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Survivin as a Radioresistance Factor in Pancreatic Cancer

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We examined whether survivin acts as a constitutive and inducible radioresistance factor in pancreatic cancer cells. Using a quantitative TaqMan reverse transcription-polymerase chain reaction for survivin mRNA in five pancreatic cancer cell lines, we found an inverse relationship between survivin mRNA expression and radiosensitivity. PANC-1 cells, which had the highest survivin mRNA levels, were most resistant to X-irradiation; MIAPaCa-2 cells, which showed the least survivin mRNA expression, were the most sensitive to X-irradiation. Our results suggested that survivin could act as a constitutive radioresistance factor in pancreatic cancer cells. To determine whether radioresistance is enhanced by induction of survivin expression by irradiation, PANC-1 and MIAPaCa-2 cells were subjected to sublethal doses of X-irradiation followed by a lethal dose. Survivin mRNA expression was increased significantly in both PANC-1 and MIAPaCa-2 cell lines by pretreatment with a sublethal dose of X-irradiation, as was cell survival after exposure to the lethal dose. In this system, enzymatic caspase-3 activity was significantly suppressed in cells with acquired resistance. These results suggest that survivin also acts as an inducible radioresistance factor in pancreatic cancer cells. Survivin, then, appears to enhance radioresistance in pancreatic cancer cells; inhibition of survivin mRNA expression may improve the effectiveness of radiotherapy.

Key words: Survivin — Pancreatic cancer cell lines — X-irradiation — Radioresistance

X-irradiation induces apoptosis in many types of cells.[1–3] Apoptosis is regulated by a delicate balance in signal transduction pathways between apoptosis-activating factors, such as p53 and caspases, and antiapoptotic factors, such as the bcl-2 family and the inhibitor of apoptosis protein (IAP) family.[4] Inhibition of apoptosis-activating factors or induction of apoptosis-inactivating factors is important in resistance to radiotherapy and chemotherapy.[1]

Survivin, a recently described member of the IAP family, contains a single baculovirus IAP repeat and lacks a carboxyl-terminal RING finger.[5, 6] Like other IAP family members, survivin binds specifically to terminal effector cell-death proteases, such as caspase-3 and -7. Survivin substantially reduces caspase activity and apoptosis in cells exposed to diverse apoptosis-triggering stimuli.[7–10]

Survivin is expressed during human fetal development, but it is not detected in normal adult tissues except for thymus and placenta. However, survivin is expressed in the cells of most cancers, including lung, stomach, colon, pancreas, breast, prostate, and bladder cancers, neuroblastomas, and lymphomas.[4, 6, 11, 12–15] In cancer cells survivin may play an important role in resistance to radiotherapy and chemotherapy. We therefore examined whether survivin acts as a constitutive and inducible radioresistance factor in pancreatic cancer cells.

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MATERIALS AND METHODS

Cells The human pancreatic cancer cell lines PANC-1, AsPC-1, Capan-1, BxPC-3, and MIAPaCa-2, were obtained from the American Type Culture Collection (ATCC; Manassas, VA). AsPC-1 cells were cultured in RPMI 1640 medium (Nipro, Osaka) supplemented with 20% heat-inactivated fetal calf serum (FCS, Biological Industries, Kibbutzbeit Haemek, Israel). PANC-1, BxPC-3 cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS. MIAPaCa-2 cells were cultured in Dulbecco's modified Eagle's medium (Nipro) supplemented with 10% heat-inactivated FCS and 2.5% heat-inactivated horse serum. Capan-1 cells were cultured in RPMI 1640 medium supplemented with 15% heat-inactivated FCS.

Quantitative RT-PCR assays for survivin Cells were trypsinized and the cell pellets were collected by centrifugation at 1000*g* for 5 min at 4°C. SepaGene RV-R (Sanko Pure Chemical, Tokyo) was used to extract total RNA from cells, and this extract was assayed for RNA with Gene Quant DNA/RNA Calculator (Pharmacia, Upsala, Sweden). Contaminating chromosomal DNA was digested with DNAse I (GIBCO-BRL, Gaithersburg, MD) accord-

ing to the manufacturer's instructions. For quantitative RT-PCR, fluorescent hybridization probes, TaqMan PCR Core Reagents Kit with AmpliTaq Gold (Perkin-Elmer, Foster City, CA), were used with the ABI Prism 7700 Sequence Detection System (Perkin-Elmer). Expression of survivin mRNA was quantified using a method similar to that reported for hTR.[16, 17] Primers and TaqMan probes for survivin mRNA were as follows. Sequences of the forward primer for survivin mRNA were 5′-AAGAACTGGCCCTTCTTGGA-3′ and the reverse primer, 5′-CAACCGGACGAATGCTTTT-3′; the sequence of the TaqMan probe was 5′-CCAGATGACGACCCCATAGAGGAACA-3′. Sequences of the forward primer for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were 5′-GAAGGTGAAGGTCGGAGT-3′ and the reverse primer, 5′-GAAGATGGTGATGGGATTTC-3′; the sequence of the TaqMan probe was 5′-CAAGCTTCCCGTTCTCAGCC-3′. Conditions of one-step RT-PCR were as follows: 30 min at 48°C (stage 1, reverse transcription), 10 min at 95°C (stage 2, RT inactivation and AmpliTaq Gold activation), and then 40 cycles of amplification for 15 s at 95°C and 1 min at 60°C (stage 3, PCR). Data for survivin mRNA were normalized to the data for GAPDH.

X-irradiation Cells (1×10^3 or 1×10^5) were cultured in tissue culture dishes 100 mm in diameter (Falcon, Oxnard, CA) containing 10 ml of culture medium. After 6 h incubation, the culture medium was changed and cells were X-irradiated with varying doses of X-rays. X-irradiation was carried out at room temperature using an MBR-1520A-TW device (20 mA, 150 kV; Hitachi Medical, Tokyo) at a dose rate of 2.089 Gy/min.

Determination of cell survival and cytotoxicity Cell survival was determined using a clonogenic assay and percent cytotoxicity was determined by trypan blue staining.[18]

Measurement of caspase-3 activity Enzymatic activity of caspase-3 was measured by fluorometric assay according to the instruction manual for apoptosis published by Pharmingen (2nd Ed., December 1998, San Diego, CA). Cells were treated with lysis buffer (10 m*M* Tris-HCl, 10 m*M* $NaH_2PO_4/NaHPO_4$ pH 7.5, 130 m*M* NaCl, 1% Triton-X-100, 10 m*M* sodium pyrophosphate) and the lysate was centrifuged at 12 000*g* for 15 min. The supernatant was collected and the protein concentration was measured by BioRad DC-protein assay. Next the lysate, fluorogenic substrate acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartic acid-α (4-methyl-coumaryl-7-amide) (Ac-DEVD-MCA, PEPTIDE) with or without caspase-3 inhibitor Ac-DMQD-CHO (PEPTIDE), and reaction buffer (40 m*M* HEPES pH 7.5, 20% glycerol, 4 m*M* dithiothreitol (DTT)) were mixed in a 96-well microtiter plate (Costar). The cleavage of the substrate was monitored in terms of amino-4-methylcoumarin (AMC) liberation using a Spectrafluor (SLT Labinstruments; excitation 360 nm, emission 465 nm) at 37°C. The fluorescence corresponding to produced AMC was measured every 1 min during a 60-min period, and the caspase-3 activity was calculated as nmol AMC release/min/mg protein.

RESULTS

Survivin mRNA expression and radiosensitivity Expression of survivin mRNA was examined during the logarithmic phase of cell growth. Survivin mRNA was expressed at a high level in PANC-1 cells, a low level in MIAPaCa-2 cells, and an intermediate level in the other three cell lines (Table I). Expression of survivin mRNA in PANC-1 was 17 times greater than in MIAPaCa-2. After exposure to 5 Gy of X-irradiation, cell survival was examined using a clonogenic assay. Among the pancreatic cancer cells examined, PANC-1 cells, which had the highest levels of survivin mRNA, were most resistant to X-irradiation. MIAPaCa-2 cells, which had the lowest level of survivin mRNA, were most sensitive to X-irradiation (Table I). Our results suggest that survivin acts as a constitutive radioresistance factor in pancreatic cancer cells. The most radiosensitive cells, MIAPaCa-2, and the most radioresistant cells, PANC-1, were selected for the following experiments.

Determination of sublethal and lethal doses of X-irradiation Viable cell numbers were examined in MIAPaCa-2 and PANC-1 cells after exposure to varying doses of X-irradiation. In both lines, viable cell numbers decreased time- and dose-dependently (Fig. 1). Sublethal doses of X-irradiation were 1 and 2 Gy for MIAPaCa-2, and 2 and 4 Gy for PANC-1 cells. Lethal doses of X-irradiation were 10 Gy for PANC-1 cells and 5 Gy for MIAPaCa-2 cells.

Survivin mRNA after exposure to sublethal doses of X-irradiation To determine whether survivin mRNA is induced by sublethal doses of X-irradiation, MIAPaCa-2

Table I. Relationship of Cell Survival after X-irradiation to Survivin mRNA Expression in Five Human Pancreatic Cancer Cell Lines

Strain	% cell survival (mean±SD)	Relative survivin mRNA expression
PANC-1	29.5±4.74	1.000
AsPC-1	21.5±3.11	0.276
BxPC-3	14.5±2.09	0.459
Capan-1	11.4±1.28	0.577
MIAPaCa-2	4.7±1.25	0.059

Cell survival was determined at 10 days following 5 Gy of X-irradiation using clonogenic assay. Cell survival was expressed as a percentage of colony-forming numbers observed in non-irradiated cells. Survivin mRNA was measured by a quantitative TaqMan reverse transcription-polymerase chain reaction assay and is indicated as values relative to PANC-1 cells.

and PANC-1 cells were given doses of 1 or 2 Gy and 2 or 4 Gy, respectively. In MIAPaCa-2 cells, expression of survivin mRNA gradually increased, peaking at 3 days after exposure to 1 or 2 Gy of X-irradiation (Fig. 2A). Similarly, expression of survivin mRNA in PANC-1 cells increased gradually, peaking at 4 days after exposure to 2 or 4 Gy (Fig. 2B).

Protection against radiation-induced cell death associated with induction of survivin mRNA and suppression of caspase-3 activity To determine whether acquired

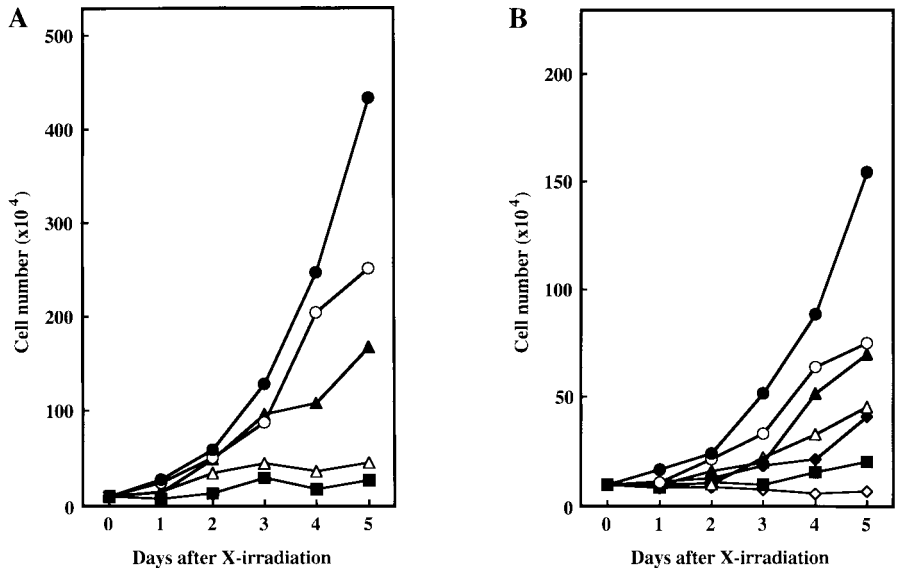

Fig. 1. Cell numbers surviving over time following exposure to varying doses of X-irradiation. In A, MIAPaCa-2 cells were exposed to 0 (●), 1 (○), 2 (▲), 3 (△), or 5 Gy (■). In B, PANC-1 cells were exposed to 0 (●), 1 (○), 2 (▲), 3 (△), 4 (◆), 5 (■), or 10 Gy (◇). Viable cell numbers were determined by trypan blue staining.

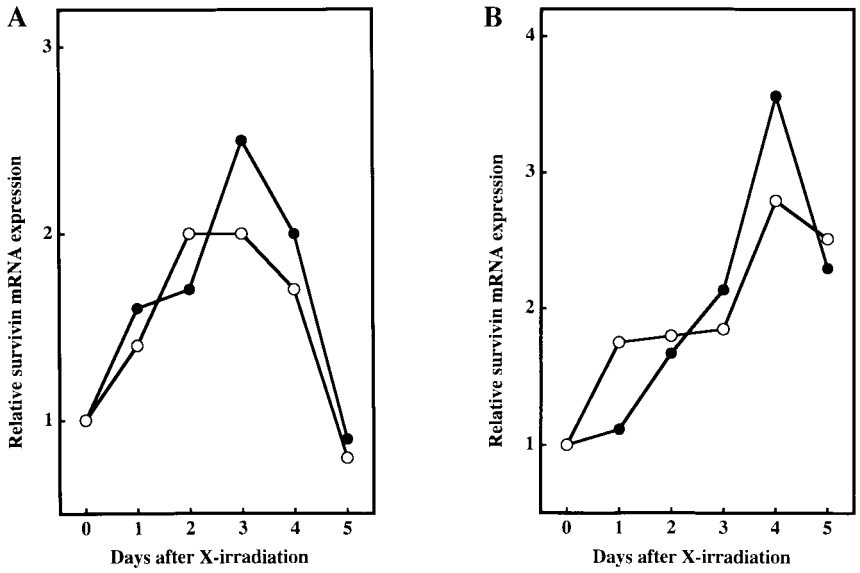

Fig. 2. Survivin mRNA expression after a sublethal dose of X-irradiation. Expression of survivin mRNA was measured by a quantitative TaqMan reverse transcription-polymerase chain reaction, and its value is represented relative to pre-irradiation expression (day 0). In A, MIAPaCa-2 cells were exposed to 1 (○) or 2 (●) Gy. In B, PANC-1 cells were exposed to 2 (○) or 4 (●) Gy.

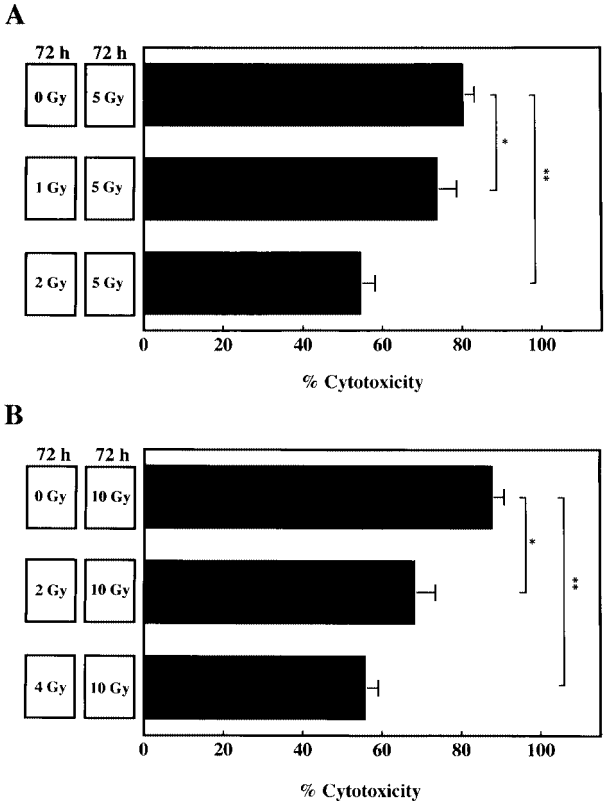

Fig. 3. Induction of radioresistance in (A) MIAPaCa-2 cells exposed to 1 or 2 Gy of X-irradiation, followed by 5 Gy; (B) PANC-1 cells exposed to 2 or 4 Gy of X-irradiation, followed by 10 Gy. Cells were cultured for 72 h after the first and second X-irradiation, and percent cytotoxicity was determined. * $P<0.05$, ** $P<0.02$ by Student's t test.

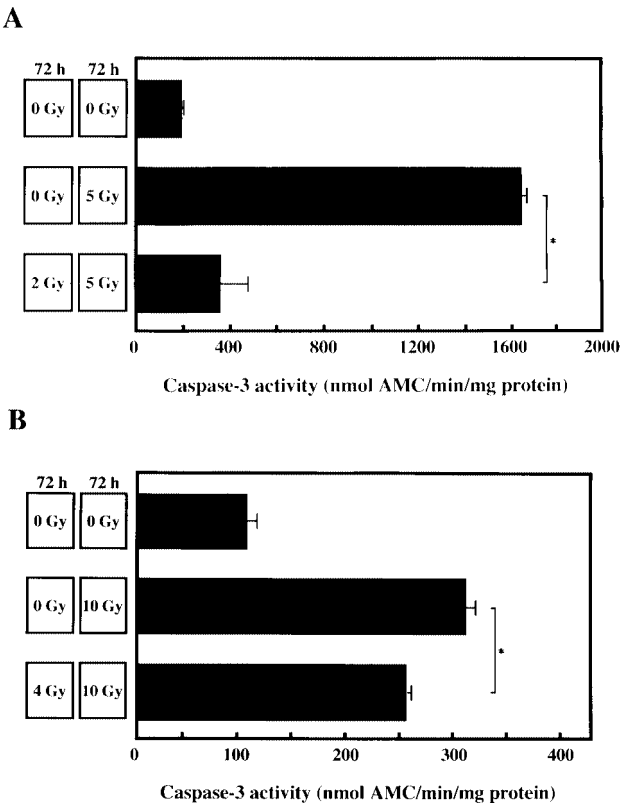

Fig. 4. Caspase-3 activity in MIAPaCa-2 (A) and PANC-1 (B) cells. Cells were X-irradiated under the indicated conditions, and cell lysates were extracted. Lysates were incubated at 37°C for 1 h, and the amount of AMC released was measured in terms of cleavage of the fluorogenic substrate Ac-DEVD-MCA (100 μM). The experiments were performed in duplicate, and the data represent the mean values±SD. * $P<0.01$ by Student's t test.

radioresistance is induced via augmentation of survivin mRNA expression in response to sublethal doses of X-irradiation, PANC-1 and MIAPaCa-2 cells were subjected to sublethal doses of X-irradiation followed by a lethal dose, after which the cytotoxicity was estimated (Fig. 3, A and B). Total cytotoxicity was decreased significantly in both cell lines by pretreatment with a sublethal dose of X-irradiation. To investigate whether this acquired resistance was induced by the function of survivin, we next examined directly enzymatic caspase-3 activity in MIAPaCa-2 and PANC-1 cells pretreated with a sublethal dose of X-irradiation. AMC release from the fluorogenic substrate Ac-DEVD-MCA increased in a time-dependent manner, and almost complete inhibition of AMC release resulted from inclusion of the specific caspase-3 inhibitor Ac-DMQD-CHO, indicating that the fluorescence detected in this assay truly represented caspase-3 enzymatic activity (data not shown). The caspase-3 activity was increased after a lethal dose of X-irradiation in MIAPaCa-2 (Fig. 4A) and PANC-1 cells (Fig. 4B), while pretreatment with a sublethal dose of X-irradiation followed by lethal X-irradiation resulted in significant suppression of caspase-3 activity.

DISCUSSION

Radiation results in DNA damage and generation of free radicals. Cells exposed to radiation die via both apoptosis and necrosis.[2] Many studies have demonstrated that p53, bcl-2, and caspase-3 are important in many responses to radiation.[1–3] These reports suggested that inhibition of apoptosis-activating factors and induction of apoptosis-inactivating factors contributed to resistance to radiotherapy. However, little has been reported about IAP family proteins as radioresistance factors in cancer cells.

Unlike other IAP family members, survivin is undetectable in most normal adult tissues, but is expressed abundantly in various human cancers.[4–6, 11, 13–15] Therefore, we

investigated whether survivin acts as a constitutive and inducible radioresistance factor in human cancer cells. First we examined the relationship between survivin mRNA expression and sensitivity to X-irradiation in pancreatic cancer cell lines. Various levels of survivin mRNA expression were detected in all five pancreatic cancer cell lines examined. PANC-1 cells showed the highest level of survivin mRNA expression and were the most resistant to X-irradiation; MIAPaCa-2 cells showed the least survivin mRNA expression and were the most sensitive to X-irradiation. This suggests that survivin acts as a constitutive resistant factor against X-irradiation. In addition, we have previously found that PANC-1 cells were the most resistant and MIAPaCa-2 cells were the most sensitive to other cellular stresses such as tumor necrosis factor α, anticancer drugs, and heat among the cell lines examined in this study.[19, 20] Consequently, survivin acts as a co-resistance factor against many types of cellular stresses. In general, adenocarcinoma, including pancreatic cancer, is considered to be radioresistant. Previous studies have demonstrated that many other kinds of cancer show some survivin gene or protein expression.[5, 6, 10] However, the relationship between radiosensitivity and survivin expression in pancreatic cancer has not been clarified, and remains to be investigated.

Next, we demonstrated that survivin mRNA expression was increased by sublethal doses of X-irradiation. Previous reports have demonstrated that intracellular resistance factors such as bcl-2, antioxidants, and heat shock proteins are upregulated in response to the cellular stresses of anticancer drug exposure, irradiation, and heat shock. These molecules were found to act as inducible resistance factors.[4, 21, 22] Our results suggested that the same was true for survivin in irradiated pancreatic cancer cell lines, since the degree of augmentation of survivin mRNA expression by sublethal doses of X-irradiation was related to the ability of the cells to survive a subsequent lethal dose. As regards protein expression of survivin, a previous study demonstrated that survivin protein level was almost parallel with the mRNA level.[8] In this study, we showed that caspase-3, which is known to be inhibited by survivin,[10] was significantly suppressed by pretreatment with a sublethal dose of irradiation. It was suggested that survivin protein acts as an anti-apoptotic molecule.

Enhanced ability of PANC-1 cells to survive a lethal dose of irradiation appeared to reflect both baseline expression and inducible expression of survivin. Since these cells showed both the highest baseline expression and the greatest inducibility of expression and radioresistance, inducibility of survivin may be related to baseline expression. In addition, PANC-1 cells also showed a lower baseline level and lower inducibility of caspase-3 activity, and this may reflect the constitutive survivin expression.

Taken together, our results suggest that survivin acts as a constitutive and inducible radioresistance factor in pancreatic cancer cells. Inhibition of survivin mRNA expression may enhance the effectiveness of radiotherapy.

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