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Radiation-induced apoptosis of tumor cells is facilitated by inhibition of the interaction between Survivin and Smac/DIABLO

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Abstract

To investigate the mechanism of radioresistance of solid tumor cells, we created two expression vectors encoding Survivin mutants, T34A and D53A. When T34A and D53A were overexpressed in NIH3T3, A549 and HeLa cells, radiation-induced apoptosis was significantly enhanced. Furthermore, we examined the binding capability of Survivin with Smac/DIABLO in the cells that overexpressed these mutants. Coimmunoprecipitation analysis revealed that mutant form of Survivin, D53A and T34A could bind to Smac/DIABLO, but with much less affinity compared to the authentic form. These results suggest that radiation-induced apoptosis of tumor cells is increased by inhibition of the interaction between Survivin and Smac/DIABLO through overexpression of T34A and D53A.

Key words: Survivin, radiation, apoptosis, dominant negative mutant, adenoviral vector

Introduction

Ionizing radiation is a useful tool for cancer therapy. In solid tumor cells derived from adenocarcinoma, squamous cell carcinoma or melanoma, it is generally considered that radiation-induced cell death mainly occurs through reproductive cell death but not apoptotic cell death. However, in hematopoietic cell lines, malignant lymphoma and leukemia cells [1-3], etc., apoptotic cell death is prone to be induced by genotoxic agents, including ionizing radiation. In the treatment combined with some antitumor drugs, radiation-induced cell death is sometimes enhanced through an increase of apoptosis even in radioresistant solid tumor cell lines [4-6]. In fact, we have reported that a novel anticancer drug, 1-(3-C-ethynyl-β-D-ribo-pentofutanosyl)cytosine (ECyd), enhances radiation-induced apoptosis in human gastric adenocarcinoma MKN45 (p53 wild type), MKN28 (p53 mutation) and murine rectum adenocarcinoma Colon26 (p53 status unknown) cells. The decrease in the radiation-induced expression of Survivin, Bcl-2, cyclin B1 and Wee1 was partly responsible for the enhancement of radiation-induced apoptosis by ECyd regardless of p53 status and cell type *in vitro* [7] and *in vivo* [8]. Furthermore, in MKN45 and MKN28 cells, purvalanol A, a cyclin-dependent kinase inhibitor, also enhances radiation-induced cell killing through an increase of apoptosis by downregulation of inhibitor of apoptosis family (IAP) family members (Survivin and XIAP), Bcl-2 family members (Bcl-X_L and Bcl-2), cyclin B1 and Wee1 [9]. Since it is generally considered that ionizing radiation promotes apoptotic signaling such as cytochrome *c* release from mitochondria, followed by activation of caspase-9, -3 or the caspase-activated deoxyribonuclease (DNase)(CAD) pathway [10,11], the constitutive or inducible anti-apoptotic proteins seem to inhibit this apoptotic signaling in the radioresistance of solid tumor cells.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family protein [12], which is intensively expressed in the G_2/M phase and potentially involved in both inhibition of apoptosis and control of cell division [13-16]. Its expression is prominently upregulated in most human cancer cells, but is undetectable or very low in normal tissues [17,18]. The high expression of Survivin in cancer cells is a marker of negative prognostic significance and a cause of tumor resistance to therapies [19-22]. The phosphorylation at threonine 34 of Survivin by Cdc2 is accepted to be essential for the anti-apoptotic activity of Survivin [23]; however, the anti-apoptotic mechanisms of Survivin have remained controversial. Early studies suggested that the Survivin directly bound to caspase-3 and suppressed apoptosis [24,25]. However, others failed to demonstrate direct effects on this protease [26-28]. As another possible mechanism, Song *et al.* [29] recently showed that Survivin bound to Smac/DIABLO, which is known as a proapoptotic protein that binds to IAPs such as XIAP, and prevents apoptosis by suppressing caspase-9 and -3. The replacement to alanine at aspartic acid 53 (D53A) of Survivin has been demonstrated to fail to associate with Smac/DIABLO and the disruption of this binding activity enhances TRAIL-, doxorubicin- and RIP3-induced apoptosis in HeLa cells [30]. However, the relationship between the phosphorylation of Survivin and its binding activity with Smac/DIABLO is still unclear.

In the present study, we examined whether ionizing radiation promoted cytochrome *c* release from mitochondria in A549 cells and Survivin was involved in inhibition of apoptotic signaling downstream of mitochondria. For this purpose, two expression vectors encoding Survivin mutants, T34A (phosphorylation-defect mutant) and D53A (defective in binding activity with Smac/DIABLO) were created. Furthermore, binding activity of Survivin with Smac/DIABLO in the cells

overexpressing these mutants was also studied.

2. Materials and methods

2.1. Reagents

Carbobenzoxy-Val-Ala-Asp-fluoromethane (Z-VAD-fmk), acetyl-Leu-Glu-His-Asp-aldehyde (Ac-LEHD-CHO), acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) and acetyl-Ile-Glu-His-Asp-aldehyde (Ac-IETD-CHO) were from Peptide Institute (Osaka, Japan). Propidium iodide (PI) and nocodazole were from Sigma Chemical Company (St. Louis, MO). ^{32}Pi (orthophosphoric acid) was from MP Biomedicals, Inc. (Irvine, CA). Protein G-Sepharose was from Amersham-Pharmacia Biotech (Buckinghamshire, UK). The following antibodies were used for western blotting and immunoprecipitation: anti-Survivin and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-cytochrome *c* (BD PharMingen, Erebodegem, Belgium), anti-Smac/DIABLO (Calbiochem) and anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA). The chemiluminescence detection kit, Western Lighting^{TM} Chemiluminescence Reagent Plus, was from Perkin Elmer (Boston, MA).

2.2. Cell culture

Mouse fibroblast cell line NIH3T3, embryonic kidney cell line 293A and human cervical carcinoma cell line HeLa were grown in DMEM medium containing 10% fetal calf serum at 37°C in 5% CO_2. Human lung carcinoma A549 cells were maintained in RPMI 1640 medium containing 10% fetal calf serum at 37°C in 5% CO_2.

2.3. PCR-mediated mutagenesis

The cDNA of Survivin was amplified by RT-PCR from total RNA of cell line HeLa and cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). To obtain the Survivin mutants, Survivin-T34A (T34A), -D53A (D53A), -T34E (T34E) and -T34D (T34D), we employed PCR-mediated mutagenesis. Mutations were further verified using the CEQ8000 DNA Analysis System (Beckman Coulter, Fullerton, CA).

2.4. Plasmid construction and transfection

The amplified cDNAs of WT and its mutants were subsequently cloned into GFP-fused mammalian expression vector pcDNA3.1/CT-GFP (Invitrogen). Transfection of NIH3T3 cells with GFP-fused plasmids was carried out using PLUS Reagent and LipofectAMINE Reagent (Invitrogen) according to the instructions provided by the manufacturer. Twenty-four hours after transfection, comparable expression of the various transfected constructs was confirmed with an EPICS ALTRA flow cytometer (Beckman Coulter) by gating the GFP-expressing population.

2.5. Adenoviral construction and transduction

The cDNAs of WT and its mutants were inserted into pAd/CMV/V5-DEST (Invitrogen). Replicant-defective adenoviral vectors pAd-LacZ, pAd-WT, pAd-T34A and pAd-D53A were generated using the Virapower Adenoviral Expression System (Invitrogen). Viral titers were determined using an Adeno-X^{TM} Rapid Titer kit (Clontech). Subconfluent cultures of the tumor cell lines were incubated with pAd vectors at MOI 50 and the virus was allowed to adhere for 1 h at 37°C. Then medium was added and the incubation was continued under the same conditions for an additional

24 h. For immunocytochemistry, the cells were fixed with 4% paraformaldehyde and analyzed for Survivin expression.

2.6. Treatment with X irradiation

At 24 h after transfection or transduction, the cells were irradiated with an X ray generator with a 1.0-mm aluminum filter at 200 kVp and 10 mA, (Shimazu HF-350, Kyoto) at a dose of 3.9 Gy/min, which was determined using Fricke's chemical dosimeter. Then they were incubated for another 48 h at 37°C. Addition of 20 μM caspase inhibitors, Z-VAD-fmk, Ac-LEHD-CHO, Ac-DEVD-CHO and Ac-IETD-CHO, was carried out at the same time as treatment with irradiation.

2.7. SDS-PAGE and Western blotting

The cells were collected 48 h after treatment with irradiation. The pellet was suspended in 50 μl of lysis buffer (20 mM HEPES [pH 7.4], 2 mM EGTA, 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml pepstatin) and kept on ice for 30 min. After centrifugation at 15,000 rpm for 15 min at 4°C, a threefold volume of Laemmli's sample buffer (0.625 M Tris-HCl [pH6.8], 10% β-mercaptoethanol, 20% SDS, 20% glycerol, 0.004% bromophenol blue) was added to the supernatant, which was then boiled for 3 min. Proteins were separated by SDS-PAGE and transferred onto nitrocellulose membranes (ADVANTEC Toyo, Tokyo, Japan). The membranes were probed with anti-Survivin, anti-cleaved caspase-3, anti-Smac/DIABLO or anti-actin in TBST buffer (10 mM Tris-HCl, 0.1 M NaCl, 0.1% Tween-20, pH 7.4) containing 5% nonfat skim milk overnight at 4^{o}C. These antibodies were detected by a method using

HRP-conjugated anti-rabbit, anti-rat or anti-goat IgG antibodies with Perkin Elmer Western Lighting^{TM}, Chemiluminescence Reagent Plus.

2.8. Assays of the release of cytochrome c from mitochondria

The cytosol of cells was prepared by a modification of the method of Bossy-Wetzel *et al.* [31]. Briefly, the cell pellet was resuspended in 300 μl of extraction buffer, containing 250 mM sucrose, 20 mM HEPES-KOH (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol (DTT) and 1 mM PMSF. After 1 h incubation on ice, cells were disrupted with a homogenizer and centrifuged at 1,000 g for 10 min at 4°C to remove nuclei and unbroken cells. The supernatant was further centrifuged at 26,000 g for 30 min at 4°C to separate mitochondria. The cytochrome *c* in the cytosol was detected using SDS-PAGE and western blotting as described above.

2.9. Coimmunoprecipitation

Cells were lysed in a Triton X-100-based lysis buffer (1% Triton X-100, 10% glycerol, 150 mM NaCl, 20 mM Tris, pH 7.5, 2 mM EDTA, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 10 μg/ml pepstatin) overnight, and the nuclear and cellular debris was cleared by centrifugation. Then the lysed cytosol was mixed with a Smac/DIABLO monoclonal antibody bound to Protein G-Sepharose. After overnight incubation at 4°C, the immunoprecipitates were washed two times in lysis buffer, and proteins were recovered by boiling beads in SDS sample buffer and analyzed by western blotting.

2.10 Detection of phosphorylated Survivin in A549 cells

A549 cells were incubated with phosphate-free medium containing 0.2 mCi/ml ^{32}Pi and 5% dialyzed FBS for 1 h, and then 100 ng/ml nocodazole was added to the medium. After incubation for 6 h, proteins in the cell lysates were immunoprecipitated by anti-Survivin antibody as described above and were separated by SDS-PAGE. Phosphorylated Survivin was visualized by autoradiography using the imaging analyzer (FUJIX BAS2500, Fuji photo Film, Tokyo, Japan).

2.11. Detection of apoptotic cells by fluorescence microscopy with propidium iodide staining and flow cytometry using annexin V

Cells were collected 48 h after treatment with irradiation. The pellet was washed twice with PBS(-) and fixed with 1% glutarardehyde/PBS(-) solution. The fixed cells were washed and resuspended in 20 μl of PBS(-) including 40μg/ml propidium iodide (PI) for 15 min in the dark to assess apoptotic cells. At least 200 cells were scored using an Olympus BX50 microscope with reflected-light fluorescence to count cells with chromatin condensation as apoptotic ones. For the detection of apoptosis using annexin V, tumor cells were collected 24 h after irradiation. For the detection of apoptotic cells, the pellets were stained with binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) containing a fluorescein isothiocyanate (FITC) conjugate of annexin V for 10 min in the dark. Then the cells were analyzed by flow cytometry.

3. Results

3.1. Radiation induces cytochrome c release from mitochondria and overexpression of Smac/DIABLO but not apoptosis

We first examined whether ionizing radiation induced apoptotic cell death. When A549 cells were exposed to 10 Gy of X rays and incubated for 12, 24, 36 and 48 h, the cells exhibited cell cycle arrest in the G_2/M phase (Fig. 1A) and enlargement of nuclei (Fig. 1B), but no increase of cells in sub-G_1 fraction or morphological changes such as nuclear condensation and fragmentation were observed. Moreover, X irradiation of A549 cells did not induce activation of caspase-3 in any incubation times as shown in Fig. 1C, though active fragments were easily observed in human leukemia MOLT-4 cells exposed to same dose of X rays. On the other hand, cytochrome *c* was released from mitochondria to cytosol in A549 cells (Fig. 1D) and the expression of the proapoptotic protein Smac/DIABLO was also increased after irradiation (Fig. 1E). It was suggested that the apoptotic signaling pathway was promoted until the mitochondrial level after irradiation, although activation of this signaling was not sufficient to achieve apoptotic cell death at the time examined.

3.2. Overexpression of Survivin mutants enhances radiation-induced apoptosis

To investigate whether constitutive Survivin in tumor cells was involved in antiapoptotic properties against Smac/DIABLO-related apoptotic signaling, which was promoted by ionizing radiation as mentioned above, we next constructed six GFP-fused plasmids: GFP, Survivin-WT/GFP (WT), Survivin-T34A/GFP (T34A), Survivin-D53A/GFP (D53A), Survivin-T34E/GFP (T34E) and Survivin-T34D/GFP (T34D). We then transfected them to evaluate their ability to induce apoptosis in mouse fibroblast NIH3T3 cells prior to experiments in tumor cells. GFP-expressing cells observed by phase-contrast and fluorescence microscopy are shown in Fig. 2A. Transfection of NIH3T3 cells with various GFP fused-vectors resulted in equally gained

intense GFP-labeling in about 70% of cells at 24 h (Fig. 2B). The overexpression of proteins was also confirmed by western blot analysis (Fig. 2C). In this SDS-PAGE pattern, the position of D53A in the gel was slightly shifted below and those of T34E and T34D were shifted above in comparison with those of WT and T34A. These differences in the mobility of Survivin mutants were explained by loss and addition of negative charge by mutagenesis, since phosphorylation and dephosphorylation of Cdc2 [32], Wee1 [33], Rb [34] and Sld1 [35] were reported to induce deceleration and acceleration in the migration in the gel, respectively. Next, we investigated the effects of overexpression of Survivin mutants on radiation-induced apoptosis in mouse fibroblast NIH3T3 cells. Cells were fixed and stained with PI 48 h after treatment with 20 Gy of X rays. Figure 3A shows fluorescent microscopic observation of morphological changes of cell nuclei, DNA fragmentation and chromatin condensation in D53A-overexpressing NIH3T3 cells with or without X irradiation. X irradiation induced nuclear condensation and fragmentation in the cells, but not in WT-overexpressing cells. No morphological changes were observed in the cells that overexpressed D53A alone. Quantitative measurements of apoptotic cells revealed that the overexpression of not only T34A but also D53A significantly enhanced X-ray-induced apoptosis. The amounts of apoptosis in D53A-overexpressing cells (27$\pm$3.1%) were higher than in T34A-overexpressing cells (20$\pm$1.9%), whereas no increase was observed in cells that overexpressed WT or in phospho-mimic mutants (T34E and T34D) (Fig. 3B).

3.3. Survivin-T34A and -D53A enhance radiation-induced apoptosis in a caspase-dependent manner

Furthermore, to examine whether enhancement of apoptosis in X-irradiated

NIH3T3 cells overexpressed with T34A and D53A was associated with caspase-3, immunoblotting for active fragments p17 of caspase-3 was performed. Fig. 3C showed that X irradiation induced active fragments of caspase-3 in the cells overexpressed with T34A and D53A but not GFP, WT, T34E and T34D. Next, we tested the effects of caspase inhibitors on the enhancement of radiation-induced apoptosis by overexpression with Survivin mutants in NIH3T3 cells (Fig. 3D). The broad spectrum caspase inhibitor Z-VAD-fmk, caspase-3 inhibitor Ac-DEVD-CHO, caspase-8 inhibitor Ac-LEHD-CHO or caspase-9 inhibitor Ac-IETD-CHO, was added to culture at the same time as X irradiation. Addition of the broad-spectrum caspase, caspase-9 and caspase-3 inhibitors, but not the caspase-8 inhibitor, to the medium suppressed the enhancement of radiation-induced apoptosis (Fig. 3D) in cells that overexpressed T34A or D53A.

3.4. Adenovirus-mediated overexpression of Survivin mutants enhances radiation-induced apoptosis in tumor cells

To achieve high-level and relatively long-term expression in tumor cells, we constructed replication-deficient adenoviral vectors encoding Survivin mutants. We used pAd-LacZ expressing β-galactosidase as a toxicity control for the virus. Transduction with the adenoviral vector pAd-WT, pAd-T34A or pAd-D53A, resulted in the strong expression of Survivin in human lung carcinoma A549 and cervical carcinoma HeLa cells as proved by western blots (Fig. 4A). Furthermore, immunocytochemistry revealed positive signals in almost all cells of both tumor lines (Fig. 4B).

To clarify whether radiation-induced apoptosis was enhanced by Survivin mutants in tumor cell lines, the apoptotic induction in tumor cells with

adenoviral-mediated expression of T34A and D53A was evaluated. Annexin V-FITC staining was employed as a marker for apoptosis. When A549 cells transduced with pAd-D53A and pAd-T34A were irradiated, a biphasic flow cytometric profile indicating the appearance of annexin V-positive cells was observed, but the cells with pAd-LacZ and pAd-WT showed no biphasic pattern after irradiation (Fig. 5A). In fluorescence microscopic observation, nuclear fragmentation and condensation were observed in A549 cells overexpressed with T34A and D53A and were irradiated with 10 Gy of X rays (Fig. 5B), whereas no apoptotic morphological changes were observed in irradiated cells expressed with LacZ and WT. Similar results were also obtained in HeLa cells (data not shown). Quantitative measurements of apoptotic cell death in A549 and HeLa cells clearly indicated that the adenoviral-mediated overexpression of D53A and T34A significantly enhanced radiation-induced apoptosis in comparison with that of the control (no transduction), pAd-LacZ and pAd-WT (Fig. 5C).

3.5. Phosphorylation of Survivin is required for its interaction with Smac/DIABLO

At first, we carried out to examine phosphorylation status of Survivin-WT, -T34A and -D53A in the cells treated with nocodazole by using *in vivo* ^{32}Pi labeling and immunoprecipitation technique. As shown in Fig. 6A, the radioactive band of 17-kDa due to phosphorylation of Survivin was observed in WT- and D53A-overexpressing cells, whereas no radioactive band was detected in T34A-overexpressing cells. To clarify the precise mechanisms by which Survivin blocked apoptosis downstream of mitochondria, we examined the binding capabilities of Survivin-WT, -T34A and -D53A with Smac/DIABLO by coimmunoprecipitation in A549 cells. After transduction with pAd-LacZ, pAd-WT, pAd-T34A or pAd-D53A, the cells were treated with 100 nM

Taxol to effectively induce the release of active mature Smac/DIABLO from mitochondria to cytosol [30]. As shown in Fig. 6B, transduction of pAd-WT, pAd-T34A or pAd-D53A resulted in intense expression of Survivin in these cells (top panel) and the amount of mature Smac/DIABLO was approximately equal regardless of which Survivin adenoviral vector was transfected (middle panel). Next, the lysates were coimmunoprecipitated using the anti-Smac/DIABLO antibody, and the final precipitation products were detected by western blotting using the anti-Survivin antibody. As shown in the bottom panel of Fig.6B, the anti-Smac/DIABLO antibody precipitated Survivin-WT but not Survivin-T34A and -D53A, indicating that Survivin-T34A and -D53A were not able to bind to Smac/DIABLO. These results suggested that T34 and D53 of Survivin were important for its binding capability with Smac/DIABLO. Furthermore, the radiation-induced active fragments (p19 and p17) of caspase-3 were increased in A549 cells transduced with Ad-T34A and Ad-D53A in comparison with Ad-WT (Fig. 6C).

Discussion

It has been reported that the Survivin mutant T34A, which is unable to be phosphorylated by Cdc2 at T34, competes with endogenous Survivin for access to kinases and thereby prevents the phosphorylation of wild-type Survivin [23]. When Survivin-T34A and -D53A were overexpressed in NIH3T3, A549 and HeLa cells, radiation-induced apoptosis was significantly enhanced (Figs. 3 and 5). In comparison with the effects of various vectors on the enhancement of radiation-induced apoptosis in the cells, the enhancement of apoptosis induced by the overexpression of these mutants in HeLa cells (p53 null) was similar to that of A549 cells (p53 wild type), indicating that

the enhancement by Survivin mutants occurred independently of p53 status (Fig. 5). The enhancement by Survivin-T34A as well as -D53A was suppressed by caspase-9 and caspase-3 inhibitors but not a caspase-8 inhibitor (Figs. 3C and D). It is well-known that caspase-9 is regulated by Apaf-1 and that cytochrome *c* released from mitochondria and caspase-8 is activated through formation of the death-inducing signaling complex (DISC) after the activation of death receptors (Fas and DR5, etc.). Though ionizing radiation was reported to activate not only the mitochondrial pathway but also the death receptor pathway without ligands in human leukemia MOLT-4 cells [3,36], the present results suggested that endogenous Survivin suppressed the caspase-3-dependent cell death via the mitochondrial pathway but not the death receptor pathway in NIH3T3, A549 and HeLa cells. In fact, the apoptotic signaling pathway was shown to be activated followed by mitochondrial downstream signaling activation such as cytochrome *c* release and expression of Smac/DIABLO after irradiation (Fig. 1D and E). However, how Survivin-T34A accelerates caspase-3 dependent apoptosis has been unknown, because of the lack of experimental evidence that the recombinant Survivin-T34A directly associated with caspase-3 and/or Smac/DIABLO through the BIR domain.

As shown in Fig. 6A and B, we demonstrated that Survivin-T34A could not also bind to Smac/DIABLO similarly to Survivin-D53A and the clear phosphorylation of Survivin-WT and -D53A but not -T34A was detected, indicating that phosphorylation at T34 was associated with binding activity to Smac/DIABLO. Since functional Survivin has been demonstrated to be a homodimer of the wild type [14,30], if dimers containing Survivin-D53A fail to interact with Smac/DIABLO, the overexpression of this dominant-negative mutant may enhance the activation of caspases induced by

apoptotic stimuli. Since phosphorylation at T34 is essential for the anti-apoptotic function of Survivin [23], phosphorylated Survivin at T34 may be necessary to bind with Smac/DIABLO in order to suppress apoptosis. Our results showed when the binding activity of Survivin with Smac/DIABLO was abrogated by Survivin mutants T34A and D53A, the formation of a Smac/DIABLO-IAPs complex was induced and then the processing of caspase-3 was facilitated by loss of the antiapoptotic properties of IAPs (Fig. 6C). This is the first report to demonstrate the relationship between a phosphorylation-defective mutant of Survivin, T34A, and Smac/DIABLO.

To explore the role of the phosphorylation at T34 in the antiapoptotic activity of Survivin, we prepared phosphomimic mutants, *i.e.*, T34E and T34D. A previous report showed that the activation of NADPH oxidase was regulated by phosphorylation at S303/S304 of $p47^{phox}$, an NADPH oxidase subunit, and this enzyme activation occurred in p47-deficient B-cells transfected with S303E/S304E but not S303D/S304D $p47^{phox}$ mutants [37]. This report suggested that the introduction of a negative charge to a protein was not always involved in the protein function after phosphorylation and that the structural change due to phosphorylation was important in the case of NADPH oxidase. Contrast to this previous data, our results showed that not only T34E but also T34D did not enhance apoptosis (Fig. 3), though the overexpression of T34A was shown to enhance radiation-induced apoptosis in NIH3T3 cells. These results suggest that the negative charge after Cdc2-dependent phosphorylation, but not the structural change in the amino acid residue, is necessary for the antiapoptotic functions of Survivin.

In summary, we demonstrated that constitutive Survivin in tumor cells was involved in antiapoptotic properties against cytochrome *c*-dependent and

Smac/DIABLO-related apoptotic signaling promoted by ionizing radiation. These results suggest that gene therapy for constitutive Survivin may be effective for therapeutic improvement in radioresistant solid tumors.

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Legends to Figures

Fig. 1. Radiation-induced G2/M checkpoint, cytochrome *c* release from mitochondria and overexpression of Smac/DIABLO but not apoptotic cell death in A549 cells. (A) A549 cells without treatment and treated with X irradiation (10 Gy) were incubated for 12, 24, 36 and 48 h, and cell cycle analysis by flow cytometry was performed. (B) Fluorescence microscopic observation after the cells were incubated for 24 h after irradiation and then stained with PI. (C) After incubation of X-irradiated cells for 12, 24, 36 and 48 h, the cleaved caspase-3 (p17 activation form) was examined by western blotting. As positive control for activation of caspase-3, the cell lysate from human leukemia cell line MOLT-4 cells exposed to 10 Gy of X rays were also analyzed as shown in right lane of (C). Cytochrome *c* release form mitochondria to cytosol (D) and expression of Smac/DIABLO (E) in the cells incubated for 24 h after irradiation were examined by western blotting.

Fig. 2. Expression of suvivin and its mutants in NIH3T3 cells transfected with GFP-fused vectors. (A) Microscopic observation 24 h after transfection with GFP-fused Survivin constructs. Typical photographs obtained by phase-contrast (Phase in left panel) and fluorescence microscopy (GFP in right panel). (B) Left panel: flow cytometric profile of NIH3T3 cells with and without transfection with pcDNA3.1/GFP. Right panel: percentages of GFP-positive cells vs. total cells in NIN3T3 cells transfected with various GFP-fused vectors carrying Survivin mutants, *i.e.*, pcDNA3.1/GFP, pcDNA3.1/WT-GFP, pcDNA3.1/T34A-GFP, pcDNA3.1/D53A-GFP, pcDNA3.1/ T34E-GFP and pcDNA3.1/T34D-GFP. (C) Western blots of Survivin (upper panel) and actin as a loading control (lower panel) in NIN3T3 cells transfected with

various GFP-fused vectors carrying Survivin mutants.

Fig. 3. Effects of overexpression of Survivin mutants on radiation-induced apoptosis in normal cells. (A) Typical photographs of nuclei stained with propidium iodide (PI). NIH3T3 cells transfected with pcDNA3.1/WT-GFP and pcDNA3.1/D53A-GFP were incubated for 48 h after treatment with X irradiation (20 Gy) and then observed under a fluorescence microscope after PI staining. Arrows: typical apoptotic cells with chromatin condensation or DNA fragmentation. (B) Radiation-induced apoptosis induction (% of total cells) in NIH3T3 cells transfected with Survivin mutants. NIH3T3 cells transfected with Survivin mutants were incubated for 48 h after treatment with 20 Gy of X irradiation. After PI staining, at least 200 cells were scored by fluorescence microscopy. Data are expressed as means ± SE for three experiments. *$p < 0.05$ vs. WT + X rays by Student's *t*-test. (C) For detection of activation of caspase-3, the transfected cells were incubated after 24 h from treatment with X irradiation and analyzed by western blotting using an anti-cleaved caspase-3 antibody as described in Fig. 1C. （D） Effects of various caspase inhibitors on the enhancement of radiation-induced apoptosis by overexpression of Survivin mutants. Various caspase inhibitors, Z-VAD-fmk, Ac-DEVD-CHO, Ac-LEHD-CHO and Ac-IETD-CHO, were added to the medium of the transfected NIH3T3 cells after treatments with irradiation and then incubated for 48 h. Fluorescence microscopic observation was performed using a protocol similar to that described as above. Data are expressed as means ± SE for three experiments. *$p < 0.05$, ** $p<0.01$ vs. X rays by Student's *t*-test.

Fig. 4. Expression of Survivin and its mutants in tumor cells transduced with adenoviral

vectors. (A) Western blots of Survivin (upper panel) and actin as a control (lower panel) in A549 cells (left panel) and HeLa cells (right panel) transduced with pAd-LacZ, pAd-WT, pAd-T34A and pAd-D53A. Expression was evaluated 24 h after transduction. (B) Expression of Survivin. At 24 h after transduction with various adenoviral vectors, A549 cells (upper panel) and HeLa cells (lower panel) were fixed with paraformaldehyde and analyzed for Survivin expression by immunocytochemistry.

Fig. 5. Effects of overexpression of Survivin mutants on radiation-induced apoptosis in tumor cell lines. (A) At 24 h after transduction with pAd-LacZ, pAd-WT, pAd-T34A or pAd-D53A, A549 cells were irradiated with 10 Gy of X rays and then further incubated for 24 h. Then the cells were stained with an FITC conjugate of annexin V for 10 min in the dark. Annexin V-positive cells indicating apoptosis were examined by flow cytometry. (B) Typical photographs of nuclei stained with propidium iodide (PI). A549 cells transfected with pAd-WT, pAd-T34A and pAd-D53A were incubated for 48 h after treatment with 10 Gy of X rays and then observed under a fluorescence microscope after PI staining. Upper panel: unirradiated control, lower panel: 10 Gy irradiation. (C) Radiation-induced apoptosis induction (% of total cells) in A549 cells (upper panel) and HeLa cells (lower panel) transduced with adenoviral vectors carrying Survivin mutants. The tumor cells transduced with Survivin mutants were incubated for 48 h after treatment with 10 Gy of X rays and then stained with PI. At least 200 cells were scored by fluorescence microscopy. Data are expressed as means ± SE for three experiments. *$p < 0.05$ vs. Ad-WT group by Student's *t*-test.

Fig. 6. Phosphorylation status and the binding capability of Survivin mutants with

Smac/DIABLO. (A) A549 cells overexpressed with pAd-WT, pAd-T34A and pAd-D53A were labeled with 0.2 mCi/ml ^{32}Pi and were treated with 100 ng/ml nocodazole. After incubation for 6 h, the cell lysates were immunoprecipitated by anti-Survivin antibody. Phosphorylated Survivin was detected by autoradiography with the imaging analyzer. (B) Lysates were prepared from A549 cells transduced with pAd-LacZ, pAd-WT, pAd-T34A and pAd-D53A, and harvested for 24 h with 100 nM Taxol to ensure the release of mature Smac/DIABLO from mitochondria to cytosol. The expression of Survivin (top) and Smac/DIABLO (middle) was detected by western blotting. Endogenous mature Smac/DIABLO was immunoprecipitated (IP) from the lysates by an anti-Smac/DIABLO antibody and examined for interactions with LacZ, Survivin-WT, -T34A and –D53A by western blotting using an anti-Survivin antibody (bottom). (C) A549 cells transduced with pAd-WT, pAd-T34A and pAd-D53A were harvested and the activation of caspase-3 was analyzed by western blotting using an anti-cleaved caspase-3 antibody.

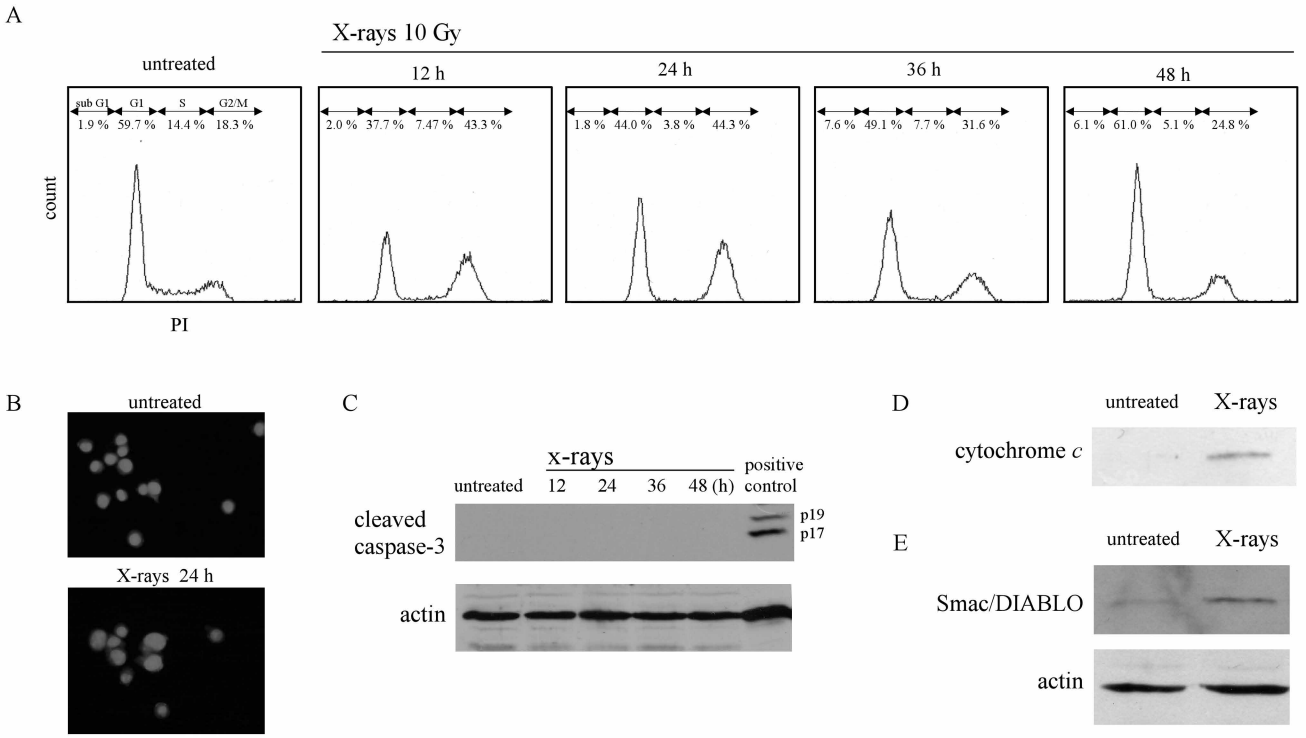


Fig. 1 Ogura *et al.*

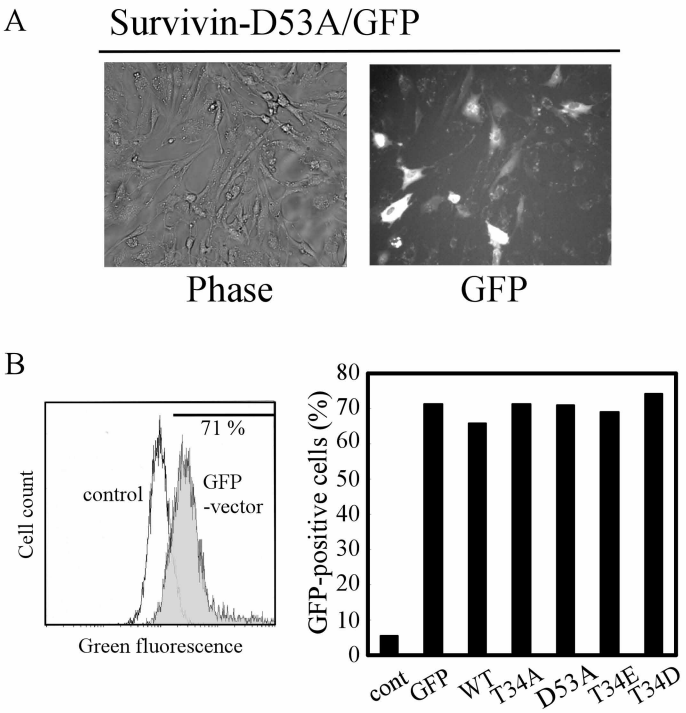


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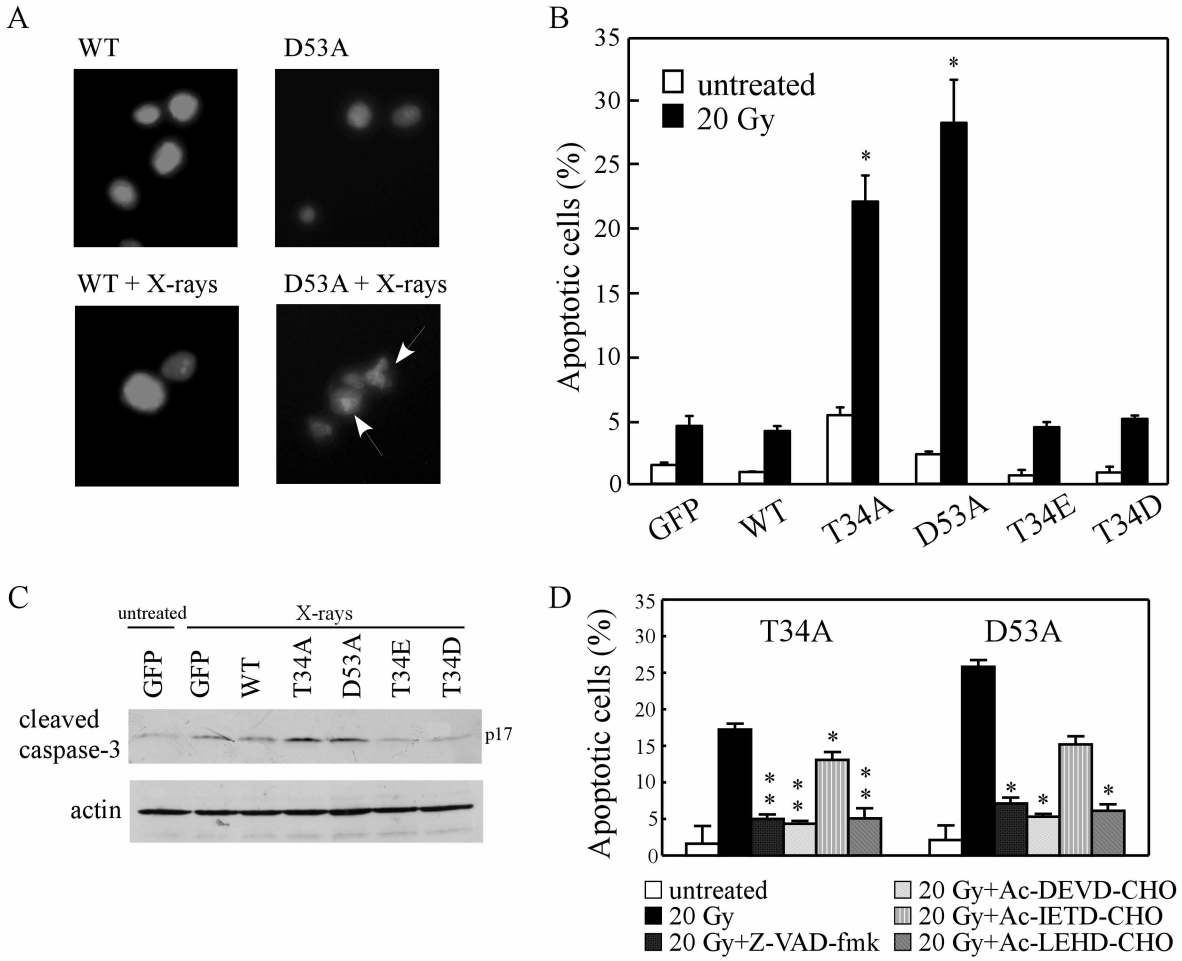


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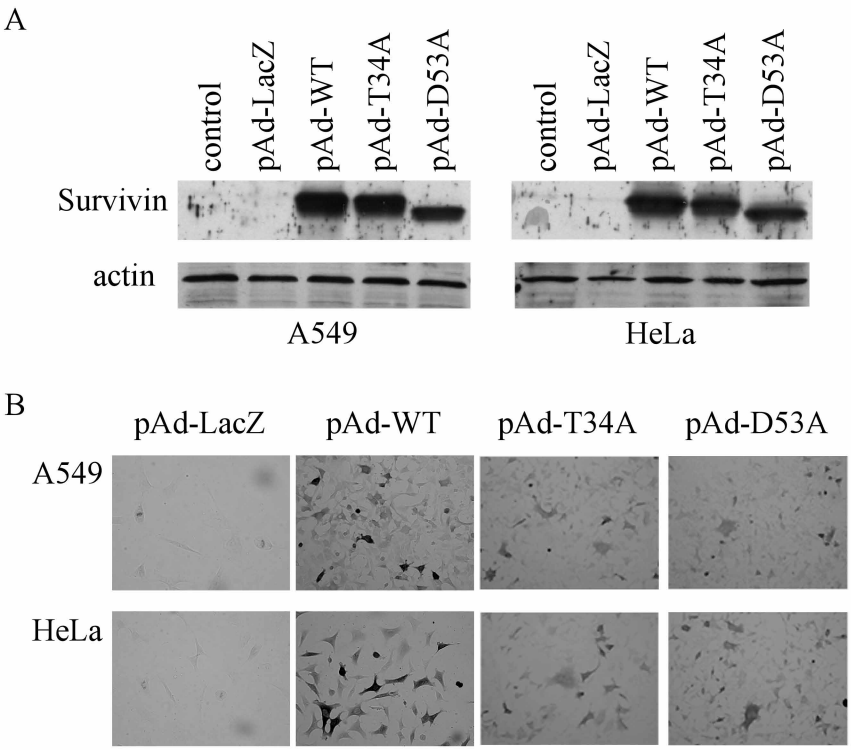


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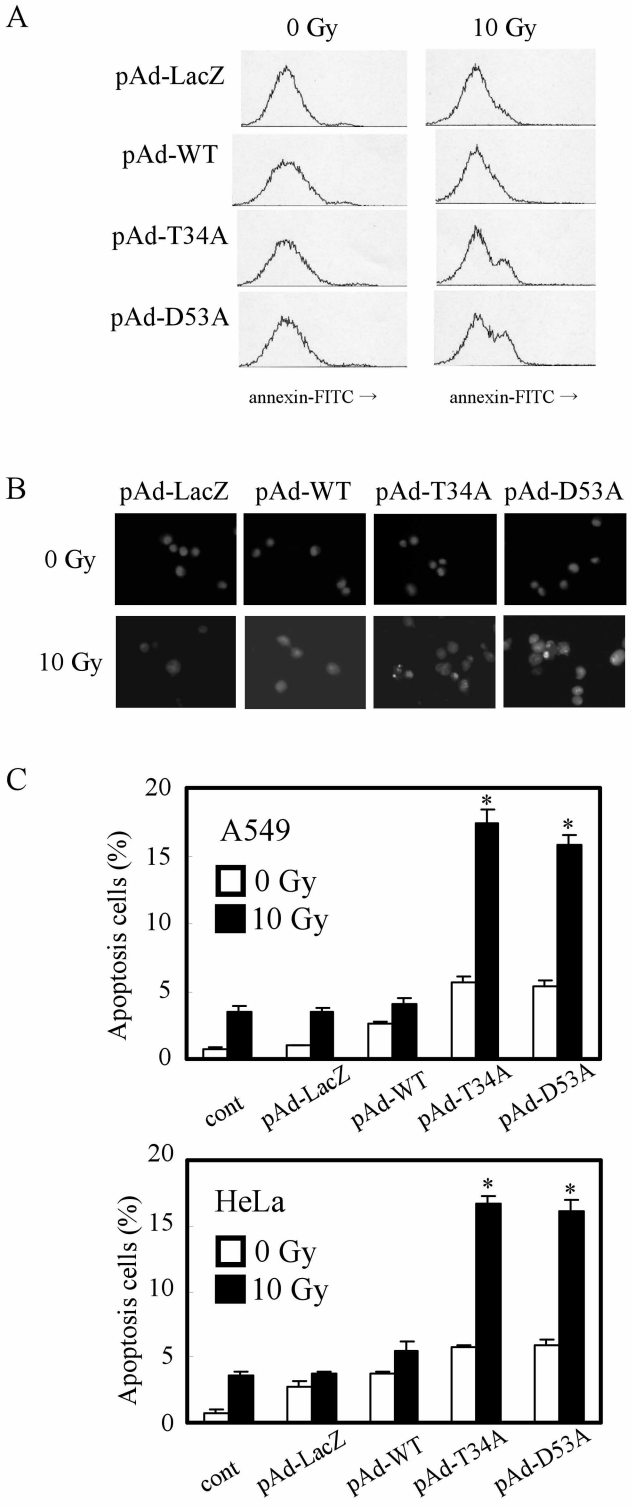


Fig. 5 Ogura *et al.*

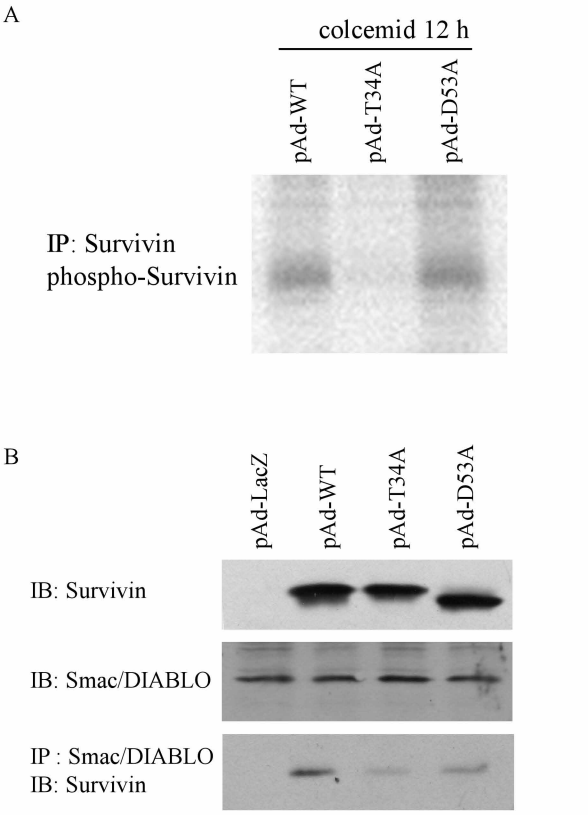


Fig. 6 Ogura *et al.*