Sensitization for γ-Irradiation–Induced Apoptosis by Second Mitochondria-Derived Activator of Caspase

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Abstract

Resistance to current treatment regimens, such as radiation therapy, remains a major concern in oncology and may be caused by defects in apoptosis programs. Because inhibitor of apoptosis proteins (IAPs), which are expressed at high levels in many tumors, block apoptosis at the core of the apoptotic machinery by inhibiting caspases, therapeutic modulation of IAPs could target a key control point in resistance. Here, we report for the first time that full-length or mature second mitochondria-derived activator of caspase (Smac), an inhibitor of IAPs, significantly enhanced γ-irradiation–induced apoptosis and reduced clonogenic survival in neuroblastoma, glioblastoma, or pancreatic carcinoma cells. Notably, Smac had no effect on DNA damage/DNA repair, activation of nuclear factor-κB, up-regulation of p53 and p21 proteins, or cell cycle arrest following γ-irradiation, indicating that Smac did not alter the initial damage and/or cellular stress response. Smac enhanced activation of caspase-2, caspase-3, caspase-8, and caspase-9, loss of mitochondrial membrane potential, and cytochrome c release on γ-irradiation. Inhibition of caspases also blocked γ-irradiation–induced mitochondrial perturbations, indicating that Smac facilitated caspase activation, which in turn triggered a mitochondrial amplification loop. Interestingly, mitochondrial perturbations were completely blocked by the broad-range caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone or the relatively selective caspase-2 inhibitor N-benzyloxycarbonyl-Val-Asp-Ala-Asp-fluoromethylketone, whereas caspase-8 or caspase-3 inhibitors only inhibited the increased drop of mitochondrial membrane potential provided by Smac, suggesting that caspase-2 was acting upstream of mitochondria after γ-irradiation. In conclusion, our findings provide evidence that targeting IAPs (e.g., by Smac agonists) is a promising strategy to enhance radiosensitivity in human cancers. (Cancer Res 2005; 65(22): 10502-13)

Introduction

The aggressive cancer cell phenotype is the result of a variety of genetic and epigenetic alterations leading to deregulation of intracellular signaling pathways (1). For example, cancer cells may become refractory to undergo apoptosis, the cell's intrinsic suicide program critical for tissue homeostasis (1). Despite intensive therapies, resistance of many tumors to current treatment protocols, including radiotherapy, due to apoptosis defects still constitutes a major problem in cancer therapy (2). Thus, current attempts to improve cancer survival will have to include strategies that specifically target tumor cell resistance to apoptosis.

Cell death by apoptosis plays a pivotal role in the regulation of various physiologic or pathologic conditions and has also been implied to mediate therapy-induced cytotoxicity (e.g., following chemotherapy, γ-irradiation, or immunotherapy; refs. 2, 3). Apoptosis pathways may be initiated through different entry sites, such as death receptors or mitochondria resulting in activation of effector caspases (3). Stimulation of death receptors of the tumor necrosis factor (TNF) receptor superfamily, such as CD95 (APO-1/Fas) or TNF-related apoptosis-inducing ligand (TRAIL) receptors, results in caspase-8 activation, which initiates direct cleavage of downstream effector caspases (4). The mitochondrial pathway is engaged by the release of apoptogenic factors, such as cytochrome c, apoptosis-inducing factor, second mitochondria-derived activator of caspase (Smac)/DIABLO, or endonuclease G, from mitochondria into the cytosol, triggering caspase-3 activation as a result of formation of the cytochrome c/Apaf-1/caspase-9–containing apoptosome complex (5). Signals originating from death receptors may be linked to mitochondria via Bcl-2 family proteins, such as BID, which triggers cytochrome c release upon cleavage by caspase-8, thereby initiating a mitochondrial amplification loop (5).

Radiation-induced apoptotic signaling can be initiated in different cellular compartments, including the nucleus, cytosolic elements, or plasma membrane (6, 7). In response to nuclear DNA damage, the tumor suppressor protein p53 plays a pivotal role in causing cell cycle arrest and in triggering DNA repair, although p53-independent pathways also exist (8). In addition, reactive oxygen species are generated on radiation, which can engage the mitochondrial pathway of apoptosis (7). The damage response to ionizing radiation also involves activation of the stress-activated protein kinase or c-Jun NH2-terminal kinase signaling pathway (7). Moreover, ionizing radiation has been shown to act on the plasma membrane where free radical species may inflict lipid oxidative damage, leading to generation of bioactive molecules, such as ceramide (7). These signaling cascades eventually fuel into a common effector phase of apoptosis characterized by activation of caspases as death effector molecules (6). However, caspase-independent apoptosis and non-apoptotic modes of cell death on irradiation have also been described (6, 7).

Defects in cell death programs, including apoptosis, may contribute to tumor progression and treatment resistance (e.g., radioresistance; ref. 2). Apoptosis signaling may be disrupted by deregulated expression and/or function of antiapoptotic or proapoptotic molecules (2). The majority of human cancers harbor high levels of inhibitor of apoptosis proteins (IAPs), such as X-linked IAP (XIAP), cellular inhibitors of IAP (cIAP) 1 and 2, survivin, or livin (9).
Aberrant expression of IAPs in tumor cells has been associated with treatment resistance and dismal prognosis (9). Because IAPs block apoptosis at the core of the apoptotic machinery by inhibiting caspases, therapeutic targeting of IAPs may overcome resistance (e.g., resistance to radiotherapy).

We recently developed Smac agonists that promoted caspase activation by antagonizing IAPs (10). Smac agonists sensitized various tumor cells in vitro for apoptosis induced by death receptor ligation or cytotoxic drugs and also enhanced the antitumor activity of TRAIL in an intracranial malignant glioma xenograft model in vivo (10). In search for novel strategies to overcome radioresistance of tumors, we investigated the role of Smac in apoptosis regulation in response to γ-irradiation in the present study. We found that Smac cooperated with γ-irradiation to enhance apoptosis and to reduce clonogenic survival in a variety of human cancers.

Materials and Methods

Cell culture, transfection, and transduction. SH-EP neuroblastoma and ASPC1 pancreatic carcinoma were maintained in RPMI 1640 (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% heat-inactivated FCS (Biochrom, Berlin, Germany), 10 mmol/L HEPES (Biochrom), and 2 mmol/L l-glutamine (Biochrom). Glioblastoma cell lines (U87MG and LN229), PaTuII pancreatic carcinoma cells, and NIH3T3 mouse fibroblast cells were maintained in DMEM (Life Technologies) supplemented with 10% heat-inactivated FCS, 10 mmol/L HEPES, and 2 mmol/L l-glutamine. C2C12 mouse myoblast cells were maintained in DMEM supplemented with 10% heat-inactivated FCS and 2 mmol/L l-glutamine. Cells (0.5 × 10⁵/mL) were cultured in 96-, 24-, or 6-well plates, 10-cm plates, or 75-cm² flasks (Falcon, Heidelberg, Germany). Cells were cultured in 96-, 24-, or 6-well plates, 10-cm plates, or 75-cm² flasks (Falcon, Heidelberg, Germany). Transduction was done using pLXIN retroviral transduction system (Sigma, St. Louis, MO) and PT67 packaging cell line (BD Biosciences Clontech, Heidelberg, Germany) according to the manufacturer’s instructions. Briefly, Smac was cloned from pBluescript KS+.
(Stratagene, Heidelberg, Germany) in pLXIN vector (pLSmacIN) using restriction enzymes XhoI and BamHI (New England Biolabs, Beverly, MA). PT67 cells were transfected with pLXIN empty vector or pLSmacIN using LipofectAMINE 2000 (Invitrogen, Karlsruhe, Germany). After selecting transfected PT67 cells with 500 μg/mL genetin (Invitrogen) for 10 days, virus-containing supernatant was filtered and transferred to LN229 glioblastoma, U87MG glioblastoma, ASPC1 pancreatic carcinoma, or PaTuII pancreatic carcinoma cells. Stable bulk cultures were generated by selection with geneticin and ectopically expressed Smac protein was controlled by Western blotting. Stable transfection of SH-EP neuroblastoma cells was done with pcDNA3 vector (Invitrogen) containing full-length Smac or pcDNA3.1 containing SmacD55 as described previously (10, 11). Clones expressing Smac or SmacΔ55 were selected by limited dilutions. NIH3T3 mouse fibroblasts or C2C12 mouse myoblasts were transiently transfected with empty pcDNA3 or with pcDNA3 containing full-length Smac using LipofectAMINE 2000. Ectopic expression of Smac was controlled by Western blot 48 hours after transfection. All chemicals were obtained from Sigma (Steinheim, Germany) unless otherwise indicated.

Determination of apoptosis, clonogenic growth, and caspase activity. Cells were treated with γ-irradiation (γ-cell 2000 Nuclear Data, CS-137, 44 Tbq, 4 Gy/min; Frankfurt, Germany) at the indicated doses and times. Apoptosis was assessed by fluorescence-activated cell sorting (FACS; Becton Dickinson, Heidelberg, Germany) analysis of DNA fragmentation of propidium iodide–stained nuclei or by Annexin V staining (Roche Diagnostics, Inc., Mannheim, Germany) as described previously (12). Clonogenic growth was assessed by crystal violet staining as described previously (13). Caspase activity was determined by fluorogenic substrates for caspase-3 (DEVD-D2R; Roche Diagnostics), caspase-8 (IETD-AFC; BioCat, Inc., Palo Alto, CA), caspase-9 (LEHD-AFC; Bachem, Heidelberg, Germany), or caspase-2 (VDADV-AFC; Bachem) according to the manufacturer’s instructions. Caspase activity was measured fluorometrically at 510 nm on a microplate fluorescence reader (1420 Victor Multilabel Counter; Wallac, Rodgau-Jugesheim, Germany). For inhibition of caspase activation, the broad-range caspase inhibitor N-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (zVAD.fmk; Bachem), the relatively specific caspase-3 inhibitor N-benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone (zDEVD.fmk; Bachem), the relatively specific caspase-8 inhibitor N-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethylketone (zIETD.fmk; Bachem), and the relatively specific caspase-2 inhibitor N-benzyloxycarbonyl-Val-Asp-Val-Ala-Asp-fluoromethylketone (zVDVAD.fmk; Sigma) were used.

Assessment of mitochondrial transmembrane potential, cytochrome c release, and Bax activation. 3,3′-Dihexyloxycarbocyanide iodide (DiOC6(3); 460 ng/mL, Molecular Probes, Karlsruhe, Germany) was used to measure the transmembrane potential. Cells were incubated for 15 minutes at 37°C in the presence of the fluorochrome, washed in PBS/1% FCS, and immediately analyzed by flow cytometry. DiOC6(3) was...
Figure 2. Continued. C, SH-EP neuroblastoma cells transfected with vector control (Neo), or mature Smac (Smac), were treated with 0, 3, 7, or 10 Gy γ-irradiation and protein expression of p53, p21, and β-actin was assessed by Western blot analysis 48 and 96 hours after irradiation. D, the ratio of G2/G1 phase is shown after 4, 8, 16, and 24 hours of γ-irradiation for SH-EP neuroblastoma cells transfected with vector control ( ), full-length Smac ( ), or mature Smac ( ). DNA content was determined by FACS analysis of propidium iodide–stained nuclei as described in Materials and Methods.

recorded in fluorescence 1. Cytochrome c release was determined in permeabilized cells using mouse anti–cytochrome c monoclonal antibody (BD Biosciences Clontech) as described previously (14). Bax activation was determined in permeabilized cells using a conformation-specific mouse anti-Bax monoclonal antibody (BD Biosciences Clontech) as described previously (15). Briefly, SH-EP neuroblastoma cells were fixed in 2% formaldehyde and incubated in the presence of 1 μg/mL mouse anti-Bax monoclonal antibody diluted in permeabilization buffer (0.5% bovine serum albumin, 0.1% saponin in PBS) for 45 minutes on ice followed by incubation with goat anti-mouse IgG-FITC (1:100; Chemicon International, Temecula, CA) for 30 minutes on ice in the dark. Cells were washed in permeabilization buffer, resuspended in PBS, and analyzed by flow cytometry for Bax conformational change.

Cell cycle analysis. Analysis of cell cycle was done by flow cytometric staining of fixed cells with propidium iodide for DNA content. Briefly, cells were resuspended in GM solution (1.1 g/L glucose, 8 g/L NaCl, 0.39 g/L Na2HPO4·12H2O, 0.4 g/L KCl, 0.15 g/L KH2PO4, 0.5 mmol/L EDTA), fixed with 3 parts of 95% nondenatured ethanol, and stained with solution S [1× PBS, 15 μg/mL propidium iodide, 50 μg/mL RNase A] overnight at 4°C. Cell cycle distribution was recorded in fluorescence 3 by flow cytometry.

Western blot analysis. Western blot analysis was done as described previously (12) using mouse anti-caspase-2 monoclonal antibody (1:5,000; BD Transduction Laboratories, Heidelberg, Germany), mouse anti-caspase-8 monoclonal antibody (1:1,000; Alexis Biochemicals, Grünberg, Germany), rabbit anti-caspase-3 polyclonal antibody (1:1,000; Cell Signaling, Beverly, MA), rabbit anti-caspase-9 polyclonal antibody (1:1,000; BD PharMingen, San Diego, CA), mouse anti-XLAP monoclonal antibody (1:1,000; clone 2B, BD Biosciences Clontech), rabbit anti-clAP2 polyclonal antibody (1:1,000; Santa Cruz Biotechnology, Heidelberg, Germany), mouse anti-p53 monoclonal antibody (1:1,000; BD Biosciences Clontech), rabbit anti-survivin polyclonal antibody (1:1,000; R&D Systems, Inc., Wiesbaden, Germany), rabbit anti-BID polyclonal antibody (1:1,000; R&D Systems), rabbit anti-p21 polyclonal antibody (1:1,000; Santa Cruz Biotechnology), mouse anti-β-actin monoclonal antibody (1:500; BD Biosciences Clontech), rabbit anti-Bax polyclonal antibody (1:1,000; Upstate Biotechnology, Lake Placid, NY), mouse anti-Flag tag monoclonal antibody (M3, 1:5,000; Sigma), or mouse anti-β-actin monoclonal antibody (1:5,000; Sigma) followed by goat anti-mouse IgG-horseradish peroxidase (HRP) or goat anti-rabbit IgG-HRP (1:5,000; Santa Cruz Biotechnology). Enhanced chemiluminescence (Amer sham Pharmacia, Freiburg, Germany) or SuperSignal West Dura Extended Duration Substrate (Pierce, Rockford, IL) was used for detection. Expression of β-actin was used to control for equal gel loading.

Intracellular staining. To analyze the phosphorylation status of histone γ-H2AX and recruitment of RAD51 to sites of DNA repair following γ-irradiation, cells were fixed with 3.7% paraformaldehyde, permeabilized with 0.1% Triton X-100, and stained with mouse anti-γ-H2AX monoclonal antibody (1:100; Upstate Biotechnology) and rabbit anti-RAD51 polyclonal antibody (1:50; Santa Cruz Biotechnology) overnight followed by incubation with goat anti-mouse IgG-FITC (1:100) and goat anti-rabbit IgG Texas red (1:100; Vector Laboratories, Burlingame, CA) for an additional hour at 4°C. Slides were mounted using Vectashield mounting medium for immunofluorescence (Vector Laboratories). Slides were analyzed using an electron scanning confocal microscope (Leica, Bensheim, Germany) and the software supplied by the manufacturer.

Nuclear protein extraction and electromobility shift assay. Adherent and floating cells (3 × 10^6/sample) were collected by scraping and centrifugation (1,000 × g for 5 minutes at 4°C). After washing once in ice-cold PBS, cell pellets were resuspended in 200 μL low-salt buffer [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl2, 10 mmol/L KCl and incubated for 10 minutes on ice. After addition of 20 μL of 10% NP40 solution, samples were mixed vigorously for 30 seconds. Nuclei were collected by centrifugation and resuspended in 50 μL high-salt buffer [20 mmol/L HEPES (pH 7.9), 420 mmol/L NaCl, 1.5 mmol/L MgCl2, 0.2 mmol/L EDTA, 25% glycerol]. Both buffers were supplemented with a protease inhibitor cocktail (Sigma), 0.2 mmol/L phenylmethylsulfonyl fluoride, 0.5 mmol/L DTT, and 1 mmol/L sodium orthovanadate before use. Nuclei were
incubated 15 minutes on ice and occasionally mixed. Nuclear extract were obtained after centrifugation at 12,500 × g for 10 minutes at 4°C and stored at −80°C. Protein determination was done with BCA Protein Assay kit (Pierce). For electromobility shift assay (EMSA), the following oligonucleotides were used: 5′-AGTGGAGGGACTTTCCCAGGC (sense) and 5′-GCTGGAAGCTCCCTCAGACT (antisense). Single-stranded oligonucleotides were labeled with T4-polynucleotide kinase (MBI Fermentas, St. Leon-Rot, Germany) and [γ-32P]ATP (Amersham Pharmacia). A 2-fold molar excess of unlabeled complementary oligonucleotides was annealed and double-stranded oligonucleotides were purified on spin columns (Micro Bio-Spin P30, Bio-Rad, Munich, Germany). Binding reactions were done for 30 minutes on ice in 20 μL buffer [1 mmol/L MgCl2, 0.5 mmol/L EDTA, 0.5 mmol/L DTT, 50 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.5), 4% glycerol] containing 5 μg nuclear extract protein, 1 μg poly(deoxyinosinic-deoxycytidylic acid) (Sigma), and 10,000 cpm labeled oligonucleotide. Binding complexes were resolved by electrophoresis in vertical nondenaturing 6% polyacrylamide gels using 0.3× Tris-borate EDTA as running buffer. Gels were dried and radioactive signals were detected by autoradiography films (Amersham Biosciences, Freiburg, Germany).

Results

Sensitization for γ-irradiation–induced apoptosis by second mitochondria-derived activator of caspase. Although Smac agonists have been reported to sensitize for apoptosis induced by death receptor ligation, chemotherapy, or killing by the immune system (10, 16–20), whether Smac may have an effect on radiation response has not yet been addressed. In search for new

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Figure 3. Effect of Smac on apoptosis regulatory molecules after γ-irradiation. SH-EP neuroblastoma cells transfected with vector control (Neo), full-length Smac (Smac), or mature Smac (SmacΔ55) were treated with 0, 3, 7, or 10 Gy γ-irradiation. Protein expression of Bax, BID, Bcl-2, XIAP, cIAP2, survivin, and β-actin was assessed by Western blot analysis 48 hours (A) and 96 hours (B) after γ-irradiation. C, Bax conformational change is shown for SH-EP neuroblastoma cells transfected with vector control (solid light gray line), full-length Smac (solid dark gray line), or mature Smac (solid black line) 24 hours after treatment with 0 or 10 Gy γ-irradiation. Patched lines represent respective isotype controls. Similar results were obtained in three independent experiments.
approaches to overcome radioresistance of cancers, we therefore investigated the antitumor effects of Smac on human tumor cell lines following γ-irradiation. As a model of radioresistance, we used the neuroblastoma cell line SH-EP shown previously to be radioresistant (21). SH-EP neuroblastoma cells were engineered to overexpress full-length, mitochondrial Smac or mature Smac, which predominantly localizes to the cytosol as determined by fluorescence microscopy (data not shown). Interestingly, we found that overexpression of full-length or mature Smac significantly enhanced γ-irradiation–induced apoptosis in a dose- and time-dependent manner compared with vector control cells (Fig. 1A-D).

Figure 4. Effect of Smac on mitochondrial membrane potential disruption and cytochrome c release upon γ-irradiation. SH-EP neuroblastoma cells transfected with vector control (white columns), full-length Smac (hatched columns), or mature Smac (black columns) were treated with 0, 3, 7, or 10 Gy γ-irradiation. A-D, mitochondrial membrane potential (MMP) was determined by DiOC₆(3) staining and flow cytometry 24, 48, 96, or 144 hours after γ-irradiation. Columns, mean of five independent experiments done in triplicates; bars, SEM. E-H, cytochrome c release was determined by flow cytometry at 24, 48, 96, and 144 hours after γ-irradiation. Columns, mean of three independent experiments done in triplicates; bars, SEM. For statistical analysis, t test was done comparing SH-EP-Neo with either SH-EP-Smac or SH-EP-SmacΔ55 (*, P < 0.05; #, P < 0.001).

Sensitization for γ-Irradiation by Smac
Ectopic expression of Smac had an effect on long-term survival following γ-irradiation (Fig. 1F). In parallel, we investigated the effect of γ-irradiation on Jurkat T-cell leukemia cells as positive control, as they have been described to be radiosensitive (22). γ-Irradiation in Jurkat cells resulted in higher levels of apoptosis compared with SH-EP neuroblastoma cells (data not shown).

Second mitochondria-derived activator of caspase has no influence on DNA damage/repair, nuclear factor-κB activation, or cell cycle arrest after γ-irradiation. To systematically gain insight into the molecular mechanisms of sensitization for γ-irradiation by Smac, we first asked whether Smac has an effect on the initial DNA damage response following γ-irradiation. To address this question, we assessed DNA damage and repair by immunofluorescent staining of phosphorylated histone γ-H2AX foci and RAD51. Phosphorylation of histone γ-H2AX at Ser139 occurs in response to DNA double-strand breaks (e.g., caused by ionizing radiation) and is proposed to concentrate repair factors, such as RAD51 at sites of DNA damage (23, 24). We found foci of phosphorylated histone γ-H2AX colocalized with RAD51 after γ-irradiation with no detectable differences between SH-EP neuroblastoma cells transfected with vector control, full-length, or mature Smac (Fig. 2A; Supplementary Fig. S2). Additionally, formation of phosphorylated γ-H2AX foci at sites of DNA damage was monitored up to 36 hours, with no apparent differences between SH-EP-Neo, SH-EP-Smac, and SH-EP-SmacΔ55 (Supplementary Fig. S3).

Next, we asked whether Smac may alter activation of nuclear factor-κB (NF-κB), a transcription factor, which is activated in response to various types of cellular stress, including γ-irradiation (25). Analysis of NF-κB DNA-binding activity by EMSA showed similar activation of NF-κB upon γ-irradiation in SH-EP neuroblastoma cells overexpressing full-length or mature Smac compared with vector control cells (Fig. 2B). This set of experiments indicates that overexpression of Smac did not alter DNA damage/repair or NF-κB activation following γ-irradiation in SH-EP neuroblastoma cells.

Because the cellular stress response to DNA damage often involves cell cycle arrest, we then went on to assess the effect of Smac on cell cycle regulatory proteins and cell cycle profiles on γ-irradiation. Notably, p53 and p21 proteins were similarly up-regulated after γ-irradiation in SH-EP neuroblastoma cells over-expressing Smac or vector control (Fig. 2C). In addition to full-length p53 protein, an immunoreactive band of a slightly lower molecular weight was detected in Smac-transfected cells, which may correspond to a cleavage fragment of p53 protein (Fig. 2C). Interaction with damaged DNA has been reported previously to induce limited cleavage of p53 protein (26). We also analyzed cell cycle profiles by flow cytometry after γ-irradiation of SH-EP neuroblastoma cells. γ-Irradiation resulted in a time- and dose-dependent arrest of cells in G2/M phase, with no detectable differences between SH-EP neuroblastoma cells overexpressing full-length or mature Smac compared with vector control cells (Fig. 2D; Supplementary Fig. S4). Our findings that SH-EP neuroblastoma cells arrest at G2/M upon γ-irradiation, although these cells harbor wild-type p53, are in line with previous studies showing impairment of the G1 checkpoint after DNA damage in wild-type p53 neuroblastoma cells, which may be due to defective wild-type p53 function because of its cytoplasmic sequestration in neuroblastoma (27, 28).

Modulation of apoptosis regulatory molecules by second mitochondria-derived activator of caspase. To gain further clues on the enhancement of apoptosis after γ-irradiation provided by Smac, we investigated key proapoptotic and antiapoptotic molecules by Western blot. Expression levels of Bax, BID, Bcl-2, XIAP, or survivin were similarly altered 48 or 96 hours after γ-irradiation in SH-EP neuroblastoma cells transfected with Smac or vector control (Fig. 3A and B). Survivin expression was down-regulated on γ-irradiation in all cell lines (Fig. 3A and B) despite G2/M arrest under these conditions (Fig. 2D). Because survivin is negatively regulated by p53, which is similarly up-regulated in all cell lines (Fig. 3A and B), these findings indicate that survivin expression may be repressed by p53. There was an increased cleavage of XIAP.

Figure 5. Effect of Smac on γ-irradiation–induced caspase activation and mitochondrial membrane disruption. A, SH-EP neuroblastoma cells transfected with vector control (white columns), full-length Smac (hatched columns), or mature Smac (black columns) were treated with 0, 3, or 10 Gy γ-irradiation. Apoptosis in the presence of 50 μmol/L zVAD.fmk was determined 144 hours after γ-irradiation by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei. Columns, mean of three independent experiments done in triplicates; bars, SEM. B, protein expression of caspase-2, caspase-8, caspase-9, caspase-3, and β-actin was assessed by Western blot analysis 48 and 96 hours after treatment with 0, 3, 7, or 10 Gy γ-irradiation in SH-EP neuroblastoma cells transfected with vector control (Neo), full-length Smac (Smac), or mature Smac (SmacΔ55).

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Figure 5. Continued. C-F, activity of caspase-2, caspase-3, caspase-8, and caspase-9 of SH-EP neuroblastoma cells transfected with vector control (white columns), full-length Smac (hatched columns), or mature Smac (black columns) was determined by enzymatic assay using fluorogenic caspase substrates 24, 48, and 96 hours after γ-irradiation as described in Materials and Methods. X-fold increase in caspase activity compared with untreated SH-EP neuroblastoma cells transfected with vector control is shown. Columns, mean of six independent experiments done in triplicates; bars, SEM. For statistical analysis, t test was done comparing SH-EP-Neo with either SH-EP-Smac or SH-EP-SmacD55 (*, P < 0.05; #, P < 0.001). G, SH-EP neuroblastoma cells transfected with vector control (white columns), full-length Smac (hatched columns), or mature Smac (black columns) were left untreated (-) or treated with 10 Gy γ-irradiation (+) in the absence or presence of 50 μmol/L zVAD.fmk, 25 μmol/L zVDVAD.fmk, 50 μmol/L zIETD.fmk, or 50 μmol/L zDEVD.fmk. Mitochondrial membrane potential was determined 96 hours after γ-irradiation by DiOC6(3) staining and flow cytometry. Columns, mean of three independent experiments done in triplicates; bars, SEM.
protein 96 hours after γ-irradiation especially in SH-EP neuroblas-
toma cells transfected with mature Smac (Fig. 3B). Additionally,
reduced expression of cIAP2 was observed in Smac-transfected SH-
EP neuroblastoma cells 96 hours after γ-irradiation (Fig. 3B). In
addition, we examined Bax activation after γ-irradiation by flow
cytometry using a conformation-specific antibody. γ-Irradiation
induced a conformational change of Bax with no detectable
differences between SH-EP neuroblastoma cells transfected with
vector control, full-length, or mature Smac (Fig. 3C).

Second mitochondria-derived activator of caspase and
γ-irradiation cooperate to trigger the mitochondrial pathway.
Next, we examined the effect of Smac and γ-irradiation on the
mitochondrial pathway. Overexpression of full-length or mature
Smac resulted in significantly enhanced breakdown of the
mitochondrial membrane potential compared with vector control
cells in a dose- and time-dependent manner (Fig. A4-D). In
addition, we determined the release of cytochrome c from the
mitochondria by flow cytometry by staining for cytochrome c in
permeabilized cells. Overexpression of full-length or mature Smac
and γ-irradiation significantly increased cytochrome c release from
mitochondria compared with vector control SH-EP neuroblastoma
cells (Fig. 4E-H). Because loss of mitochondrial membrane
potential preceded cytochrome c release on γ-irradiation, perme-
ability transition pore activation may be involved in the release of
cytochrome c under these conditions (29). Alternatively, mito-
chondrial cytochrome c release on γ-irradiation may occur by
mechanism(s) not involving permeability transition (29). Together,
this set of experiments shows that Smac cooperated with
γ-irradiation to trigger the mitochondrial pathway.

Second mitochondria-derived activator of caspase and
γ-irradiation cooperate to activate caspases. To investigate
whether apoptosis was the result of caspase activation, we used the
broad-range caspase inhibitor zVAD.fmk. Apoptosis in response to
γ-irradiation was almost completely blocked in the presence of
zVAD.fmk in SH-EP neuroblastoma cells irrespective of Smac
expression (Fig. 5A), showing that apoptosis required activation of
caspases. We then analyzed activation of the caspase cascade by
monitoring cleavage of individual caspases by Western blot analysis
and by enzymatic caspase assay using fluorogenic caspase
substrates. Notably, Western blot analysis revealed that Smac
overexpression, particularly mature Smac, enhanced processing of
caspase-2, caspase-8, caspase-9, and caspase-3 (Fig. 5B). Addition-
ally, significantly increased activity of caspase-2, caspase-3, caspase-8,
or caspase-9 following γ-irradiation was detected in SH-EP
neuroblastoma cells overexpressing full-length or mature Smac
compared with vector control cells (Fig. 5C-F). Detection of caspase-
8 activity by enzymatic assay before detection of caspase-8 cleavage
fragments by Western blot may indicate that the unprocessed
proenzyme-8 can be induced to be enzymatically active, in line
with previous findings (30, 31). Alternatively, the differential
detection of caspase-8 activation by caspase assay may be due to
the higher sensitivity of the enzymatic assay compared with
Western blot analysis. Together, our findings show that Smac
promoted activation of the caspase cascade on γ-irradiation in SH-
EP neuroblastoma cells.

Second mitochondria-derived activator of caspase enhances
mitochondrial perturbations in a caspase-dependent manner.
To further dissect the sequence of events leading to caspase
activation and mitochondrial perturbations in γ-irradiation–
duced apoptosis in Smac-transfected cells, we used relatively
selective chemical inhibitors of individual caspases and examined
mitochondrial membrane perturbations after γ-irradiation. Addi-
tion of the pan-caspase inhibitor zVAD.fmk completely blocked loss
of mitochondrial membrane potential in SH-EP neuroblastoma
cells transfected with Smac or vector control (Fig. 5G). Interestingly,
the relatively specific chemical inhibitor for caspase-2 zVDVAD.fmk
also completely inhibited disruption of mitochondrial membrane
potential in these cells (Fig. 5G), indicating that caspase-2 was
acting as an initiator caspase upstream of the mitochondria on
γ-irradiation in SH-EP neuroblastoma cells. In contrast, the
relatively selective caspase-8 inhibitor zLETD.fmk or the relatively
selective caspase-3 inhibitor zDEVD.fmk only reduced the increased
drop in mitochondrial membrane potential provided by Smac on
γ-irradiation (Fig. 5G). These findings indicate that Smac
potentiates activation of caspase-3 and caspase-8, which can serve
as a feedback loop for further disruption of the mitochondrial
membrane potential.

Effect of second mitochondria-derived activator of caspase
on γ-irradiation–induced apoptosis in malignant and non-
malignant cells. We then extended our studies to additional
human cancer types to exclude that the sensitization effect of Smac for
γ-irradiation was restricted to a particular cell type. U87MG
glioblastoma, LN229 glioblastoma, ASPC1 pancreatic carcinoma,
and PaTuII pancreatic carcinoma cells were engineered to over-
express full-length Smac or empty vector control (data not shown).
Importantly, overexpression of Smac significantly enhanced
γ-irradiation-induced apoptosis of U87MG glioblastoma, LN229
glioblastoma, ASPC1 pancreatic carcinoma, or PaTuII pancreatic
carcinoma cells in a dose-dependent manner (Fig. 6A-D). In addition,
overexpression of Smac cooperated with γ-irradiation to reduce
clonogenic growth of U87MG glioblastoma and PaTuII pancreatic
carcinoma cells (Supplementary Fig. S5).

Moreover, we also extended our studies to nonmalignant cells
to investigate whether Smac has an effect on normal cells upon
γ-irradiation. We found that ectopic expression of full-length Smac
did not significantly alter γ-irradiation–induced apoptosis of mouse
NIH3T3 fibroblasts or mouse C2C12 myoblasts compared with
vector control cells (Fig. 6E-F). Of note, mouse DIABLO and human
Smac are identical in amino acid sequence in the NH2-terminal
region responsible for binding to BIR3 of XIAP (32). Thus, Smac
enhanced apoptosis and reduced clonogenic survival following
γ-irradiation in different cancer cell lines but not in nonmalignant
cells, indicating some tumor specificity.

Discussion
Due to intrinsic resistance of many tumors to established
therapies, including radiotherapy, current attempts to improve the
survival of cancer patients largely depend on strategies to target
tumor cell resistance (1, 2). Because induction of apoptosis in
target cells is a key cytotoxic mechanism of most antitumor
therapies, including γ-irradiation, defects in apoptosis programs
may cause resistance (2). In recent years, several approaches have
been pursued for specific modulation of defined cellular death
pathways to increase the therapeutic efficacy of radiation therapy
and/or to reduce radiation-mediated side effects (6). For example,
the combination of both radiation and agents, which directly
induce cell death, such as the death ligand TRAIL, or strategies
to alter the threshold for death induction by inhibition of
antia apoptotic signals generated by mitogenic stimuli (e.g., phos-
phatidylinositol 3-kinase/Akt or mitogen-activated protein kinase
survival pathways) have been tested to increase radiation response
IAPs are expressed at high levels in many tumors and have been reported to contribute to the resistance of cancers (e.g., resistance to radiotherapy; ref. 9). Because inhibition of caspases by IAPs occurs at the core of the apoptotic machinery, therapeutic modulation of IAPs could target a key control point in deciding cell fate. Smac is a mitochondrial protein that is released into the cytosol in response to apoptotic stimuli and promotes caspase activation by antagonizing IAPs (5). Recently, we found that Smac agonists sensitized even resistant tumor cells for apoptosis induced by death receptor ligation or anticancer drugs (10). Here, we provide for the first time evidence that Smac significantly enhanced γ-irradiation-induced cytotoxicity in a variety of human cancers. Overexpression of full-length or mature Smac markedly increased apoptosis and reduced clonogenic survival on γ-irradiation in neuroblastoma, glioblastoma, or pancreatic carcinoma cells. Analysis of signaling pathways mediating this cooperative effect of Smac and γ-irradiation revealed that overexpression of full-length or mature Smac enhanced caspase activation, loss of mitochondrial membrane potential, and cytochrome c release from mitochondria (Fig. 7). Because Smac increased activation of caspase-3 and caspase-2 before promoting caspase-8 and caspase-9 activation, these findings indicate that Smac antagonized XIAP-mediated inhibition of caspase-3, which has been related to the ability of the Smac dimer to bind to the BIR2 domain (34). Smac-triggered increase in caspase activity initiated an amplification loop acting back on the mitochondria to induce further mitochondrial perturbations. Accordingly, the increased drop of mitochondrial membrane potential provided by Smac was blocked by inhibition of caspase-3 or caspase-8. Smac may initiate the mitochondrial amplification loop by promoting activation of effector caspases, such as caspase-3, which in turn can cleave caspase-8, BID, and caspase-2 to trigger additional mitochondrial damage (35). Whether Smac may enhance caspase-2 activation independently of caspase-3 is unknown at present. Interestingly, loss of mitochondrial

Figure 6. Effect of Smac on γ-irradiation–induced apoptosis in malignant and nonmalignant cells. U87MG glioblastoma (A), LN229 glioblastoma (B), ASPC1 pancreatic carcinoma (C), and PaTuII pancreatic carcinoma (D) cells transduced with control virus (white columns) or full-length Smac virus (black columns) were treated with 0, 10, or 20 Gy γ-irradiation. Apoptosis was determined at 96 hours (C and D) or 144 hours (A and B) after γ-irradiation by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei. Columns, mean of three independent experiments done in triplicates; bars, SEM. For statistical analysis, t test was done comparing vector control with Smac-transduced cells (*, P < 0.05; #, P < 0.001). E and F, NIH3T3 mouse fibroblasts (E) or C2C12 mouse myoblasts (F) were transiently transfected with vector control (white columns) or full-length Smac (black columns) and treated with 0, 10, or 20 Gy γ-irradiation. Apoptosis was determined 96 hours after γ-irradiation by FACS analysis of DNA fragmentation of propidium iodide–stained nuclei. Expression of Flag-tagged Smac (Smac-FT) in NIH3T3 mouse fibroblast cells and C2C12 mouse myoblast cells transiently transfected with vector control (N) or full-length Smac (S) was determined by Western blot analysis 48 hours after transfection (insets in E and F). Columns, mean of three independent experiments done in triplicates; bars, SEM.
membrane potential on γ-irradiation was completely blocked in the presence of the caspase-2 inhibitor zVDVAD.fmk and also by the broad-range caspase inhibitor zVAD.fmk but not by caspase-3 or caspase-8 inhibitors, indicating that caspase-2 was acting upstream of mitochondria upon ɣ-irradiation. Recent evidence suggests that caspase-2 is required for mitochondrial permeabilization in response to cellular stress, such as DNA-damaging drugs or UV irradiation, at least in certain cell types (36–38). Our findings provide evidence that caspase-2 activity is also involved to engage the mitochondrial pathway on γ-irradiation, at least in certain cell types (36–38). Our findings provide evidence that caspase-2 activity is also involved to engage the mitochondrial pathway on γ-irradiation, in addition to DNA-damaging drugs or UV irradiation described previously (36, 37). However, because commercially available chemical inhibitors of caspases lack absolute specificity (39), the role of caspase-2 in controlling mitochondrial functions following γ-irradiation remains to be confirmed in future studies. Because we found that caspases are largely dispensable for irradiation-induced loss of mitochondrial membrane potential in Jurkat T-cell leukemia cells (data not shown), in line with previous reports (40, 41), our findings that caspases are required to engage the mitochondrial pathway on γ-irradiation in SH-EP neuroblastoma cells point to cell type–specific differences in the regulation of mitochondrial integrity. Ectopic expression of either full-length or mature form of Smac enhanced γ-irradiation-induced apoptosis, although mature Smac was more effective compared with full-length Smac.

Interestingly, Smac did not alter DNA damage/repair, activation of the transcription factor NF-κB, or cell cycle arrest caused by γ-irradiation. Thus, Smac enhanced radiation response by altering the threshold for death execution rather than affecting the damage to DNA or the initial cellular stress response elicited by γ-irradiation. This highlights the notion that susceptibility of human cancer to cytotoxic therapies, such as γ-irradiation, critically depends on intact apoptosis pathways. Thus, targeting defects in apoptosis programs may significantly alter treatment response. In addition to apoptosis, nonapoptotic forms of cell death may also be involved in γ-irradiation–induced cytotoxicity. Irradiation-induced cell death is considered to involve damage to distinct cellular structures, including damage to DNA, cellular membranes, or mitochondria, which all eventually lead to activation of caspases as death effector molecules (6, 7). IAPs block the apoptosis machinery at a central point by antagonizing activation and activity of effector caspases (9). There is mounting evidence that IAPs determine sensitivity to radiotherapy in human cancers (42–44). High levels of XIAP or survivin have been associated with resistance to radiotherapy in lung or colorectal carcinoma (42–45). Recently, inhibition of survivin or XIAP using antisense oligonucleotides was shown to enhance the efficacy of radiotherapy by reducing survival and increasing apoptosis of lung cancer cells (42, 43). Compared with these strategies to knockdown individual members of the IAP proteins, targeting IAPs by Smac agonists may prove to be especially effective to promote cell death in response to radiotherapy, because Smac simultaneously antagonize several members of IAP proteins, including XIAP, cIAP1, cIAP2, survivin, or livin (5). Importantly, our findings show for the first time that Smac significantly potentiated γ-irradiation-induced apoptosis in different human cancers, known to be notoriously resistant to radiation, such as neuroblastoma, glioblastoma, or pancreatic carcinoma. Notably, Smac enhanced apoptosis upon γ-radiation in cancer cells but not in nonmalignant cells, indicating some tumor specificity. The underlying mechanism(s) for the differential sensitivity of malignant versus nonmalignant cells toward the sensitization effect by Smac is presently unknown and is subject to further investigation. Previously, Smac agonists have been reported to potentiate the antitumor activity of death receptor ligands, anticancer drugs, or killing by the immune system (10, 16–20). Besides Smac agonists, peptidic or nonpeptidic small-molecule antagonists of IAPs were developed to target IAPs (46–49). Which of the currently available strategies to knockdown IAPs is best to increase the efficacy of radiotherapy in cancer remains to be tested in future studies.

Clinically, resistance to apoptosis is a major cause of primary or acquired nonresponsiveness of cancers leading to treatment failure. By showing that Smac sensitized a variety of tumors for γ-irradiation–induced apoptosis, our findings have important implications for the development of novel strategies in radiotherapy.

Figure 7. Illustration of apoptosis pathways modulated by Smac after γ-irradiation. See text for details.
Thus, targeting IAPs (e.g., by Smac agonists or small-molecule IAP antagonists) is a promising novel approach to enhance radiosensitivity of human cancers.

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References

Sensitization for γ-Irradiation–Induced Apoptosis by Second Mitochondria-Derived Activator of Caspase

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