent with our results, this stable clone formed colonies under permissive conditions (active p53 and ras mutation), whereas colony formation was blocked under non-permissive conditions (inactive p53 and ras mutation). Lohr et al. [8] have reported that SV40-immortalized bovine pancreatic duct cells could acquire tumorigenicity after transduction of constitutive active Ki-ras.

In conclusion, we established conditionally immortalized pancreatic epithelial cells from *H-2K^b-ts*A58 transgenic mice. These cells mainly showed the characteristics of duct cells and could proliferate continuously in culture under permissive conditions, whereas p53 was turned off under non-permissive conditions and proliferation ceased following G2/M arrest. Furthermore, ras activation and p53 inactivation could cooperatively induce anchorage-independent cell growth of IMPE cells, although ras activation or loss of p53 function alone did not lead to transformation. This in vitro model of conditionally immortalized pancreatic epithelial cells may be useful for systematic analysis of genetic alterations implicated in pancreatocarcinogenesis and might serve as a platform for the identification of early disease markers and for testing of novel therapies.

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N-Cadherin Expression and Epithelial-Mesenchymal Transition in Pancreatic Carcinoma

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ABSTRACT

Purpose: **Loss of intercellular adhesion and increased cell motility promote tumor cell invasion. In the present study, E- and N-cadherin, members of the classical cadherin family, are investigated as inducers of epithelial-to-mesenchymal transition (EMT) that is thought to play a fundamental role during the early steps of invasion and metastasis of carcinomas. Cell growth factors are known to regulate cell adhesion molecules. The purpose of the study presented here was to investigate whether a gain in N-cadherin in pancreatic cancer is involved in the process of metastasis via EMT and whether its expression is affected by growth factors.**

Experimental Design: **We immunohistochemically examined the expression of N- and E-cadherins and vimentin, a mesenchymal marker, in pancreatic primary and metastatic tumors. Correlations among the expressions of N-cadherin, transforming growth factor (TGF)β, and fibroblast growth factor 2 was evaluated in both tumors, and the induction of cadherin and vimentin by growth factors was examined in cultured cell lines.**

Results: **N-cadherin expression was observed in 13 of 30 primary tumors and in 8 of 15 metastatic tumors. N-cadherin expression correlated with neural invasion (P = 0.008), histological type (P = 0.043), fibroblast growth factor expression in primary tumors (P = 0.007), and TGF expression (P = 0.004) and vimentin (P = 0.01) in metastatic tumors. Vimentin, a mesenchymal marker, was observed in a few cancer cells of primary tumor but was substantially expressed in liver metastasis. TGF stimulated N-cadherin and vimentin protein expression and decreased E-cadherin expression of Panc-1 cells with morphological change.**

Conclusion: **This study provided the morphological evidence of EMT in pancreatic carcinoma and revealed that overexpression of N-cadherin is involved in EMT and is affected by growth factors.**

INTRODUCTION

Cadherins, calcium-dependent cell adhesion molecules, are involved in maintaining the epithelial structure of a variety of tissues and play important roles in embryonic development and maintenance of normal tissue architecture (1). It has been well established that E-cadherin plays a role in tumor progression and metastasis, because loss of E-cadherin expression has been found to correlate with an invasive and undifferentiated phenotype in many carcinomas including pancreatic carcinoma (2–7). N-cadherin (neural cadherin), another adhesion molecule, is associated with a heightened invasive potential in cancer. A recent study demonstrated that overexpression of N-cadherin in breast carcinoma correlates with invasiveness as a result of N-cadherin-mediated interactions between cancer and stromal cells (8). The phenotype of breast cancer cell lines was found to undergo dedifferentiation from epithelial to mesenchymal as a result of N-cadherin transfection without a loss of E-cadherin expression (9). In squamous epithelial cells, expression of N-cadherin produced a scattered phenotype with an epithelial-to-mesenchymal transition (EMT) in association with a reduction in E- and P-cadherins (10). In N-cadherin transfected breast cancer cells, N-cadherin promotes motility and invasion, but the reduction in the expression of E-cadherin does not necessarily correlate with either of these two (11). These findings indicate that N-cadherin, functioning as adhesion molecules, may be more important than E-cadherin for metastasis and invasion.

Changes in cell adhesion, regulated by environmental signals such as growth factors, appear to be necessary for dynamic cellular movement and maintenance of tissue patterning. Growth factors and cytokines can modulate expression of E-cadherin; for example, transforming growth factor (TGF)β induces dedifferentiation of the phenotype of normal mammary epithelial cells from epithelial to fibroblastic, which correlates with a reduction in the expression of E-cadherin (12). Fibroblast growth factor (FGF)-1 and FGF-2 enhance E-cadherin-mediated cell-cell adhesion and reduce *in vitro* invasion in cancer cells (13, 14). Furthermore, N-cadherin-dependent motility may be mediated by FGF receptor signaling, but the mechanism of regulating cadherin expression is not known (8, 11).

Pancreatic cancer has a very poor prognosis, and the 5-year survival rate for patients who underwent surgical resection is reported to be only 8.1–24.0% (15–18). The reasons for such poor prognosis are a high incidence of local recurrence, lymph node metastasis, hepatic metastasis, and peritoneal dissemination. As pancreatic cancer progresses, a high rate of neural

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invasion, which is associated with poor prognosis, is observed and increases even more as the cancer becomes undifferentiated (19–21). One of the reasons that pancreatic cancer extends along the neural bands is probably due to the abundance of nerves inside and around the pancreas. Another possibility is that the adhesion molecules, which define the affinity of cancer cells to neural band, subsequently affect the motility of cancer cells. One study of the relationship between neural cell adhesion molecule expression and neural invasion found no correlation (22). Because N-cadherin is highly prevalent in neuronal tissues and is also found in fibroblasts, muscles, vascular endothelium, and peritoneal mesothelial cells (23–26), it is important to investigate the association between the expression of N-cadherin in pancreatic cancer and its invasiveness including neural invasion.

The purpose of this study presented here was to investigate whether a gain in N-cadherin in pancreatic cancer is involved in the process of metastasis via EMT and whether its expression is affected by growth factors. To this end, the expression of N- and E-cadherins and vimentin, a mesenchymal marker, was immunohistochemically examined in pancreatic primary and metastatic tumors. In addition, clinicopathological parameters including patient prognosis were assessed in relation to N-cadherin expression. Correlations among the expressions of N-cadherin, TGFβ, and FGF were evaluated in both primary and metastatic tumors. Finally, the induction of cadherin and vimentin by growth factors was examined in cultured cell lines.

MATERIALS AND METHODS

Antibodies and Growth Factors. Monoclonal mouse immunoglobulin (IgG) antibodies to N-cadherin were purchased from Zymed Laboratories Inc. (San Francisco, CA), E-cadherin from Takara Bio Inc. (Shiga, Japan), and vimentin from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies to FGF2 and TGFβ were obtained from Santa Cruz Biotechnology. Human recombinant FGF-2 and TGFβ were obtained from R&D Systems (Minneapolis, MN).

Patients and Paraffin-Embedded Tissue Sample. Thirty tissue samples were obtained from patients with primary pancreatic cancer who were operated on at the Department of Surgery and Surgical Basic Science of Kyoto University Hospital (Kyoto, Japan) between January 1997 and June 2000. The average age at surgery was 66.3 years (range, 46–76). We chose only those patients who had survived at least 60 days after surgery to exclude perioperative mortality-related bias. Follow-up data were updated on December 31, 2002 (median follow-up was 10.1 months; range, 3.0–43.9). Tissue samples were fixed with 10% formaldehyde in PBS, embedded in paraffin, and cut into consecutive 4-μm-thick sections. All of the tumors were diagnosed and confirmed as invasive ductal adenocarcinomas at the Department of Pathology, Kyoto University Hospital. Pancreatic cancer was staged according to the Putnam (Unio Internationale Contra Cancrum) system (27) and additionally characterized with the Japan Pancreas Society classification (28). Fifteen samples of hepatic metastasis were collected separately. A total of 45 samples were used for immunohistochemistry of N- and E-cadherins, vimentin, TGFβ, and FGF-2.

Immunohistochemistry. Because the avidin-biotin complex method using various dilution series of primary and secondary antibodies did not lead to any positive N-cadherin immunoreaction, the Catalyzed Signal Amplification System was implemented. The Catalyzed Signal Amplification System is up to 1000 times more sensitive than the usual immunoenzymatic detection systems and allows for the detection of small amounts of antigen with monoclonal antibodies, which are normally considered unsuitable for paraffin sections (29). The standard immunoperoxidase technique was used for E-cadherin, TGFβ, FGF-2, and vimentin.

Paraffin sections were dewaxed in three changes of xylene, followed by rinsing in graded ethanol and finally three courses of dehydration with double-distilled water. For antigen retrieval, the slides were pretreated in a Target Retrieval Solution (S3307; DAKO, Carpinteria, CA), heated in a hot water bath for 20 min at 95°C, followed by cooling down at room temperature for 20 min. Next, they were soaked in 3% H_2O_2 for 10 min and then treated with an endogenous biotin blocking reagent (X0590; DAKO) to block endogenous peroxidase activities. Next, the sections were incubated for 10 min at room temperature with 50 mM Tris-HCl buffer containing 0.15 M NaCl and 0.1% Tween 20 (TBST). The N-cadherin antibody diluted to 1:1000 with antibody dilution solution (DAKO) was applied to the section followed by incubation for 15 min at room temperature. The sections were washed with TBST three times for 5 min at room temperature, after which the Catalyzed Signal Amplification System (K1500; DAKO) was used to detect N-cadherin. Staining was completed with 30-s incubation with diaminobenzidine-tetrahydrochloride. E-cadherin, TGF β, FGF-2 and vimentin primary antibodies were diluted to 1:100 and incubated at 4°C overnight. After being washed three times in PBS, the sections were incubated with the appropriate peroxidase-labeled secondary antibodies for 1 h at room temperature and incubated with streptavidin-peroxidase complex. The sections were then washed again and developed for 1–10 min with diaminobenzidine-tetrahydrochloride in 50 mM Tris-buffered saline containing 20 μl of 30% H_2O_2 as the substrate. Finally, all of the sections were rinsed with distilled water and counterstained with Mayer's hematoxylin and mounted. To confirm the specificity of the results, we exposed nonspecific IgG as the primary antibody to several samples, and none of them showed any immunoreaction.

Evaluation of Immunostaining. Two investigators (S. N., S. T) simultaneously assessed the results of immunostaining without knowledge of the patient clinicopathological details. The intensity of staining was evaluated with the method described previously (5, 22, 30–32). The samples were then divided into two groups based on the intensity of staining of N-cadherin in cancer cells, a low N-cadherin group in which ≤20% of the cancer cells were stained and a high N-cadherin in which >20% were stained. E-cadherin expression in the tumors was graded according to the proportion of positive cells. E-cadherin expression was considered to be normal if >90% of cancer cells exhibited a staining pattern similar to that in normal epithelial cells, and sections with <10% of the cancer cells stained or with complete absence of staining were classified as reduced pattern. The intensities of FGF-2, TGFβ, and vimentin

staining were also divided into two groups in the same way as that of N-cadherin staining.

Cells. Five human pancreatic cancer cell lines, AsPC-1, BxPC-3, Capan-2, Miapaca-2, and Panc-1, were purchased from American Type Culture Collection (Rockville, MD). Cells were grown in monolayer culture in RPMI 1640 (Life Technologies Inc., Gaithersburg, MD) containing 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 37°C in a humidified atmosphere composed of 95% air and 5% CO_2.

After the cells had been incubated for 24 h at 37°C, fresh serum-free medium was added alone or supplemented with 5 or 10 ng/ml of FGF-2 or TGFβ. The cells were then kept for an additional 24–48 h at 37°C. For the activation of the FGF receptor, 1 μg/ml of heparin was added to the FGF-2. Protein expression of N-, E-cadherin, and vimentin with or without FGF and TGFβ treatment was evaluated by using Western blot and immunocytochemical analysis.

Protein Extraction and Western Blotting. Cells were harvested and lysed with radioimmunoprecipitation assay buffer [10 mM PBS (pH 7.4), 0.1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS containing 1 mM of phenylmethylsulfonyl fluoride and gabexate mesilate]. Total extracts were cleared by centrifugation at 14,000 for 10 min at 4°C, and the extracted protein was then subjected to Western blotting as described previously (3). Fifty-μg aliquots of protein were loaded onto 7.5% SDS-polyacrylamide gels and transblotted to a 0.45-μm immoblin-P transfer membrane (Millpore, Bedford, MA). The blots were blocked at 4°C overnight with 5% nonfat milk in TBST [50 mM Tris-HCL (pH 7.4), 150 mM NaCl, and 0.2% Tween-20] and reacted with appropriately diluted primary antibody solutions (1:100) for 1 h at room temperature. The enhanced chemiluminescence system (Amersham Life Sciences, Amersham, United Kingdom) was used for the detection of bound antibodies. Primary antibody-bound membranes were incubated for 1 h at room temperature with horseradish peroxide-conjugated, antimouse IgG diluted with TBST. After washing with TBST and Tris-buffered saline, the membranes were treated with enhanced chemiluminescence reagents according to the manufacturer's protocol. The membranes were exposed to X-ray film for 1–15 min. Protein expression was measured with the ATTO spot analyzer system AE-6920M (ATTO Corporation, Tokyo, Japan). The quantity of target protein was calibrated by that of β-actin, and relative intensities were obtained.

Immunocytochemical Analysis. After the cells had been grown on glass coverslips to 50% confluence, they were washed with PBS and fixed with 100% ethanol and 100% acetic acid (9:1) for 10 min on ice. Only for vimentin processing, the cells were incubated with 2% Triton X in PBS. For all of the other processes, they were incubated with the N-, E-cadherin, and vimentin monoclonal antibody for 1 h at room temperature. Nonspecific protein was blocked with 2% normal goat serum in PBS for 30 min. After washing, Cy3-conjugated secondary antimouse IgG was applied in the dark followed by incubation for 1 h at room temperature. Finally, the cells were observed under a fluorescence microscope.

Statistical Analysis. Relationships between the clinicopathologic characteristics of the 30 patients with high and low N- and E-cadherins were examined with the χ^2 test or Fisher's exact probability test. Survival rates were calculated with the Kaplan-Meier method, and the differences between high and low N-cadherin expression groups were evaluated with the log-rank test. The results in *in vitro* experiments are expressed as the mean value ± SD. Statistical differences among each time point were assessed by ANOVA. The Turkey-Kramer test for post-hoc multiple comparisons was used when ANOVA was significant. P values < 0.05 were considered statistically significant.

RESULTS

Overexpression of N-Cadherin and Reduced Expression of E-Cadherin in Pancreatic Cancer Tissue. The staining of N-cadherin in primary pancreatic cancer tissue was mainly identified in the cytoplasm of cancer cells, infiltrating cells, and neural bands (Fig. 1*A*). In noncancerous tissues, acinar, ductal, and islet cells were not stained with N-cadherin. Thirteen of the 30 pancreatic cancers (43%) were positive for N-cadherin expression. In metastatic liver tumors, N-cadherin immunoreactivity was strongly identified in noncancerous hepatic cells as well as in the cytoplasm of metastatic cancer cells (Fig. 1, *B* and *C*). Eight of 15 metastatic liver tumors (53%) were positive for N-cadherin expression.

In primary cancer tissues, E-cadherin expression in cancer cells was heterogeneous or negative compared with that in normal epithelial tissues and was characterized by patterns with variable degrees of membrane and cytoplasmic staining (Fig.

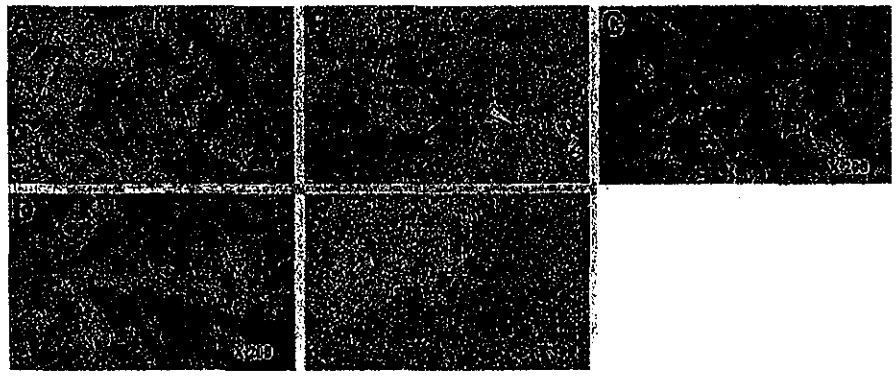

Fig. 1 Immunohistochemical staining of N-cadherin and E-cadherin in primary pancreatic cancer and hepatic metastasis. *A*, N-cadherin expression in primary tumor; *B* and *C*, N-cadherin in hepatic metastasis (*B*, ×100; *C*, ×200); *D*, reduced expression of E-cadherin in primary tumor and (*E*) in hepatic metastasis. Staining of N-cadherin was mainly observed in the cytoplasm of cancer cells and was also found in neural bands (▲) and in cell membrane of hepatocytes (△)

Table 1 Relationship between N- and E-cadherin expression and clinicopathological factors

Parameters	N-cadherin expression			E-cadherin expression		
	Negative	Positive	P^a	Normal	Reduced	P^a
UICC[b] classification						
Tumor extent (pT)			0.962			0.758
1 or 2	3	2		1	4	
3	7	6		5	8	
4	7	5		4	8	
Node involvement (pN)			0.936			0.398
0	5	4		2	7	
1	12	9		8	13	
Distant metastasis (pM)			0.193			0.584
0	13	7		6	14	
1	4	6		4	6	
Histological grading (G)			0.043			0.193
1	2	1		2	1	
2	14	6		5	15	
3	0	5		3	2	
4	1	1		0	2	
Stage			0.880			0.429
Stage I or II	7	5		5	7	
Stage III or IV	10	8		5	13	
Other tumor characteristics[c]						
Tumor size			0.778			0.862
≤2 cm	2	1		1	2	
2<, ≤4 cm	8	5		5	8	
>4 cm	7	7		4	10	
Lymphatic invasion			0.558			0.283
Negative	7	4		5	6	
Positive	10	9		5	14	
Venous invasion			0.098			0.429
Negative	9	3		3	9	
Positive	8	10		7	11	
Nerve invasion (intrapancreatic)			0.008			0.760
Negative	7	0		2	5	
Positive	10	13		8	15	
Nerve invasion (extrapancreatic)			0.269			0.121
Negative	10	5		7	8	
Positive	7	8		3	12	

[a] P was calculated by χ^2 test or Fisher's exact test.
[b] UICC, Unio Internationale Contra Cancrum.
[c] Japan Pancreas Society classification.

1*D*). Twenty pancreatic cancers (66%) were found to have reduced expression of E-cadherin. This expression was preserved in the noncancerous hepatic cells but reduced in the metastatic cancer cells of the metastatic liver tumors (Fig. 1*E*). Reduced expression of E-cadherin was also detected in 11 metastatic liver tumors (73%).

Correlation between N- and E-Cadherin Expression and Clinicopathological Features Including Survival Analysis. Table 1 summarizes the relationship between N- and E-cadherin expression and the clinicopathological features of the pancreatic cancers. N-cadherin expression in primary tumors significantly correlated with the extent of intrapancreatic nerve invasion and histological grade: tumors with positive nerve invasion and poorly differentiation had higher expression of N-cadherin. The survival rates for the 13 patients with N-cadherin-positive tumors and 17 with N-cadherin-negative tumors were not significantly different (Fig. 2). Moreover, there was no significant correlation between reduced E-cadherin expression and any of the clinicopathological factors.

Correlations among N-cadherin, E-cadherin, FGF-2, TGFβ, and Vimentin Expression in Pancreatic Cancer Tissue. TGFβ and FGF expressions were observed in fibroblasts, islet cells, and acinar cells in noncancerous tissue, but those in cancer cells were heterogeneous (Fig. 3, *A, B, D,* and *E*). Vimentin, a mesenchymal marker, was mainly observed in fibroblasts that surrounded the cancer cells and in a few cancer cells in primary tumors (Fig. 3*C*). However, vimentin expression was substantially in cancer cells of hepatic metastasis (Fig. 3, *F* and *G*).

The relationship between the expression of N-cadherin staining and those of E-cadherin, FGF2, TGFβ, and vimentin was analyzed on the basis of expressions only in cancer cells. In primary tumors, there was a significant correlation between N-cadherin expression and FGF-2: tumors with a higher expression of FGF-2 also showed higher expression of N-cadherin (Table 2). Metastatic liver tumors demonstrated significant correlations between N-cadherin and TGFβ and vimentin: tumors with a higher expression of N-cadherin and vimentin also showed a higher expression of TGFβ (Table 3). When expression of these factors in primary tumors and hepatic metastases

were compared, the expression of N-cadherin and vimentin was higher in the latter than in the former, but the difference did not reach statistical significance. No correlation could be established between overexpression of N-cadherin and reduced expression of E-cadherin.

Up-Regulation of N-Cadherin by Growth Factors in Cancer Cells. N- and E-cadherins and vimentin protein expression levels in pancreatic cancer cell lines were evaluated by Western blot analysis (Fig. 4). N-cadherin and E-cadherin were detected as a single band corresponding to the respective molecular sizes of 136 kDa and 123 kDa, which is consistent with their known molecular weight. Expression levels of N- and E-cadherin varied among five pancreatic cancer cell lines. N-cadherin was expressed in BxPC-3, Panc-1, and more strongly in Capan-2, whereas E-cadherin expression was observed in the four cell lines except MIAPaCa-2. Vimentin was detected as a single band corresponding to the molecular size of 56 kDa and was expressed in the four cell lines except BxPC-3.

Changes in the expression of N-cadherin, E-cadherin, and vimentin as a result of TGFβ or FGF-2 treatment was examined by Western blot analysis and immunocytochemistry. TGFβ treatment (5 ng/ml) significantly increased N-cadherin and vimentin protein expression and decreased E-cadherin expression in Panc-1 cells (Fig. 5, *A* and *C*). FGF-2 treatment (10 ng/ml) also increased N-cadherin expression in BxPC-3 cells, but E-cadherin expression was not markedly changed (Fig. 5*B*). Immunocytochemistry confirmed changes in N- and E-cadherin and vimentin in Panc-1 cells in response to changes in TGFβ and N-cadherin and in BxPC-3 cells in response to changes in FGF-2 (Fig. 6). Immunoreactivity for N- and E-cadherin was mainly observed in cell membrane and for vimentin in cytoplasm (Fig. 6). It was noted that TGFβ treatment caused Panc-1 cells to form scattered appearance of cell clusters. Other cell lines were refractory to the treatment with TGFβ and FGF-2.

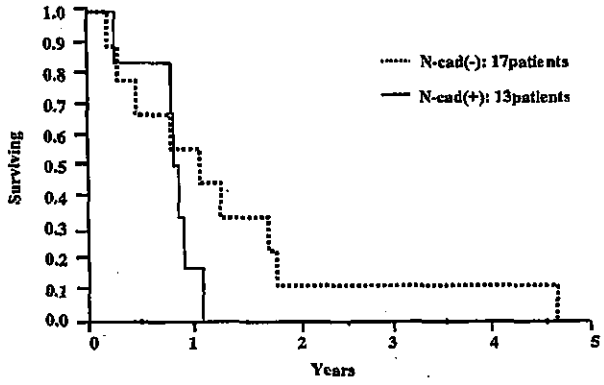

Fig. 2 Kaplan-Meier survival curves of patients with positive and negative N-cadherin expression. There was no statistical difference between the two groups (log-rank $P = 0.199$).

Table 2 N-cadherin expression in primary pancreatic cancer

	N-cadherin		
	Negative ($n = 17$)	Positive ($n = 13$)	P
E-cadherin			0.794
Normal ($n = 10$)	6	4	
Reduced ($n = 20$)a	11	9	
TGFβα[a]			0.176
Low ($n = 11$)	8	3	
High ($n = 19$)	9	10	
FGF			0.007
Low ($n = 13$)	11	2	
High ($n = 17$)	6	11	
Vimentin			0.712
Low ($n = 27$)	15	12	
High ($n = 3$)	3	1	

[a]TGF, transforming growth factor; FGF, fibroblast growth factor.

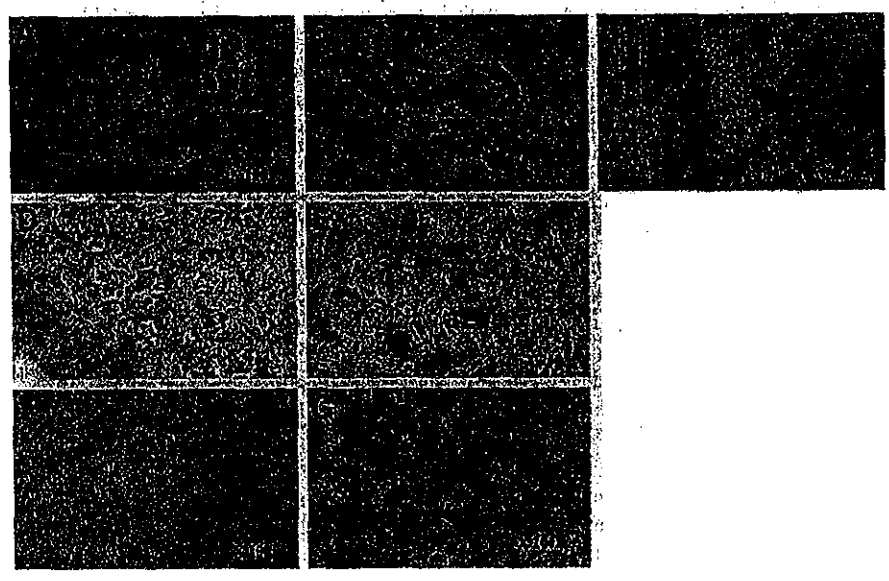

Fig. 3 Immunohistochemical staining of transforming growth factor (TGF)β, fibroblast growth factor (FGF)2, and vimentin in primary pancreatic cancer and hepatic metastasis. *A* and *D*, TGFβ; *B* and *E*, FGF2; *C*, *F*, and *G*, vimentin (*F* and *G* were same staining; *F*, ×100; *G*, ×200). *A–C*, primary cancer tissue; *D–G*, hepatic metastasis. TGFβ and FGF2 expressions in cancer cells were heterogeneous. Vimentin was mainly observed in fibroblasts that surrounded the cancer cells and in a few cancer cells in primary tumors.

Table 3 N-cadherin expression in hepatic metastasis.

	N-cadherin		
	Negative (n = 7)	Positive (n = 8)	P
E-cadherin			0.184
Normal (n = 4)	3	1	
Reduced (n = 11)	4	7	
TGFβ[a]			0.004
Low (n = 7)	6	1	
High (n = 8)	1	7	
FGF2			0.398
Low (n = 6)	2	4	
High (n = 9)	5	4	
Vimentin			0.010
Low (n = 10)	7	3	
High (n = 5)	0	5	

[a]TGF, transforming growth factor; FGF, fibroblast growth factor.

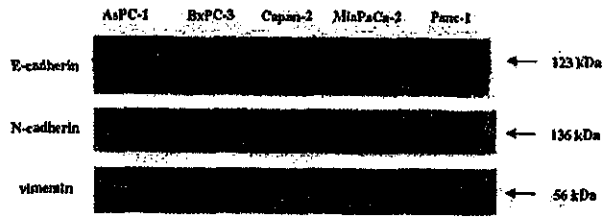

Fig. 4 Western blot analysis of E-cadherin, N-cadherin, and vimentin in five pancreatic cancer cell lines. Thirty μg of total proteins extracted from cancer cells were loaded onto each lane.

DISCUSSION

Important steps in the development of metastasis and local recurrence *in vivo* have been linked to enhanced cell-cell adhesion or cell-matrix adhesion in the tumor itself or to enhanced cancer cell extrication at different sites (4). Analysis of adhesion molecules in human cancer cell lines suggested that those molecules might influence the migration of tumor cells (33). To infiltrate host tissues, cancer cells of epithelial origin have to separate from the tumor mass by breaking their cell-cell contacts, also known as adherens functions (34, 35). Various studies of clinical tumor tissue samples and tumor cell lines demonstrated that reduced expression of E-cadherin is associated with tumor progression and enhanced cell invasiveness (36–38).

Acquisition of metastatic phenotype of cancer cells consists of multiple steps including EMT. Changes in cadherin expression patterns may play a role in the process of EMT and cellular motility (39). Nonepithelial cadherin, including N-cadherin, was found to induce a mesenchymal-scattered phenotype associated with reduced E- and P-cadherin in squamous epithelial cells (10). In prostate cancer, especially undifferentiated tumors and metastases, E-cadherin was mostly negative, and all of the cancer cells were positive for N-cadherin in what is called "the cadherin switch" (40). The purpose of the current study was to investigate whether a gain in N-cadherin in pancreatic cancer is involved in the process of metastasis via EMT and whether its expression is affected by growth factors. In epithelial cells the resultant loss of E-cadherin and the increase in N-cadherin expression means that the tumor cells have been converted to a metastatic phenotype, for example, EMT. In the study presented here, we could not find any correlation between N- and E-cadherin expression in primary pancreatic or in metastatic tumors. An N-cadherin transfection study of breast cancer cells demonstrated recently that N-cadherin promotes motility and invasion and that a reduced expression of E-cadherin does not necessarily correlate with motility or invasion (11). N-cadherin itself might have the potential to promote tumor progression and metastasis, because in our study overexpression of N-cadherin and reduced expression of E-cadherin was much more evident in metastatic than in primary tumors. In addition, vimentin, a mesenchymal marker, was strongly expressed in cancer cells of hepatic metastasis, which in turn was significantly associated with the expression of N-cadherin. Although it is very difficult to provide firm evidence of EMT in cancer tissue, these results suggest that during the metastatic process, EMT may occur, and pancreatic cancer cells may convert to a metastatic phenotype so that the process is related to the changes in cadherin expression.

A number of studies have shown that epithelial cells can be induced to scatter in response to environmental signals such as growth factors (35, 41, 42). It was shown that TGFβ induces a mesenchymal transdifferentiation and modulates E-cadherin expression in epithelial cells (34, 43, 44). Transfection of N-cadherin into breast cancer cells resulted in increased cell migration and invasion, which was greatly enhanced by the

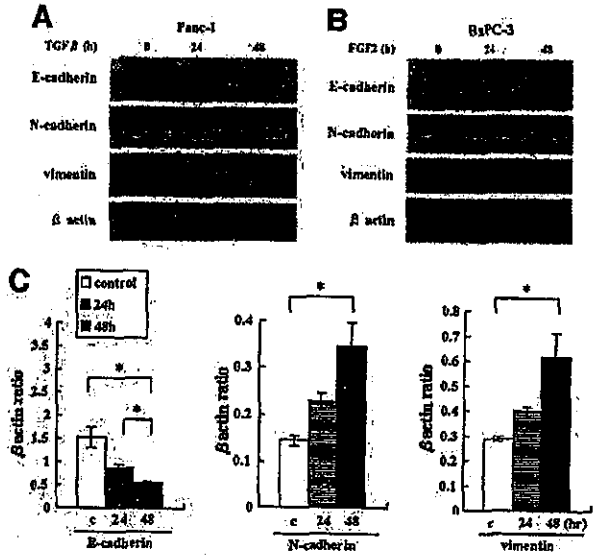

Fig. 5 Western blot analysis of changes in N-cadherin, E-cadherin, and vimentin expression by in transforming growth factor (*TGF*)β and fibroblast growth factor (*FGF*)2 treatment in pancreatic cancer cells. *A*, Panc-1 cells were incubated with 5 ng/ml TGFβ for 0, 24, and 48 h. *B*, BxPC-3 cells were incubated with 10 ng/ml FGF2 for 0, 24, and 48 h. Fifty μg of total proteins were loaded. *C*, quantitative analysis with image intensifier. n = 3; *, significant changes against control (ANOVA). N-cadherin and vimentin expressions were significantly induced, and E-cadherin expression was reduced by 48 h of TGFβ in Panc-1 cells. N-cadherin expression was induced, but E-cadherin and vimentin expressions were not changed by 24 h of FGF2 in BxPC-3 cells; *bars*, ±SD.

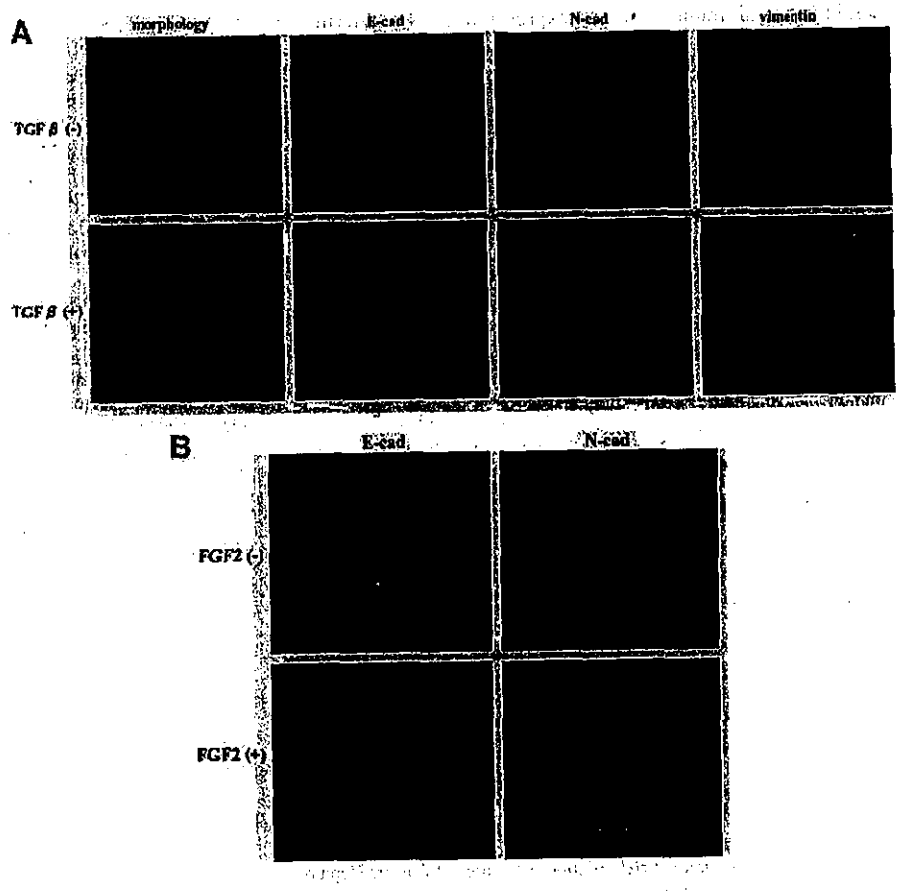

Fig. 6 Immunocytochemical analysis of changes in N- cadherin, E-cadherin and vimentin expression by transforming growth factor (*TGF*)β and fibroblast growth factor (*FGF*)2 treatment in pancreatic cancer cells. *A*, Panc-1 cells were incubated with 5 ng/ml TGFβ for 48 h. *B*, BxPC-3 cells were incubated with 10 ng/ml FGF2 for 24 h. Reduced expression of E-cadherin and high expression of N-cadherin and vimentin was found in Panc-1 cells, and high expression of N-cadherin was observed in BxPC-3 cells. Note that TGFβ treatment resulted in scattered appearance of cell clusters.

presence of FGF-2 and accompanied by up-regulation in matrix metalloproteinase-9 activity (8, 11). In our study, we investigated the correlation between the expression of growth factors and cadherin in pancreatic cancer cells in connection with EMT. In primary tumors, there was a significant correlation between N-cadherin expression and FGF-2. In metastatic liver tumors, there were also significant correlations between N-cadherin and TGFβ and vimentin: metastatic tumors with a higher expression of TGFβ also had a higher expression of N-cadherin and vimentin. Our *in vitro* study using Western blot analysis and immunocytochemistry demonstrated that the cell morphology of TGFβ-treated Panc-1 cells became spindle shaped. This change was associated with a reduction in E-cadherin expression and an increase in N-cadherin and vimentin expression. FGF-2 also induced high N-cadherin expression, whereas E-cadherin expression remained unchanged in BxPC-3. Up-regulation of N-cadherin may well be the result of EMT induced by TGFβ or may be directly effected through its signaling pathway, for example, up-regulation in matrix metalloproteinase-9 through FGF receptor.

Studies in neurite extension indicated that N-cadherin promotes cell motility that is dependent on the adhesive function of N-cadherin (45). Pancreatic cancer easily extends along the abundant nerve shafts inside the pancreas. Several studies demonstrated that the extent of perineural invasion correlates with tumor differentiation in pancreatic cancer (18–22). Our results show that N-cadherin expression significantly correlates with intrapancreatic neural invasion and tumor differentiation. In prostate cancer, N-cadherin was found to be exclusively expressed in the poorly differentiated area (40). These results indicate that N-cadherin may be responsible for pancreatic cancer extension through the intrapancreatic nerve bundles as an early step in extrapancreatic invasion.

In our study, N-cadherin expression of cancer cells was predominantly observed in a cytoplasmic but not a membranous pattern in primary pancreatic tumors. N-cadherin was ubiquitously present in the cell membrane of noncancerous hepatic cells, but it was present in the cytoplasm of the cancer cells in hepatic metastasis as well. N-cadherin showed an intense presence in the regions of cell-cell contact in mesothelioma, but staining was characterized by a cytoplasmic pattern in the spindle cell area. This difference between cadherin expression in epithelioid and spindle cell areas may reflect differences in the adhesive nature of the tumor cell population (30). The extracellular domain of a cadherin promotes cell-cell adhesion, whereas the cytoplasmic domain serves to link the cadherin to the cytoskeleton via interactions with catenin and is critical for the adhesive function of the cadherin (46). This suggests that cytoplasmic cadherin has a possibility to promote cell motility and strengthen cell-cell adhesion.

In conclusion, the study reported here provided morphological evidence of the occurrence of EMT in pancreatic

carcinoma and found that overexpression of N-cadherin is involved in EMT and is affected by growth factors. Because EMT is an important process in the invasion and metastasis of malignant tumor cells (31, 47, 48), it is possible that N-cadherin is the adhesion molecule not only to acquire the fibroblastic morphology of EMT but also to obtain invasive and metastatic potential. To confirm this, it will be necessary to perform an N-cadherin transfection study with an invasion and motility assay.

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ORIGINAL ARTICLE

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Surgical resection deteriorates gemcitabine-induced leukopenia in pancreatic cancer

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Abstract
Background. Gemcitabine hydrochloride (GEM) is one of the most effective chemotherapeutic agents for pancreatic cancer; however, factors affecting GEM-induced leukopenia have not been clarified yet. In the present study, we analyzed the relationship between patients' backgrounds and GEM-induced leukopenia.
Methods. Thirty-eight patients with pancreatic cancer were analyzed for correlation between the dose of GEM and the blood leukocyte number. Moreover, we compared leukopenia in resected and non-resected patients.
Results. The incidence of grade 3 or 4 leukopenia was 25% in the non-resected patients, whereas equivalent leukopenia was observed in 57% of the resected patients ($P = 0.048$ by the χ^2 test). The relative decrease in blood leukocytes induced by GEM administration was more severe in resected patients (41.3 ± 9.9%), as compared to non-resected patients (52.6 ± 16.0%; $P = 0.023$ by t-test).
Conclusion. In the present study, we found that the administration of GEM to patients after surgical resection caused more severe leukopenia, as compared to findings in non-resected patients. These data suggested that more frequent monitoring of the leukocyte count and prolonged intervals between GEM administrations are necessary for resected patients with pancreatic cancer.

Key words Gemcitabine · Leukopenia · Pancreatic cancer · Cancer chemotherapy · Surgical resection

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Introduction

Pancreatic cancer is one of the most refractory cancers, and is often diagnosed at advanced stages. Surgical resection is a first-line treatment to prolong survival time; however, the rate at which surgical resection is performed is approximately 10%–15%.[1] In most patients, relapse, such as liver metastases or local recurrence, is detected even after radical surgical resection. To improve survival time, multidisciplinary treatment, such as adjuvant cancer chemotherapy or radiotherapy combined with surgery is recommended.[2] Recently, a number of studies have reported that the anticancer agent, gemcitabine hydrochloride (GEM) significantly prolonged survival rate and attenuated various symptoms in pancreatic cancer patients, as compared with 5-fluorouracil (5-FU), which has most commonly been used.[3] Currently, GEM is recognized as the first-line chemotherapeutic agent for the treatment of pancreatic cancer in Europe, the United States, and Japan.[4,5]

GEM was approved by the Ministry of Health, Labour, and Welfare of Japan as a chemotherapeutic agent for pancreatic cancer in April 2001. In Japan, a phase I clinical study included only 11 patients, and patients who underwent surgical resection were not included in this study.[5] Therefore, the information necessary for determining appropriate chemotherapy for surgically resected patients was insufficient. Based on this background, we monitored the adverse effects of GEM in 14 patients with primary and recurrent pancreatic cancer, and reported that the incidence of leukopenia was relatively high.[6] In addition, we evaluated the appropriate usage of an agent for leukopenia, granulocyte colony-stimulating factor (G-CSF), and reported that the leukocyte count could be favorably controlled by G-CSF during chemotherapy with GEM.[6]

Subsequently, we have continued to monitor the adverse effects of GEM, and found that the frequency and the degree of GEM-induced leukopenia differed markedly among patients, i.e., some patients did not show leukopenia, while others readily developed severe leukopenia. In this study, we investigated the backgrounds of patients treated with