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A Role for Survivin in Radioresistance of Pancreatic Cancer Cells

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Using gene-transduced pancreatic cancer cells, we examined whether survivin expression is directly involved in regulation of radiosensitivity. Ordinarily radiosensitive MIAPaCa-2 cells transduced with wild-type *survivin* gene (MS cells) proliferated more rapidly than cells transduced with control vector. MS cells were significantly less radiosensitive than control vector-transduced cells. Radiation-induced activity of caspase-3, but not caspase-7, was significantly inhibited in MS cells. On the other hand, transduction of a dominant-negative mutant *survivin* gene into radioresistant PANC-1 cells augmented radiosensitivity. Further, the radiation-induced increase in caspase-3 activity was enhanced, indicating that survivin function was truly inhibited. These results indicate that survivin expression directly down-regulates radiosensitivity.

Key words: Survivin — Pancreatic cancer — Radioresistance — Caspase-3

Survivin, a member of the inhibitor of apoptosis protein (IAP) family, contains a single baculovirus IAP repeat and lacks a carboxyl-terminal RING finger.[1,2] Survivin is expressed during human fetal development, but is not detectable in normal adult tissues, except for the thymus and placenta. Survivin is expressed in most malignant cells, including those of pancreatic, gastric, colonic, lung, breast, prostatic, and bladder cancers, neuroblastomas, and lymphomas.[2–8] In these malignant diseases, previous studies established that survivin is an important mediator in carcinogenesis, acting as a resistance factor by inhibiting caspase activity in cells exposed to diverse genotoxic stresses such as anticancer drugs and Fas-ligand.[9–12] The actions of survivin in irradiated cells, however, are not fully understood. Mechanisms underlying radioresistance have been investigated in pancreatic cancer, which is known to be radioresistant. We recently found that survivin can act as both a constitutive and an inducible radioresistance factor in pancreatic cancer cells.[13] However, the degree to which survivin contributes to radioresistance is unknown. We therefore examined the effect of *survivin* gene expression on cell survival and caspase-3 activity using radiosensitive MIAPaCa-2 cells transduced with a wild-type *survivin* gene. Further, we sought to augment radiosensitivity by inhibition of survivin function, using radioresistant PANC-1 cells transduced with a dominant-negative mutant of the *survivin* gene. As the MIAPaCa-2 and PANC-1 cells used in this study both harbor a mutant-type p53, effects from several molecules that interact with caspase-3 in cells with wild-type p53 could be excluded.

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MATERIALS AND METHODS

Cells The human pancreatic cancer cell lines MIAPaCa-2 and PANC-1, were purchased from the American Type Culture Collection (ATCC; Manassas, VA). MIAPaCa-2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nipro, Osaka) supplemented with 10% heat-inactivated fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS) and 2.5% heat-inactivated horse serum and grown at 37°C under 5% CO_2 and 95% humidified air. PANC-1 cells were cultured in RPMI 1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated FBS.

Quantitative reverse transcription polymerase chain reaction (RT-PCR) assays of mRNA for wild-type and dominant negative mutant of survivin Cells were homogenized and total RNA was extracted by ISOGEN (Nippon Gene, Tokyo). The extract was assayed for RNA concentration with the Gene Quant DNA/RNA Calculator (Pharmacia, Uppsala, Sweden). Survivin mRNA and 18S ribosomal RNA (18S rRNA) were determined by a quantitative RT-PCR using an ABI PRISM 7700 sequence detector system (Perkin-Elmer Applied Biosystems, Foster City, CA).[14,15] Gene-specific primers and fluorescent hybridization probes for survivin used in quantitative RT-PCR were as follows: forward primer, 5′-AAG AAC TGG CCC TTC TTG GA-3′; reverse primer, 5′-CAA CCG GAC GAA TGC TTT T-3′; and probe, 5′-CCA GAT GAC GAC CCC ATA GAG GAA-3′.[13,16] To determine the dominant-negative mutant of survivin mRNA, we changed the sequence of the reverse primer to 5′-TTG ACA GAA AGG AAA GCG GC-3′. To compare amounts of mRNA encoding survivin in different samples, the quantity of specific mRNA was normalized as a ratio to the amount of 18S

rRNA,[17] which was determined using TaqMan 18S Ribosomal Control Reagents Kit (Perkin-Elmer Applied Biosystems) according to the manufacturer's protocol. Quantitative RT-PCR was performed by a one-step method using a TaqMan Core Reagent Kit (Perkin-Elmer Applied Biosystems).

Transfection of wild-type survivin and dominant-negative mutant of survivin into pancreatic cancer cells A plasmid encoding human survivin, pcDNA3-myc-survivin, was kindly provided by Dr. J. C. Reed (The Burnharm Institute, La Jolla, CA). This plasmid was constructed by ligation of human survivin cDNA obtained from Jurkat T cells into pcDNA3-myc.[12] We used PCR to construct a dominant-negative mutant of survivin by using the survivin complementary DNA (cDNA) as a template. Two overlapping complementary oligonucleotide primers designed to produce a T-to-G substitution at nucleotide 354 and a G-to-C substitution at nucleotide 355 included a forward primer, 5′-GCA TTC GTC CGG TGC CGC TTT CC-3′, and a reverse primer, 5′-GGA AAG CGG CAC CGG ACG AAT GC-3′. PCR products were digested with *Xba*I and *Not*I and ligated into pcDNA3-myc. This plasmid, designated pcDNA3-myc-C84A-survivin, encodes a mutant form of survivin with substitution of a cysteine residue at amino acid 84 by an alanine residue. Transfections were performed using LipofectAmine Plus reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Briefly, 10^6 cells were seeded in each well of a 6-well culture plate (Costar, New Bedford, MA) in 3 ml of cultured medium, and incubated for 24 h. The cells were washed two times with serum-free medium, then 800 μl of medium and 200 μl of DNA-Plus-LipofectAmine reagent complexes containing either 3 μg of pcDNA3-myc-survivin, pcDNA3-myc-C84A-survivin, or pcDNA3-myc, were added to six wells each. After 3 h of incubation, 2 ml of medium supplemented with 15% FBS was added to each well, and cells were incubated for an additional 24 h. At the end of incubation, the cells were washed two times with serum-free medium, and 3 ml of medium containing 10% FBS, and 800 μg/ml of geneticin sulfate (Invitrogen) was added. Geneticin-resistant clones were obtained by the limiting dilution method. Expression of survivin mRNA and dominant-negative mutant of survivin mRNA was determined by a quantitative RT-PCR method as described above.

X-irradiation Cells (2×10^3 or 5×10^5) were cultured in 96-well microplates (Costar, Tokyo) and tissue culture dishes 100 mm in diameter (Costar) containing 200 μl and 10 ml of culture medium, respectively. After 24 h incubation, the culture medium was changed and the cells were irradiated with 5 Gy 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 Viable cell numbers were estimated using a Cell Counting Kit (Dojindo, Kumamoto). Briefly, 10 μl of WST-1 stock solution (5 m*M* WST-1, 20 m*M* HEPES, 0.2 m*M* 1-methoxy PMS in H_2O) was added to each well of the 96-well microplates and incubated for 1 h. Then, the absorbance of the culture supernatants was measured with a microculture plate reader (Easy Reader EAR400, SLT Labinstruments, Grodig, Austria) at 450 nm.

Measurement of caspase-3 activity Enzymatic activity of caspase-3 was measured by a 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-methylcoumaryl-7-amide) (Ac-

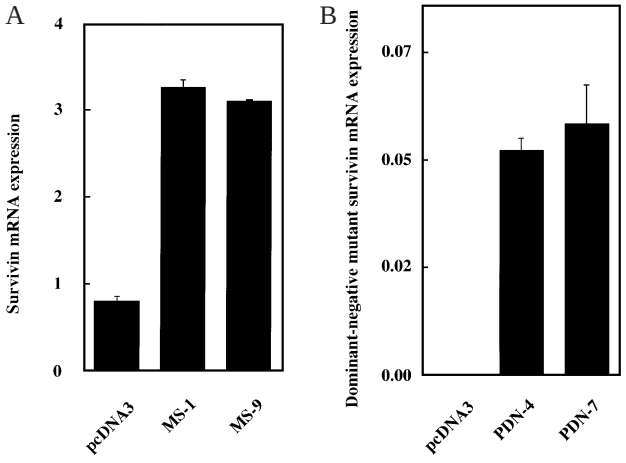

Fig. 1. A: Survivin mRNA expression by gene-transduced MIAPaCa-2 cells. Total RNA was extracted and mRNA expression for survivin and 18S rRNA was measured by TaqMan RT-PCR as described in "Materials and Methods." Survivin mRNA expression was quantitated relative to expression of 18S rRNA. Data represent the mean±SD of three independent samples. B: Expression of mRNA for the dominant-negative mutant of survivin in gene-transduced PANC-1 cells. Total RNA was extracted, and mRNA expression for the dominant-negative mutant of survivin and 18S rRNA was measured by TaqMan RT-PCR. Expression of the dominant-negative mutant of survivin was quantitated relative to expression of 18S rRNA. Data represent the mean±SD of three independent samples. pcDNA3, cells transduced with control vector; MS, cells transduced with wild-type *survivin* gene expression vector; PDN, cells transduced with the dominant-negative mutant of survivin; RT-PCR, reverse transcription-polymerase chain reaction.

DEVD-MCA, Peptide Institute, Osaka) with or without the specific caspase-3 inhibitor acetyl-L-aspartyl-L-methionyl-L-glutaminyl-L-aspartyl-aldehyde (Ac-DMQD-CHO, Peptide Institute), and reaction buffer (40 m*M* HEPES pH 7.5, 20% glycerol, 4 m*M* DTT) were mixed in 96-well microtiter plates (Costar). The cleavage of the substrate was monitored in terms of amino-4-methylcoumarin (AMC) liberation in a Spectrafluor (SLT Labinstruments; excitation 360 nm, emission 465 nm) at 37°C. The fluorescence due to produced AMC was measured every 1 min during a 60-min period, and the caspase-3 activity was calculated as nmol AMC released/min/mg protein.[15)]

RESULTS

Growth potential in cells transduced with wild-type and dominant-negative mutants of the *survivin* gene We first examined proliferation in MIAPaCa-2 cells transduced with wild-type *survivin* gene to assess the effect of overexpression in the absence of irradiation. MIAPaCa-2 cells transduced with wild-type *survivin* gene expression vector (pcDNA3-myc-survivin) showed an approximately 4-fold increase in survivin mRNA and a higher proliferation rate than cells transduced with control vector (pcDNA3; doubling time (h)=55.9 for pcDNA3, 42.2 for MS-1, and 45.4 for MS-9; Figs. 1A and 2A). This proliferation-enhancing effect was particularly evident as a 1.3- to 1.4-fold increase at 3 days after initiating incubation of transduced cells. Meanwhile, in PANC-1 cells transduced with a dominant-negative mutant of the *survivin* gene expression vector (pcDNA3-myc-C84A-survivin), the transduced gene was demonstrated to be expressed. Transduced cells showed similar or lower proliferation rates compared to cells transduced with control vector (pcDNA3; doubling time (h)=47.3 for pcDNA3, 49.4 for PDN-4, and 50.6 for PDN-7; Figs. 1B and 2B). No difference was observed in morphological features or cell cycle distribution between these transduced clones and control cells (data not shown).

Alterations in radiosensitivity and caspase-3 activity in transduced cells To clarify whether resistance to X-irradiation can be acquired by overexpression of the *survivin* gene, we first examined cell survival in irradiated MIAPaCa-2 cells transduced with wild-type *survivin* gene expression vector. As shown in Fig. 3A, the sensitivity of *survivin* gene-transduced cells was significantly lower than that of cells transduced with control vector ($P<0.01$ and 0.05). We next examined cell survival in PANC-1 cells transduced with a dominant-negative mutant of the *survivin* gene expression vector, to clarify whether inhibition of survivin function could augment radiosensitivity. The aberrant survivin protein encoded by the mutant gene is

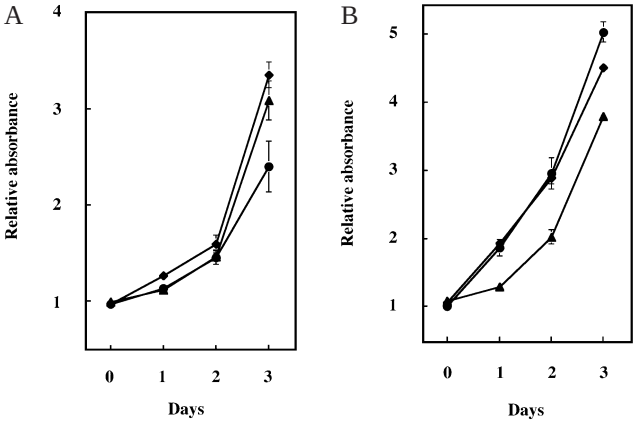

Fig. 2. Growth curve of cells transduced with wild-type *survivin* gene (A) and a dominant-negative mutant of survivin (B). Cell viability was estimated by a colorimetric assay as described in "Materials and Methods." Absorbance, which reflects cell number, is stated relative to the value for cells at day 0. Data represent the mean±SD of three independent samples. In A, MIAPaCa-2 cells transduced with pcDNA3 are represented as ●; cells with wild-type *survivin* gene are ◆, MS-1; and ▲, MS-9. In B, PANC-1 cells transduced with pcDNA3 are shown as ●; cells with the dominant-negative mutant of survivin are ◆, PDN-4; and ▲, PDN-7.

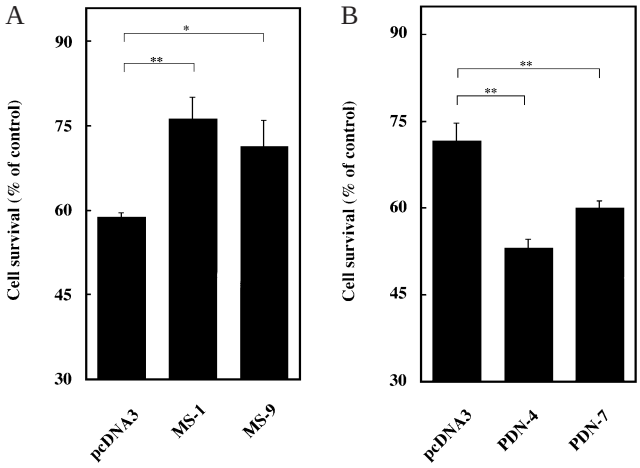

Fig. 3. Effect of transduction of wild-type *survivin* gene into MIAPaCa-2 (A) and the dominant-negative mutant of survivin into PANC-1 (B) on cell survival after 5 Gy of X-irradiation.[13)] Cells were cultured for 72 h after the irradiation and cell survival was assessed by a colorimetric assay as described in "Materials and Methods." Cell survival is stated relative to the value for controls without irradiation. Data represent the mean±SD of three independent samples. The term "pcDNA3" refers to cells transduced with control vector. In A "MS" indicates cells transduced with wild-type *survivin* gene expression vector. In B "PDN" indicates cells transduced with the dominant-negative mutant of survivin. * $P<0.05$, ** $P<0.01$ by Student's *t* test.

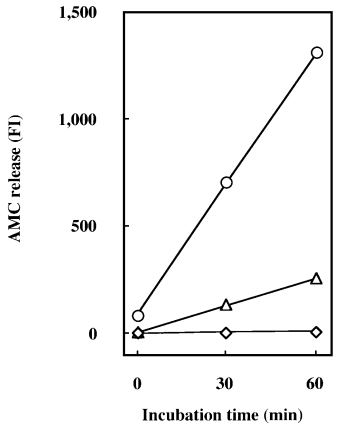

Fig. 4. Effect of a specific caspase-3 inhibitor, Ac-DMQD-CHO, on fluorogenic AMC release in lysates of MIAPaCa-2 cells. Lysates were incubated at 37°C for 60 min, and the amount of AMC released (FI) by cleavage of the fluorogenic substrate Ac-DEVD-MCA (100 μM) was measured. Data represent the mean value of three independent samples. X-irradiated cell lysate without Ac-DMQD-CHO, ○; control cell lysate without Ac-DMQD-CHO, △; and X-irradiated cell lysate with Ac-DMQD-CHO, ◇. Ac-DMQD-CHO, acetyl-L-aspartyl-L-methionyl-L-glutaminyl-L-aspartyl-aldehyde; AMC, amino-4-methylcoumarin; FI, fluorescence intensity.

Table I. Caspase-3 Activity in Gene-transduced Cells

Strain	0 Gy	5 Gy	Ratio[a)]
pcDNA3 (MIAPaCa-2)	120.1±14.1	666.8±10.9	5.55
MS-1	109.9±13.9	448.8±5.9	4.08
MS-9	117.4±6.2	438.4±2.8	3.73*
pcDNA3 (PANC-1)	129.6±8.6	219.9±16.1	1.69
PDN-4	108.1±12.9	514.9±6.1	4.76**
PDN-7	116.2±2.0	489.9±6.2	4.22**

a) Relative ratio to cells without X-irradiation.
Data represent mean±SD (nmol AMC/min/mg protein) of three independent lysates.
* $P<0.05$, ** $P<0.01$ by Student's *t* test.

not able to inhibit the activation of caspases, because the mutation is located in the baculovirus inhibitors of apoptosis repeats (BIR) domain, which is essential for interaction between caspases and survivin protein.[18)] Sensitivity was enhanced in these cells compared to control vector-transduced cells (Fig. 3B). To confirm that the transduced survivin participates in the signaling pathway of irradiated cells, caspase-3 activity was measured following X-irradiation. AMC release from the fluorogenic substrate Ac-DEVD-MCA for caspase-3 and -7 increased in a time-dependent manner. A specific caspase-3 inhibitor, Ac-DMQD-CHO, completely inhibited AMC release, indicating that the fluorescence detected in this assay truly represented caspase-3 enzymatic activity (Fig. 4). In cells transduced with *survivin* gene, the increase of caspase-3 activity in response to X-irradiation was significantly inhibited (Table I). In PANC-1 cells, constitutive caspase-3 activity was lower than MIAPaCa-2 cells, but transduction of a dominant-negative mutant of the *survivin* gene augmented caspase-3 activity by 2.5- to 2.8-fold.

DISCUSSION

In this study we investigated whether sensitivity to X-irradiation could be affected by survivin expression using cells transduced with wild-type *survivin* gene or a dominant-negative mutant of the *survivin* gene. Cells transduced with the wild-type *survivin* gene showed higher growth potential than control vector-transduced cells. Recently, Ito *et al.* examined the effect of survivin on cell proliferation in human hepatocellular carcinoma cells transduced with wild-type *survivin* gene.[19)] They demonstrated that overexpression of survivin resulted in faster cell proliferation via a decrease in the duration of the G0/G1 phase and an increase in the S phase. They also found that survivin interacts with cyclin-dependent kinase 4 (Cdk4) and that p21/WAF-1/Cip1 was released from Cdk4 by overexpression of survivin using an immunoprecipitation method. In our present experiments, no difference in constitutive caspase-3 activity was seen between cells transduced with *survivin* gene and cells transduced with control vector (Table I). This suggested that the increased proliferation associated with overexpression of *survivin* gene resulted from activation of the cell cycle, not inhibition of the apoptotic signaling pathway.

In this study we obtained the first evidence that survivin expression can decrease sensitivity to X-irradiation via inhibition of caspase-3 activity. Since we demonstrated that inhibition of caspase-3 activity by a specific caspase-3 inhibitor augmented radiosensitivity, caspase-3 may be an important molecule for the radiation-induced cytotoxicity.[20)]

Grossman *et al.* recently examined whether overexpression of survivin affects ultraviolet-B (UVB)-induced cytotoxicity by using transgenic keratinocytes, and found that the percentage of sunburned cells and the extent of UVB-induced apoptosis were decreased in *survivin* gene-transduced keratinocytes.[21)] These results suggest that survivin expression is involved in regulation of sensitivity to several types of radiation, not only in cancer cells, but also in normal cells. In addition, in our experiments, overexpression of survivin resulted in partial prevention of irradiation-induced cytotoxicity, supporting findings that other resistance-related molecules such as bcl-2, bcl-XL, and c-IAP-1 also act as resistance factors against irradiation by inhibiting caspase activity.[22–24)] Generally, survivin acts as

a resistance factor against various genotoxic stresses via not only caspase-3, but also caspase-7. In our experiments, caspase-7 activity was not increased in X-irradiated cells, indicating that resistance induced by survivin expression was caused mainly by inhibition of caspase-3 activity. Among other IAP family members, HIAP1, HIAP2, XIAP, and livin have been reported to inhibit activation of caspase-3, like survivin.[12, 25–27] Therefore, these molecules also may act as radioresistance factors.

Whether inhibition of survivin function increases radiosensitivity has been unclear, although previous studies showed sensitization of cells to other genotoxic stresses such as anticancer drugs.[11, 28] We therefore transduced a dominant-negative mutant *survivin* gene into radioresistant PANC-1 cells, and found that this resulted in augmentation of radiosensitivity. It has been reported that the aberrant survivin protein encoded by the mutant gene is unable to inhibit activation of caspases.[18] We found significant enhancement of an irradiation-associated increase in caspase-3 activity in cells transduced with the dominant-negative mutant.

In general, adenocarcinomas including pancreatic cancer tend to be radioresistant. Previous studies have demonstrated that many types of cancer show some degree of *survivin* gene or protein expression.[2–8] Consequently, inhibition of *survivin* gene expression or protein function may be an effective mode of radiosensitization therapy in various cancers. Since irradiation has major dose-dependent side effects, including tissue fibrosis and bone marrow suppression, inhibition of survivin may increase the efficacy of low doses and thus, minimize adverse effects.

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