Chemosensitivity of human pancreatic carcinoma cells is enhanced by IκBα super-repressor

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Pancreatic cancer has an unfavorable prognosis; surgery and chemotherapy at present have only limited value. To improve the prognosis of pancreatic cancer, effective non-surgical therapy is necessary. NF-κB is reported to be related to resistance to apoptosis, but its role in chemosensitivity remains controversial. We examined the effects on chemosensitivity of inhibition by induction of the super-repressor IκBα in pancreatic cancer cell lines, BxPC-3, Capan-1 and Panc-1. IκBα protein was transduced by infection of adenovirus vector AxCAhIkBΔN. Sensitivity to VP-16 and doxorubicin was increased significantly by IκBα induction in all three pancreatic cell lines. To investigate molecular events during IκBα induction, we examined the changes in expression of drug-resistance-related genes by real-time RT-PCR and those in apoptosis-related genes by cDNA microarray. There was no common change of gene expression before and after IκBα induction among the three pancreatic cancer cell lines, except for mdm2. Further examination of other genes is necessary for a better understanding of the molecular mechanisms of enhancement of chemosensitivity through IκBα induction. However, we have confirmed that IκBα induction leads to an increase of chemosensitivity of pancreatic cancer. Many problems remain before clinical application of this adenoviral system will be feasible, but our results may ultimately lead to an improved therapy of pancreatic cancer. (Cancer Sci 2003; 94: 467–472)

Resistance of tumors to chemotherapy is a common clinical problem in human cancer. Pancreatic cancer is considered to be one of the malignancies most resistant to therapy. Overall 5-year survival rate is only 8.1% in Japan[1]. Despite many advances in the diagnosis of pancreatic cancer, only a small minority of patients are candidates for curative surgical resection. However, even for resected patients, the 5-year survival rate is only 18.9%,[1] because of post operative recurrence.

Accordingly, to improve the prognosis of pancreatic cancer, there is an urgent need to develop effective non-surgical treatment for this disease. Radiotherapy for pancreatic cancer has limited value in clinical practice, as does chemotherapy, due to the lack of any individual agent with potent activity.[2, 3]

Apoptosis seems to be the main mechanism whereby chemotherapy induces the killing of tumor cells. Apoptosis also serves as a cell-killing mechanism induced by cytokines such as tumor necrosis factor α (TNFα). Apoptosis induced by TNFα and by chemotherapy seems to require at least partial activation of the caspase cascade, leading to proteolytic cleavage of key proteins, and ultimately cleavage of cellular DNA. Chemotherapy and other stimuli activate the transcription factor NF-κB, and this response potently suppresses the apoptotic potential and protects cells from apoptosis.

There are several reports suggesting that inhibition of NF-κB in cancer cells enhances the sensitivity to anti-cancer drugs.[5–10] However, some authors found that reduction of NF-κB did not alter the chemosensitivity.[11–14] The role of NF-κB in chemosensitivity remains controversial.

To ascertain the role of NF-κB in chemosensitivity of pancreatic cancer, we examined the effect of the inhibition of NF-κB by the induction of the IκBα super-repressor, and analyzed the molecular changes accompanying IκBα induction.

Materials and Methods

Cell lines. Human pancreatic cancer cell lines, BcPC-3, Capan-1 and Panc-1 were purchased from American Type Culture Collection (Manassas, VA), and cultured in RPMI1640 medium (GIBCO, Gaithersberg, MD) supplemented with heat-inactivated 10% fetal bovine serum (FBS; GIBCO). A human breast cancer cell line, MCF-7 and a human embryonic kidney cell line, 293, were obtained from Riken Gene Bank, and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. All cells were kept in a humidified atmosphere of 5% CO_2, at 37°C .

Drugs. VP-16 and doxorubicin were purchased from Sigma Chemical Co. (St. Louis, MO).

Immunoblot analysis. One million cells were lysed by incubating them for 15 min on ice in modified RIPA buffer (50 m*M* Tris-HCl, pH 7.4, 1% NP40, 0.25% sodium deoxycholate, 150 m*M* NaCl, 1 m*M* glycoletherdiaminetetraacetic acid, 1 m*M* phenylmethanesulfonyl fluoride, 1 μg aprotinin, leupeptin, pepstatin, 1 m*M* Na_3VO_4, 1 m*M* NaF), and after centrifugation, the protein content of the supernatant was determined by Bradford protein assay (Bio-Rad Laboratories, Hercules, CA). An equal volume of 2× loading buffer (100 m*M* Tris-HCl pH 6.8, 200 m*M* dithiothreitol, 4% sodium dodecyl sulfate, 10% glycerol, 0.2% bromophenol blue) was added to the supernatant, and this was boiled for 5 min. Equal amounts of protein from each extract (15 μg per lane) were separated by electrophoresis on a 10% polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane, Immobilon P (Millipore, Eschborn, Germany). After having been blocked with 5% dried milk in TBS (10 m*M* Tris-HCl pH 7.5, 150 m*M* sodium chloride), the membranes were incubated with anti-IκBα mouse monoclonal antibody, H-4 (Santa Cruz Biotech., Inc., Santa Cruz, CA) for 24 h at 4°C. After washing, the membranes were incubated with a ×500 dilution of horseradish peroxidase-conjugated rabbit anti-mouse IgG, EnVision+ (Dako, Carpinteria, CA). Staining was carried out using the ECL kit (Amersham, Buckinghamshire, England), according to the manufacturer's instructions. Signals were measured using a Light Capture (ATTO, Tokyo), and analyzed with the supplied CS-Analyzer software (ATTO). Data are expressed as relative light units per microgram of protein.

Adenoviral vectors. The adenoviral vector for the IκBα super-repressor (AxCAhIκBΔN[4]) was kindly provided by Dr. Hirofumi Hamada of Sapporo Medical College. Control empty expression vector, AxCAwt, was kindly provided by Dr. Izumu Saito of the Institute of Medical Science, University of Tokyo.

Adenovirus preparation. To obtain high-titer viral stocks, 2×10^8 HEK293 cells were infected at a multiplicity of infection

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(MOI) of 5–10. The infected cells were grown for 3 days until a cytopathic effect was seen. Viral particles were released by freezing and thawing of cells four times. Virus preparation was subjected to CsCl banding for further purification. Determination of infectivity and viral concentration was performed by standard plaque assay using 293 cells.

Infection of adenoviral vector and exposure to chemotherapeutic agents for morphological change. During the exponential growth phase, cancer cells were plated in a 6-well culture plate (Becton Dickinson, San Jose, CA) at a density of 5×10^5 cells/well. Twenty-four hours later, a suspension of adenovirus at MOI 10:1 was distributed on the cell monolayers. After an additional 48 h, cells were observed microscopically.

MTT-dye reduction assay. Cells were seeded in 96-well plates (100 μl/well at a density of 1×10^5 cells/ml), grown for 24 h, and exposed to various concentration of anti-cancer agents after infection with AxCAhIκBΔN or AxCAwt or neither. The survival fraction was estimated with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide] dye reduction assay as described by Mosmann[15] with some modifications. Briefly, after 72 h incubation with anti-cancer agents, MTT solution (10 mg/ml in phosphate-buffered saline) was added (10 μl/well). Plates were further incubated for 4 h at 37°C. Thereafter, the formazan crystals formed were dissolved by adding 110 μl of 0.04 *N* HCl in 2-propanol. Absorption was measured with a plate-reader, Titertek Multiscan (Titertek, Huntsrill, AL) at 540 nm. For each concentration and combination, experiments were repeated four times. RPMI1640 medium (100 μl) with 10 μl of MTT solution and 110 μl of 2-propanol was used as blank solution.

RNA preparation. Total RNA from cell lines was extracted using an RNeasy Mini Kit (QUIAGEN, Inc., Valencia, CA). The quality and concentration of RNA were determined on the basis of absorbance measurement at 260 and 280 nm using an Agilent 2100 Bioanalyzer (Agilent Technologies, Inc., Palo Alto, CA).

Real-time PCR. Using TaqMan real-time PCR, the samples were checked for genes considered to be related to drug resistance. Using the Primer Express software (Applied Biosystems, Foster City, CA), we designed specific primers and fluorescence-labeled probes (TaqMan probes; Applied Biosystems) for the drug resistance-related genes and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene (internal standard; GenBank Accession No. B1977763), as shown in Table 1. The RNA sample was subjected to reverse transcription using TaqMan Reverse Transcription Reagents (Applied Biosystems) to prepare cDNA. Reverse transcription was performed under the following conditions: 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. Then, cDNA was subjected to 40 cycles of the PCR using a TaqMan PCR Reagent Kit, with each cycle consisting of reactions at 50°C for 2 min, 95°C for 10 min 15 s, and 60°C for 1 min. Threshold cycle (Ct) , was obtained from PCR reaction curves monitored by the ABI PRISM 7700 (Applied Biosystems) for drug resistance genes and GAPDH, followed by calculation of differences after IκBα induction. The Mann-Whitney *U* test was used to test the significance of differences. $P<0.05$ was regarded statistically significant.

cDNA microarray.

RNA reverse transcription, labeling and hybridization: Extracted total RNA was subjected to reverse transcription using an RNA Fluorescence Labeling Core Kit (TaKaRa Bio, Inc., Shiga). RNA from cells infected by AxCAhIκBΔN was labeled with green fluorescent dye, Cy5-dCTP, while RNA from cells infected by AxCAwt was labeled with red fluorescent dye, Cy3-dCTP. After purification, hybridization of the samples on a Human Cancer CHIP Version 3.0 (638 genes) and a Human Apoptosis CHIP Version 1.1 (164 genes), both products of TaKaRa Bio, Inc., was conducted with an automated hybridizer, Hybstation (Genomic Solutions, Inc., Ann Arbor, MI).

Data analysis: After hybridization, images were obtained with an Affymetrix428 Array Scanner (Affymetrix, Inc., Santa Clara, CA) and were numerically converted using ImaGene Version 4.1 (BioDiscovery, Inc., Marina Del Rey, CA). This corrected the numerical data by the global normalization method, to select genes whose expression showed a twofold or greater difference between RNA from IκBα-induced cells and control cells.

Results

Induction of IκBα protein by AxCAhIκBΔN. After infection with AxCAhIκBΔN or AxCAwt or neither, proteins from each cell line were tested by western blotting. A single band due to IκBα protein of 36 kd was induced in all cell lines after infection with AxCAhIκBΔN (Fig. 1). At least ten times more IκBα pro-

Table 1. Sequence of TaqMan probe for drug resistance related genes and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*)

Gene	Sequence	Corresponding cDNA sequence
Topo1	FAM-TATCTGGACCCTAGGATCACAGTGGCTTGG-TAMRA	2413–2442
MDR1	FAM-TGAAACTGCCTCATAAATTTGACACCCTGG-TAMRA	1657–1686
MRP-1	FAM-TTGTTTTCGGGTTCCCTCCGAATGAA-TAMRA	4331–4356
LRP1	FAM-AACCCTCATCACCGATGGCTCCACT-TAMRA	2592–2616
GAPDH	JOE-CAAGTTCCCGTTCTCAGCC-TAMRA	243–262

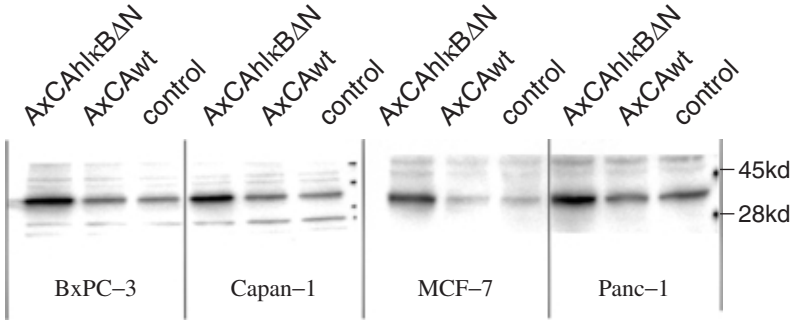

Fig. 1. Expression of IκBα protein induced by AxCAhIκBΔN (western blotting). Proteins from cells after infection of AxCAhIκBΔN or AxCAwt or neither (control) were examined.

tein was induced in cells infected with AxCAhIκBΔN as compared to cells infected with AxCAwt or cells without adenovirus infection.

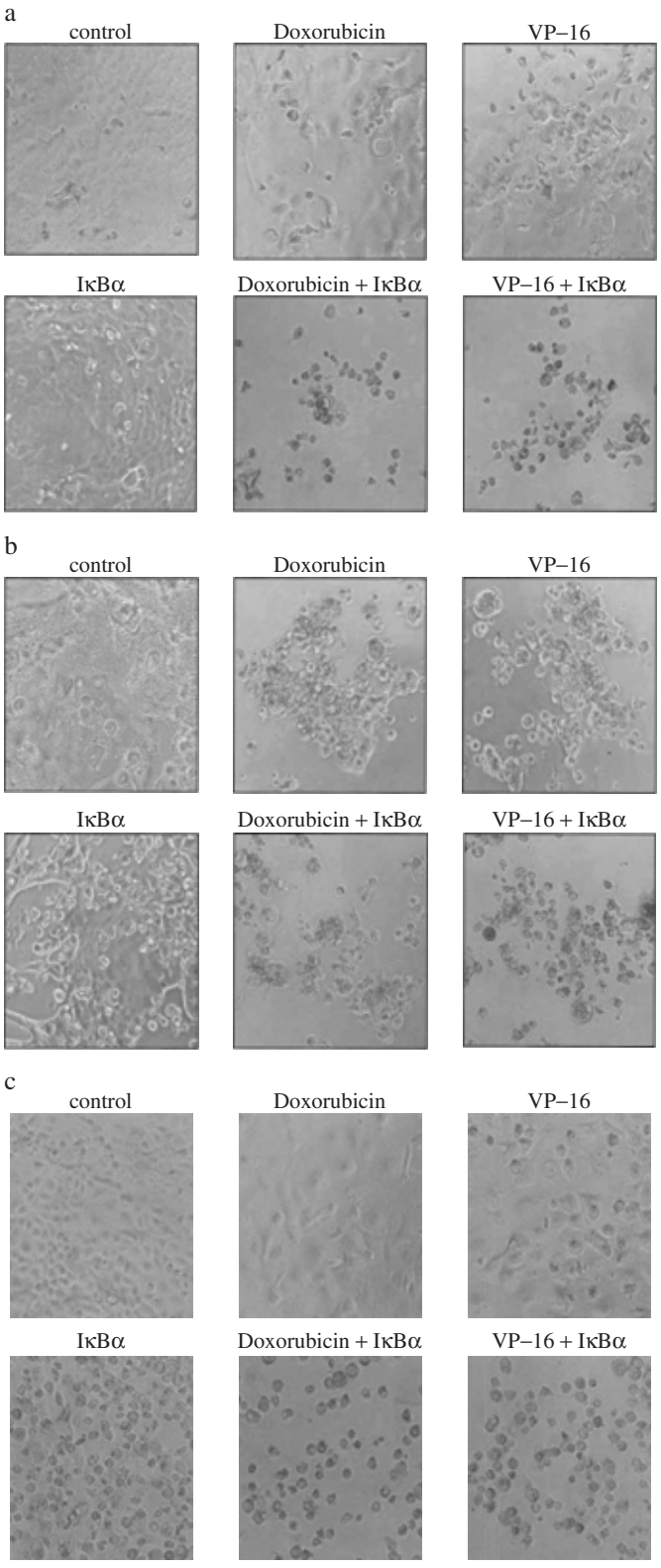

Fig. 2. Morphological change of pancreatic cell lines after treatment with anti-cancer drugs combined with/without IκBα induction (×40). a) BxPC-3, b) Capan-1, c) Panc-1.

Enhancement of chemosensitivity.

Morphological changes: Cells of all cell lines became attached to the plate without anti-cancer drug treatment. Seventy-two hours after exposure to doxorubicin at 0.2 *μM*, about 40% of BxPC-3 cells became round and some cells were floating (Fig. 2a). The cell rounding seen here as a response to drug treatment leads to cell detachment, with some cell fragments left attached to the substratum, indicating sites of previously detached cells. Simultaneous infection of AxCAhIκBΔN, at MOI 10:1, had greater cytopathic effects than the anti-cancer drug alone. No significant change was observed after infection of AxCAwt (data not shown).

Likewise, treatment with 20 *μM* VP-16 resulted in an increase of cytopathic effects of the combination of anti-cancer drug and induction of IκBα. Similar morphological changes were observed in Capan-1 cells (Fig. 2b) and Panc-1 cells (Fig. 2c). Cytopathic effects were increased by the combination of anti-cancer drugs and infection of AxCAhIκBΔN.

MTT assay: The pancreatic carcinoma cell lines, BxPC-3, Capan-1, Panc-1 and a breast cancer cell line, MCF-7 were exposed to VP-16 and doxorubicin at various concentrations for 72 h. In a chemosensitive cell line, BxPC-3, the inhibition by 20 *μM* VP-16 was 52.7%, and the cytotoxic effects were dose-dependent (Fig. 3a). The induction of IκBα significantly increased the inhibition rate by VP-16, especially at low concen-

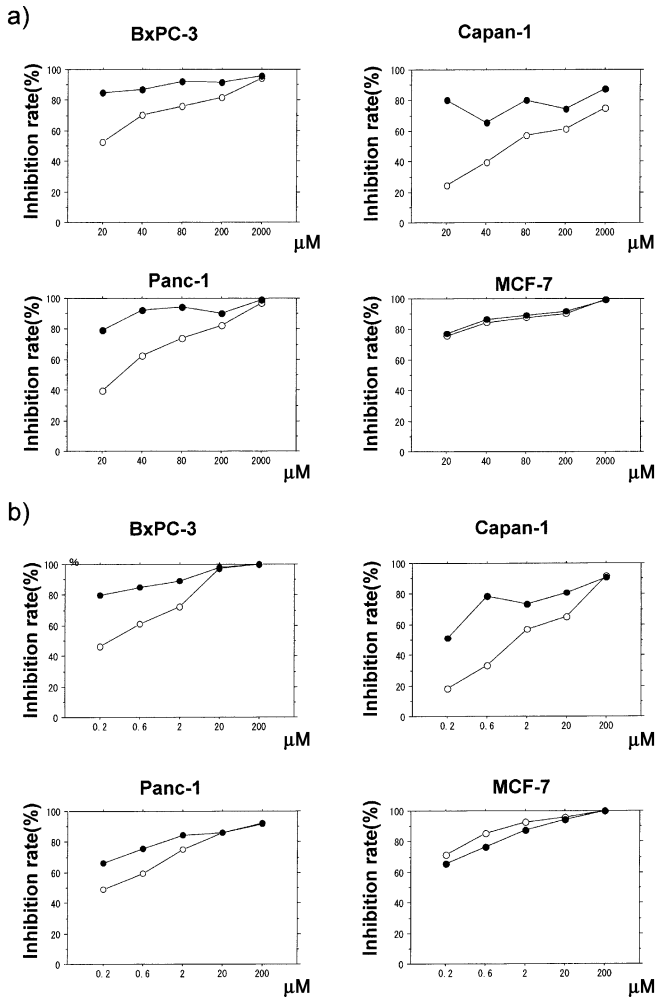

Fig. 3. MTT dye-reduction assay. Cell growth inhibition rate (inhibition rate) at various concentration of a) VP-16 and b) doxorubicin. ● with IκBα induction, ○ without IκBα induction.

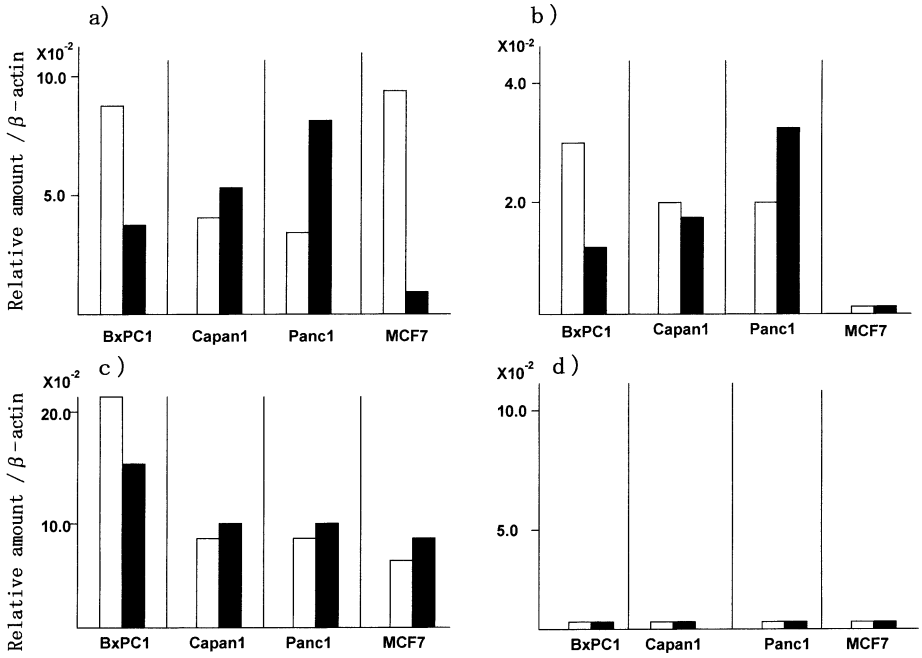

Fig. 4. Expression of drug resistance related genes (TaqMan realtime RT-PCR). Relative amounts of RNA for β-actin are shown. a) topo1, b) LRP, c) MRP-1, d) MDR1. ■ with IκBα induction, □ without IκBα induction.

tration, which is close to the concentration in peripheral tissue during clinical use. In a chemoresistant cell line, Capan-1, similar results were obtained. Inhibition rates of Capan-1 were as low as 24.6% and 39.6% at 20 and 40 *μM* VP-16, but inhibition rates were increased to 80.4% and 65.6% by the induction of IκBα. In another pancreatic cell line, Panc-1, results were similar. However, in a breast cancer cell line, MCF-7, no increase of cytotoxic effects was observed by the induction of IκBα at any concentration of VP-16.

In each cell line, cell growth was inhibited by doxorubicin in a dose-dependent manner, as by VP-16.

Similar results were obtained with the combination of doxorubicin and AxCAhIκBΔN (Fig. 3b). BxPC-3 cells showed 46.3% inhibition at 0.2 *μM* doxorubicin, and the inhibition rate was increased to 79.8% by the combination of doxorubicin and IκBα induction. Inhibition rates of Capan-1 were as low as 18.5% and 37.6% at 0.2 and 0.6 *μM* doxorubicin. Upon induction of IκBα, the inhibition rates were increased to 51.3% and 78.9% at 0.2 and 0.6 *μM* doxorubicin. In another pancreatic cancer cell line, Panc-1, the results were similar. However, in a breast cancer cell line, MCF-7, no increase of cytotoxic effects was observed by the induction of IκBα at any concentration of doxorubicin as well as VP-16.

AxCAwt infection did not cause such an increase of inhibition rate even if it was combined with anti-cancer drug (data not shown).

Changes of mRNA amount of drug resistance-related genes after induction of IκBα protein. In BxPC-3 cells, mRNA for topo1, multidrug resistance protein (MRP) 1 and lung resistance-related (LRP) were decreased by AxCAhIκBΔN infection (Fig. 4). By contrast, mRNAs for topo1 and LRP were increased in Panc-1 by AxCAhIκBΔN infection. There was no common change of the amount of mRNA for *topo1*, *MRP1* and *LRP* genes among the three pancreatic cancer cell lines. mRNA for MDR1 was not detectable in any cell line.

Alteration of gene expression monitored by cDNA microarray. Among 762 genes which we tested in this study, there was no common change of gene expression in all three pancreatic cancer cells after AxCAhIκBΔN infection, except for mdm2 and tumor necrosis factor (TNF). Expression of the *mdm2* gene was reduced to about one-fourth in BxPC-3, Capan-1 and Panc-1 after AxCAhIκBΔN infection. TNF was increased in not only pancreatic cancer cells, but also MCF-7 (Fig. 5).

The amount of mRNA of p53 was not changed by IκBα induction.

Discussion

Activated NF-κB has anti-apoptotic function[16] and inhibition of NF-κB increased cell death and chemosensitivity.[5–10] However, there are some conflicting reports.[11–14] The role of NF-κB in chemosensitivity remains controversial. In our study, induction of IκBα protein by an adenoviral vector induced an increase of sensitivity to cytotoxic drugs in all pancreatic cancer cell lines, and these results are consistent with a recent report that inhibition of NF-κB sensitized carcinoma cells to apoptosis.[6]

On the other hand, MCF-7 did not show a change of chemosensitivity after IκBα induction. This result is consistent with another report.[6] In MCF-7, NF-κB was not expressed either before or after treatment with anti-cancer drugs (data not shown). Deficiency of NF-κB seemed to account for the lack of enhancement of chemosensitivity by IκBα induction in MCF-7.

MRP1, LRP and MDR1 are known to be related to resistance to doxorubicin and VP-16.[17–21] We first checked the changes of such drug resistance-related genes to examine the molecular mechanism of this phenomenon. Quantitative RT-PCR revealed no common change in expression of these genes among the pancreatic cancer cell lines.

Recently, cDNA microarray for gene expression monitoring has been applied to many biological and medical studies, including identification of a predictive marker for drug sensitivity or detection of a chemoresistance pathway.[22, 23] Following the real-time RT-PCR, we used the cDNA microarray method to analyze the differences in gene expression, between before and after IκBα induction. Among 792 genes tested, there was no common and pancreatic cancer-specific change of gene expression caused by IκBα induction, except for mdm2, which was reduced to about one-fourth.

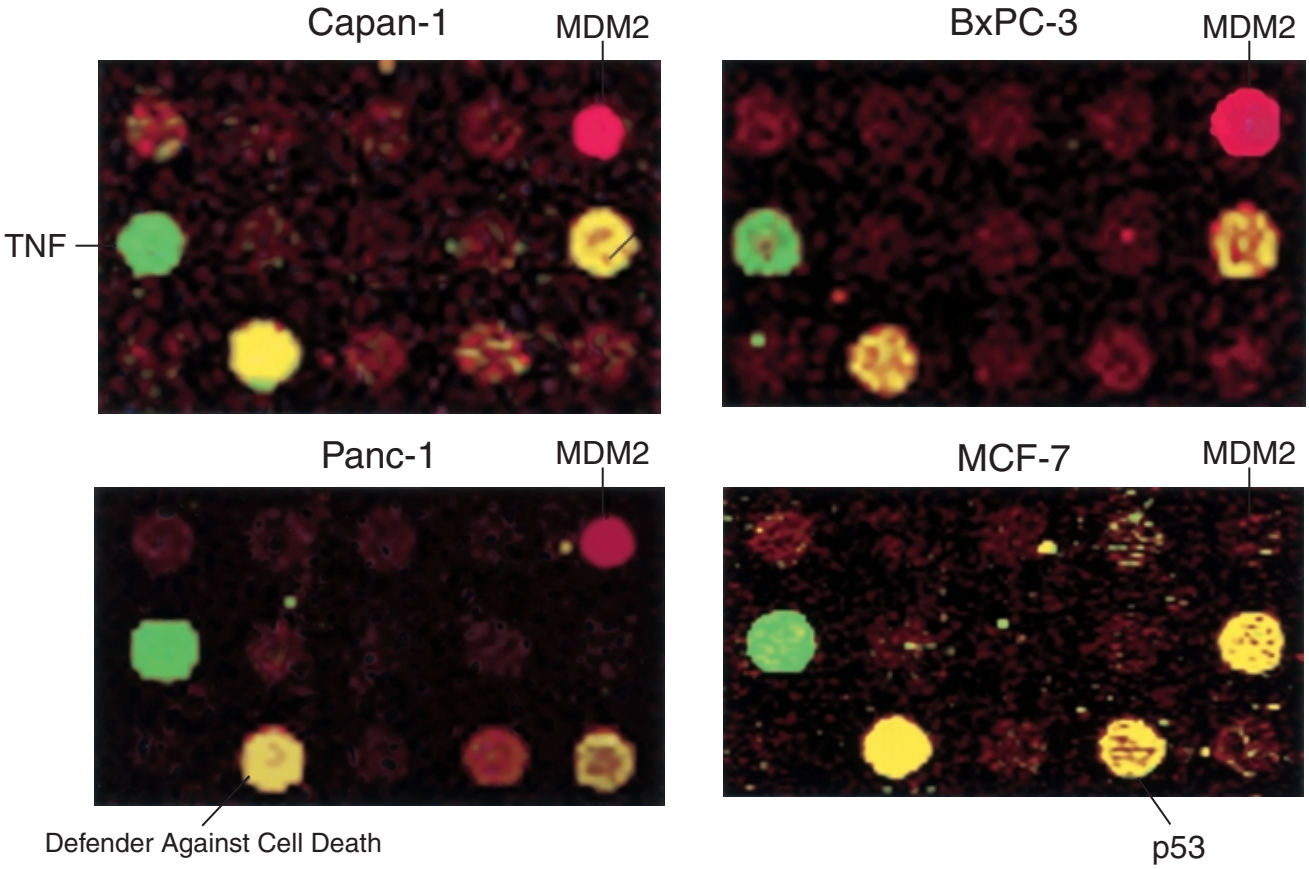

Fig. 5. Changes of expression of apoptosis-related genes (cDNA microarray). Up-regulated genes appear in green and down-regulated genes in red.

Mdm2 is a cellular attenuator of p53. It forms a complex with p53 which is critical for the fate of the cell, and is degraded by ubiquitin proteosome. Most anti-cancer drugs, including VP-16 and doxorubicin, induce DNA breaks[24] and activate p53.[25] Accumulated p53 increases the transcription of many genes, including *p21*, *GADD45*, *Bax*, and *Fas/APO1*, and cells are G1-arrested or undergo apoptosis.

There are some reports indicating that mdm2 is related to human malignancies and chemosensitivity of cancer cells. Mdm2 expression in esophageal squamous cell carcinoma is a novel marker for lack of response to chemo-/radiotreatment.[26] Increased mdm2 levels in fibroblasts decreased doxorubicin-induced p53 stabilization and cell death.[27] Moreover, mdm2 can induce p53-independent doxorubicin resistance in acute lymphoblastic leukemia.[28]

According to these reports, increased mdm2 may play an important role in the enhancement of chemosensitivity by IκBα induction. However, the mechanisms of the effect remain unclear. Further investigation on the associated molecular events is necessary to understand the mechanism of enhancement of chemosensitivity in parallel with IκBα induction.

There are some differences in clinical features between histological types of pancreatic cancer. As compared with other types of pancreatic cancer, anaplastic ductal carcinoma is the most malignant. When anaplastic carcinoma is found, the tumor is already advanced in most cases, and is rarely resectable. In our study, we tested three pancreatic cancer cell lines of different histological types; Capan-1 from well-differentiated adenocarcinoma, BxPC-3 from moderately to poorly differentiated adenocarcinoma, and Panc-1 from anaplastic ductal carcinoma. Chemosensitivity was enhanced by IκBα induction in all three types of pancreatic carcinoma cell lines. Our system should therefore be useful for most types of pancreatic cancer, including anaplastic ductal carcinoma.

Application of these findings to human cases will be a long way off. Pancreatic cancer *in vivo* is hypovascular. Delivery of the adenoviral vector to pancreatic cancer might be difficult. However, recently, endoscopic injection of adenoviral vector into pancreatic cancer combined with intravenous administration of gemcitabine was reported to be effective to treat unresectable pancreatic cancer.[29]

We have demonstrated here that IκBα induction enhances the chemosensitivity of pancreatic cancer cells, at least under some conditions. We hope that this work will provide clues towards an advance in the treatment of pancreatic cancer.

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