Predominant Suppression of Apoptosome by Inhibitor of Apoptosis Protein in Non-Small Cell Lung Cancer H460 Cells: Therapeutic Effect of a Novel Polyarginine-conjugated Smac Peptide

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ABSTRACT

The inhibitor of apoptosis proteins (IAPs) plays a central role in repressing caspase-mediated cell death. However, little is known about the actual role of endogenously expressed IAPs in cancer cells. We found that the cytochrome c/apoptotic protease-activating factor-1 (apoptosome)-dependent caspase activation is deficient in human non-small cell lung cancer (NSCLC) NCI-H460 cells. This dysfunctional apoptosome activity was not correlated with any decrease of apoptosis component factors, but it was linked to an increased X-linked inhibitor of apoptosis protein (XIAP). In H460 cells, the overexpressed XIAP, but not c-IAP1, bound to the processed form of caspase-9 and suppressed the activation of downstream effector caspases. Moreover, the defect in apoptosome activity in H460 cells was dramatically restored by the IAP-targeting SmacN7 peptide, which disrupted XIAP-caspase-9 binding, indicating an essential role of the IAP in the apoptosome inhibition. However, the SmacN7 did not show any striking effect on the apoptosome activity of normal lung fibroblast cells, although these cells also expressed modest amounts of IAP. To explore the therapeutic approach, we additionally developed SmacN7(R)8, a newly designed cell permeable peptide. The SmacN7(R)8 selectively reversed the apoptosis resistance of H460 cells, and when in combination with chemotheraphy, regressed the tumor growth in vivo with little toxicity to the mice. Our results indicate that IAP-dependent suppression of apoptosome predominantly occurs in IAP-overexpressing tumor, and the IAP-targeting Smac peptide is an effective molecule to increase tumor cell death induced by chemotherapy in vitro and in vivo.

INTRODUCTION

Apoptosis is a genetically regulated cell death mechanism that plays an essential role in various biological processes (1). Abnormalities in apoptosis that lead to early cell death or the absence of normal cell death have been linked not only to a variety of human diseases, including neurodegenerative disorders and cancer (2), but also to cancer cell resistance to chemotherapy (3). Currently, there are two well-characterized apoptotic pathways, one initiated through the engagement of cell surface death receptors by their specific ligands (4), and the other triggered by changes in internal cellular integrity, mitochondrial pathway (5). Both pathways eventually converge, resulting in activation of caspases, cysteine proteases that comprise the effector arm of the apoptotic process (6). Chemotherapeutic agents, UV irradiation, and other agents induce caspase activation mainly via the mitochondrial pathway, which involves mitochondrial integration of apoptotic signals and subsequent release of cytochrome c into the cytosol. The liberated cytochrome c, along with adenosine nucleotides, initiates formation of an apoptosome consisting of Apaf-1 oligomers. This apoptosome recruits and activates caspase-9, which in turn activates the executioner caspases, caspase-3 and caspase-7 (7, 8). Thus, the activated caspases kill the cells by proteolysis of key cellular substrates. IAPs are BIR-domain-containing protein members that suppress apoptosis induction (9). The antiapoptotic activity of several IAPs has been attributed especially to their ability to inhibit caspases (10–12). The XIAP is a prototype IAP family member that directly binds and inhibits several effector caspases (13, 14). IAPs are themselves regulated by proteins that block their antiapoptotic activity (15). In Drosophila, Reaper (RPR), Hid, Grim, and, more recently, Sickle are reported to be proapoptotic proteins (16–21). They activate caspase-dependent cell death pathways by suppressing the ability of IAPs to inhibit caspases. It has been shown that they can physically interact with the fly members of IAP family, including D-IAP1 and D-IAP2, and antagonize the binding of the IAP proteins to caspases. Smac/DIABLO performs a similar function to these proteins in mammals (22, 23). The only sequence homology among insect Reaper, Hid, Grim, Sickle, and human Smac is in the four NH2-terminal residues of the active proteins (19–21, 24–28). This short peptide fits into a hydrophobic pocket on the surface of the BIR domain of IAPs and is essential for binding IAPs and blocking their caspase-inhibitory activity (29–31).

Lung cancer is the leading cause of cancer-related death worldwide, and NSCLC is the most common histological cell type and often presents in an advanced stage. Although cisplatin and taxol are established antitumor agents for the treatment of advanced NSCLC, chemoresistance is still a major cause of treatment failure, and the molecular mechanisms involved are poorly understood. Chemotherapy resistance has been associated with decreased susceptibility to apoptosis, introducing the possibility that cell death determinants may influence the outcome of treatment. Recent studies have demonstrated that dysfunctional apoptosome activity is related to chemoresistance in human ovarian cancer (32, 33), but the mechanisms have not been fully clarified. Here, we show that the apoptosome deficiency was observed in chemoresistant NSCLC NCI-H460 cells. No deficient apoptosome components, such as Apaf-1, cytochrome c, or caspase-9, were detected. However, we found that they overexpressed XIAP. The newly synthesized peptide that contains seven amino acids at the NH2 terminus of active, processed Smac conjugated with a cell membrane permeable polyarginine (SmacN7(R)8) strongly reversed the apoptosis resistance of H460 cells. Moreover, the tumor-specific therapeutic effect of the peptide was addressed.
MATERIALS AND METHODS

Design and Synthesis of Peptides. On the basis of the fact that argininerich peptides have efficient translocation activities through cell membranes (34), SmaeN7 (H-AVPIAQK-OH), SmaeN7 conjugated with six arginine residues (H-AVPIAQK-GGGRGRRGGC-OH) or eight arginine residues (H-AVPIAQK-GGGRGRRGGRC-OH) and R8 (NH2-GGGRGRRR-RRGC-COOH) peptide, as well as their respective fluorescein-tagged counterparts, were chemically synthesized. The purity of the peptides as determined by reversed phase-high performance liquid chromatography analysis was >95%. Cisplatin and taxol were purchased from Nippon Kayaku Co., Ltd. (Tokyo, Japan) and Sigma (St. Louis, MO), respectively. Acetyl-Asp-Glu-Val-Asp-(4-methylcoumaryl-7-amide) and ZVAD-fmk were from Peptide Institute, Inc. (Osaka, Japan).

Purification of Recombinant Protein. XL1blue bacteria, which harbored a plasmid pPROEX HTa encoding His-tagged wild-type XIAP, was induced to express recombinant His6-XIAP by the addition of 1 mM isopropyl-1-thio-β-β-n-galactopyranoside to culture media. His-tagged XIAP protein was purified as described elsewhere (35).

Cell Lines and Cell Culture. Human normal lung fibroblast TIG3 (36) and human NSCLC, NCI-H460, H23, H226 cell lines were grown in RPMI 1640, and 293T cells in DMEM (Nissui Co., Ltd., Tokyo, Japan), with 10% heat-inactivated fetal bovine serum and 100 μg/ml kanamycin in a humidified atmosphere of 5% CO2 and 95% air. The three tumor cell lines express normal retinoblastoma proteins (37). As for p53 status, H23 and H226 are p53 mutant, whereas H460 is p53 wild-type (38). As for K-ras status, H23 and H460 are K-ras mutant, whereas H226 is K-ras wild-type (39).

Estimation of Peptide Internalization. To test the internalization efficiencies of the synthesized peptides, 10 μM FITC-SmaeN7, FITC-R8, FITC-SmaeN7(R)6, and FITC-SmaeN7(R)8 were added into the medium and continued to incubate for 3 h. Cells were washed three times with PBS. The distribution of fluorescein-labeled peptides was analyzed by an Olympus CK 40 fluorescence microscope.

Cell-free Assays and Measurement of Caspase Activity. Cell lysates were prepared by homogenizing cells in buffer A (20 mM HEPESS-KOH (pH 7.5), 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA containing 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM DTT) and then centrifuged at 12,000 × g for 20 min. The supernatant was used as the cytosolic fraction. Each lysate sample (100 μg of protein) was initiated with 10 μM cytochrome c plus 1 mM of dATP. After incubated at 30°C for various time periods (see figure legends), DEVDase activity was measured as described previously (40). In some experiments, cell extracts were mixed with recombinant, purified His-XIAP, His-XIAP plus SmaeN7 or SmaeN7(R)8 before cytochrome c and dATP were added. For drug-treated cells, lysates were prepared in standard buffer [10 mM Tris-HCl (pH 8.0), 5 mM DTT, and 1 mM phenylmethylsulfonyl fluoride] by three rounds of freezing and thawing, and then centrifuged at 12,000 × g for 20 min. The same supernatant was also subjected to Western blot analysis to detect active caspase fragments.

Measurements of Cell Growth Inhibition and Flow Cytometry Analysis. Cells were plated in 96-well microplates for measurement of cell growth inhibition and in 10-cm dishes for flow cytometry analysis 24 h before treatment. The sensitivity of tumor cells to drugs was evaluated by cell growth inhibition with various agents as indicated in the figure legends and the treatment. The sensitivity of tumor cell lines to drugs was evaluated by cell growth inhibition with various agents as indicated in the figure legends and the treatment. The sensitivity of tumor cell lines to drugs was evaluated by cell growth inhibition with various agents as indicated in the figure legends and the treatment.

Immunoprecipitation Experiment. Cytosolic extracts were prepared in buffer A as described above. Both control and cytochrome c/dATP-activated cell lysates were precleared with protein G-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden) at 4°C for 2 h, incubated with a monoclonal antibody to XIAP (MBL, Nagoya, Japan) at 1:200 dilution at 4°C overnight, and then followed by protein G-Sepharose pulldown at 4°C for 2 h. XIAP-depleted supernatants were then subjected to Western blot analysis.

Western Blot Analysis. Anti-XIAP (1:500) was from MBL, anti-cIAP-1 and anti-cIAP-2 (1:1000) from Genzyme/Technne (Minneapolis, MN), anti-cleaved caspase-9 (D330 and D315) (1:500), and anti-cleaved caspase-3 (D175) (1:500) from Cell Signaling Technology, Inc. (Beverly, MA), anti-Apaf-1(N) (1:1000) from QED Bioscience, Inc. (San Diego, CA), anti-caspase-9 (1:500) and anti-caspase-3 (1:500) from Transduction Laboratories (Lexington, KY), monoclonal antibody to cytochrome c (1:500) from Pharamingen (San Diego, CA), monoclonal anti-a-tubulin (1:1000) from Sigma. In all Western blots, anti-a-tubulin immunoblotting was done as loading controls. Cell lysates were subjected to standard SDS-PAGE and then transferred to a nitrocellulose membrane. After blocking at room temperature for 1 h in blocking buffer (PBS(-)5% skim milk — 0.1% Tween 20), the membranes were incubated with antibodies diluted (as described above) in blocking buffer at room temperature for 2 h, followed by three washes in washing buffer (PBS-) followed by incubation with antimouse or antirabbit immunoglobulin peroxidase conjugated (1:500; Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) at room temperature for 1 h. After three washes, the bands were visualized by ECL Western Blotting Detection Reagents (Amersham, Tokyo, Japan).

Antitumor Activity of Chemotherapeutic Agent with SmaeN7(R)8 against Xenografts. H460 cells (3 × 105 cells/mouse) were implanted s.c. in the right flank region of nude mice, BALB/CaJcl-nu (Clea Japa, Inc., Tokyo, Japan). Therapeutic experiments (six mice/group) were started (day 0) when the tumor reached 50–100 mm3. Cisplatin and taxol were administrated i.v. at doses of 7 mg/kg and 60 mg/kg, respectively, and SmaeN7 and SmaeN7(R)8 were administrated by intratumoral injection in 50 μl of 10 mM solution on day 0. Control animals received the same volume of a saline. The length (L) and width (W) of the tumor mass were measured, and the tumor volume (TV) was calculated as: TV = (L × W2)/2. Statistical evaluations were performed using the Student’s t test. Tumor tissue lysates were prepared by homogenizing in buffer A as described above. The supernatant of each sample was subjected to anticleaved caspase-3 Western blot analysis.

RESULTS

Chemoresistant H460 Has a Diminished Apoptotic Activity. NSCLC lines, NCI-H460, H23 and H226, were incubated with cisplatin or taxol for 16 and 24 h, stained with propidium iodide, and analyzed by a flow cytometer. As shown in Fig. 1A, when H23 or H226 cells were treated with cisplatin, a significant portion of the cells underwent apoptosis (43 and 19% at 24 h, respectively). In contrast, H460 cells, under the same conditions, resisted apoptosis (6% at 24 h). Taxol-treated H460 cells also showed resistance to apoptosis compared with H23 and H226, although not so great as in the case of cisplatin. To examine the cytochrome c/dATP-dependent caspase activation in lung cancer, we prepared cytosolic extracts from these cells and from normal lung fibroblast TIG3 cells and examined cell-free apoptotic activity. The H460 cell line displayed a diminished level of caspase activation, as compared with H23, H226 and normal cell line TIG3 (Fig. 1B). We next examined apoptosome proteins to determine whether their decreased expressions could account for the defective caspase activation observed in H460 cell line. Western blot analysis showed that H460, like other cell lines, expressed intact apoptosome components, such as cytochrome c, Apaf-1, caspase-9, and caspase-3 (Fig. 1C). Reverse transcription-PCR analysis additionally revealed that H460 expressed active Apaf-1L isoform comparable with other cell lines (data not shown).

Predominant Role of IAPs in the Low Apoptotic Activity of H460. We next examined expression of IAPs to determine their involvement in the defective caspase activation observed in H460 cells. As shown in Fig. 2A, H460 displayed a high XIAP level. No differences were detected in expressions of cIAP-1, and the expression of cIAP-2, which was detected in some human glioma cells (data not shown), was not significantly observed in these cell lines. In addition, the processing of caspase-9 was induced after the addition of cytochrome c/dATP in all three cell lines, and the processed caspase-9 disappeared after XIAP-depletion in H460 but not in H23 and H226 cells (Fig. 2B), indicating that XIAP, but not cIAP-1, selectively bound to the processed form of caspase-9 in H460 cells. The NH2-terminal residues of mature Smac/DIABLO is essential for binding IAP and blocking their caspase-inhibitory activity (23). To determine
SELECTIVE TARGETING OF IAP IN H460 CELLS

Fig. 1. Chemoresistant H460 cells have diminished cytochrome c-dependent caspase activation. A, H460, H23, and H226 cells were treated with cisplatin (10 μg/ml) or taxol (250 ng/ml) for the indicated periods, and propidium iodide–labeled cells were analyzed by flow cytometry. B, cytosolic extracts prepared from H460, H23, H226, and TIG3 cell lines were treated with buffer or cytochrome c (10 μM) plus dATP (1 mM) for 60 min at 30°C and then assayed for activity against the fluorogenic caspase substrate DEVD-MCA (20 μM). Results represent the mean ± SD for at least three independent experiment. C, whole cell lysates (30 μg of protein) were analyzed by Western blotting with antibodies against cytochrome c, Apat-1, caspase-9, caspase-3, and α-tubulin for loading control.

the role of the overexpressed IAP in the apoptosis defect of H460 cells, we additionally examined the effect of chemically synthesized NH₂-terminal seven amino residues of mature Smac/DIABLO. SmacN7, on the apoptosis activity of H460 cells and saw the low apoptosis activity dramatically reversed (Fig. 2C). Surprisingly, however, the peptide had little effect on the functional apoptosis activity-containing cells, including H23, H226, and normal TIG3. SmacN7 disrupted binding of XIAP to processed caspase-9 and then initiated caspase-3 processing in H460 cells but had little effect on H23 and H226 cells (Fig. 2D). These results indicate that overexpressed XIAP plays a predominant role in the inactivation of apoptosis in H460 cells.

Arginine-rich Peptide-conjugated NH₂ Terminus of Smac Acts as a Cell-permeable IAP Inhibitor. To conduct the NH₂-terminal Smac peptide into cells, we designed peptides to be conjugated with several arginine residues (Fig. 3A) and estimated their accumulation in cells. We saw that without the arginine residues, SmacN7 did not accumulate in cells, but both SmacN7(R)6 and SmacN7(R)8 showed sufficient amount of accumulation in cells (Fig. 3B). SmacN7(R)8 showed higher internalization efficiency than SmacN7(R)6. We additionally tested whether SmacN7(R)8 could functionally relieve XIAP inhibition of caspases in 293T cytosolic extracts. We found that it was able to recover the XIAP-suppressed caspase activity in a dose-dependent manner to nearly the same extent as SmacN7 (Fig. 3C).

SmacN7(R)8 Reverses Apoptosis Resistance of H460 Cells and Potentiates Chemotherapy-induced Apoptosis. To investigate whether SmacN7(R)8 in the cytosol would make H460 cells sensitive to chemotherapeutic agent-induced cell death, cells were exposed to cisplatin or taxol alone or combined with SmacN7(R)8 and cell survivals were determined. As shown in Fig. 4A, cisplatin or taxol alone led to insufficient cell death. It should be noted that H460 cells were shown to be resistant to cisplatin-induced apoptosis at 16 and 24 h (Fig. 1A), but nevertheless, we observed certain levels of growth inhibition at 48 h after the drug treatment (Fig. 4A). This could be attributable to the contribution of cell cycle arrest because H460 cells have wild-type p53, and G1 arrest was actually induced after exposure to the drugs (Fig. 1A). Combining the drugs with SmacN7(R)8 caused increased cell death in a dose-dependent manner [69, 60, and 36% of cell survivals for cisplatin with SmacN7(R)8; 62, 44, and 28% for taxol with SmacN7(R)8]. SmacN7(R)8 alone, however, had no or little effect on cell survival. Neither the transduction domain R8 alone nor SmacN7 alone, which was not able to internalize into cells, could significantly affect H460 cell sensitivity to cisplatin or taxol (Fig. 4A). Moreover, the peptide did not significantly affect the drug sensitivity of H23, H226, and normal TIG3 cells that had high apoptosis activities (Fig. 4B). These results indicate that the cell permeable Smac peptide potentiates chemotherapy-induced cell death in apoptosis-defective tumors that overexpress IAPs.

SmacN7(R)8 Potentiates Cell Death by Activating Caspase-dependent Pathway. To determine whether SmacN7(R)8 could enhance caspase-mediated apoptosis, we examined the activation of DEVDase after cells were treated with drugs for various time periods. As shown in Fig. 5, A and B, exposure of H460 cells to cisplatin or taxol in combination with SmacN7(R)8 resulted in an increased DEVDase activation, as compared with cisplatin or taxol alone, and the increased DEVDase activation was also accompanied by an enhanced cleavage of caspase-3 precursor to its active products (Fig. 5, C and D). Moreover, the broad-spectrum caspase inhibitor zVAD-fmk totally abrogated DEVDase activity as well as the cleavage of caspase-3 induced by cisplatin or taxol in combination with SmacN7(R)8 (Fig. 5, A–D). zVAD-fmk also suppressed cell death caused by the drugs (data not shown), confirming that SmacN7(R)8 potentiates cell death by restoring the caspase-dependent pathway.

In Vivo Antitumor Activity of SmacN7(R)8 in Combination with Chemotherapeutic Agent against Human H460 Cancer Xenografts. To test whether SmacN7(R)8 could increase chemotherapeutic agent cytotoxicity in vivo, we developed NCI-H460 xenografts in nude mice. Mice were treated with saline (PBS), SmacN7, SmacN7(R)8, and cisplatin alone or cisplatin in combination with PBS, SmacN7, or SmacN7(R)8. As shown in Fig. 6A, treatment with SmacN7 or SmacN7(R)8 alone did not have any apparent tumor-suppressive effect, whereas cotreatment with cisplatin plus SmacN7(R)8 showed a synergistically enhanced inhibitory effect on the growth of H460 xenografts, as compared with cisplatin alone or cisplatin combined with SmacN7 (Fig. 6, B and C). Caspase-3 cleav-
It has been reported that the Smac NH$_2$-terminal peptide of Smac that targets IAPs and antagonizes their function disrupted binding of XIAP to the processed caspase-9 and dramatically restored apoptosome activity in H460 cells. Moreover, the NH$_2$-terminal peptide of Smac that targets IAPs and antagonizes their function disrupted binding of XIAP to the processed caspase-9 and dramatically restored apoptosome activity in H460 cells. These results strongly indicate that IAP plays a predominant role in suppressing apoptosome in H460 cells. Our data show that XIAP as well as other IAPs were also expressed, at least to a certain extent, in the cells with high apoptosome activity, such as H23, H226 and TIG3. Surprisingly, however, the NH$_2$ terminus of the Smac peptide was exclusively effective in reversing the apoptosome defect in H460 cells but not in other cell lines. These observations suggest that despite the wide distribution of IAPs, inhibition of the apoptotic pathway could be predominant in some tumor cells. There is more than one explanation for the results. First, there could be a threshold at which IAPs bind and suppress caspases. This notion could be supported by our unpublished data that showed XIAP did not suppress cytochrome c-dependent caspase activation in vitro until its concentration reached a certain level. On the other hand, we cannot exclude a second possibility that XIAP could be regulated by some posttranscriptional modification such as phosphorylation in apoptosome-defective H460 cells. Additional investigations are needed to clarify these points.

It has been reported that the Smac NH$_2$ terminus, as short as seven amino acids (SmacN7(R)8, can antagonize IAPs and promote caspase activation in vitro (29). Thus, we chemically synthesized SmacN7(R)8, a peptide that contains seven amino acids at the NH$_2$ terminus of the active, processed Smac conjugated with a membrane permeable arginine repeat, and we tried to find new ways to study the effect of combination therapy in relation to the activated apoptotic mechanism. Although the mechanism of efficient internalization is not completely clear, eight arginines (R8) was the optimal number and mechanism. Although the mechanism of efficient internalization is not completely clear, eight arginines (R8) was the optimal number and...
the function of SmacN7(R)8 was as effective as SmacN7 in eliminating caspase inhibition by XIAP. The SmacN7 peptide efficiently restored the low apoptosome activation of H460 to levels as high as seen in normal cells. This equivalence in efficacy makes a strong case for the idea that short peptides derived from the NH2 terminus of mature Smac or its synthetic mimetics could be potential coactivators of caspases in vivo to kill cancer cells with an apoptosome defect caused by IAP overexpression.

As shown in Fig. 5, the caspase activation was induced at 36–48 h in cells, whereas, in cell free system, it occurred more quickly (Fig. 2). As reported previously, several upstream signaling steps including activation of stress-activated protein kinases are required for cytochrome c release to cytosol to activate apoptosome (cytochrome c/Apaf-1/caspase-9) pathway in intact cells (45). These steps could cause the delay of caspase activation in intact cells as compared with that in cell free system.

At present, it is not clear that chemotherapy-induced apoptosis occurs in vivo in solid tumors and that necrosis may also occur as a result of chemotherapy treatment. Our xenograft experiment shows that cisplatin induces caspase activation in vivo in H460 solid tumor, and SmacN7(R)8 potentiates the tumor-suppressive activity of chemotherapy through enhancing caspase activation. In this study, we used the active SmacN7(R)8 peptide (10 mM in 50 μl) given intratumorally. As for clinical application, additional studies could be needed to test i.v. administration of the Smac peptide. On the other hand, searching for smaller compounds that mimic the action of Smac peptide could also make a strong idea for the combination treatment with chemotherapy to promote clinical application.

As shown in Fig. 4A and Fig. 5, taxol seemed to induce cell death somewhat more effectively than cisplatin did in H460 cells. A recent report has shown that in H460 cells, DNA-damaging agents induce caspase-dependent cell death, whereas taxol, the antimicrotubule agent, can trigger a caspase-independent pathway (46). The unidentified caspase-independent pathway could explain the effectiveness of taxol in the apoptosome-defective H460 cells. Our unpublished results also indicate that SmacN7(R)8 enhances sensitivity of H460 to cis-

[Fig. 3. Polyarginine-conjugated SmacN7 acts as a cell-permeable IAP inhibitor. A, structure of SmacN7 peptides used in this study. B, fluorescence microscopic observation of the cells treated with 10 μM of eight arginine (R8) residues, SmacN7, six or eight arginine-conjugated SmacN7 peptides [SmacN7(R)6 or SmacN7(R)8]; all labeled with fluorescein) for 3 h, followed by three washes with PBS. C, 293T cytosolic extracts were mixed with purified XIAP (100 nM) and then stimulated with cytochrome c (10 μM) plus dATP (1 mM) in the presence of the indicated increasing amounts of SmacN7 or SmacN7(R)8 (10, 20, or 40 μM) for 60 min at 30°C and then assayed for activity against the fluorogenic caspase substrate DEVD-MCA (20 μM). Extracts without XIAP(-) were used as control. The DEVDase cleavage activity was plotted as a percentage of the activity without XIAP (100%). Results represent the mean ± SD for at least three independent extract preparations.

\[\text{SmacN7} \quad \text{H-AVPIAQK-OH}\]
\[\text{SmacN7(R)6} \quad \text{H-AVPIAQKGGGRRRRRC-OH}\]
\[\text{SmacN7(R)8} \quad \text{H-AVPIAQKGGGRRRRRRGRC-OH}\]
\[\text{R8} \quad \text{H-GGGRRRRRRRGRC-OH}\]

\[\text{XIAP~}^{-}\]
\[\text{XIAP~}^{+}\]
\[\% \text{DEVD-MCA Cleavage}\]

\[\text{SmacN7}\]
\[\text{SmacN7(R)8}\]
platin, which can be totally abrogated by zVAD-fmk. In contrast, zVAD-fmk cannot completely suppress cell death induction by taxol alone or in combination with SmacN7(R)8. Notably, SmacN7(R)8 was still effective in enhancing taxol-induced cell death even in the presence of zVAD-fmk. These findings suggest that SmacN7(R)8 potentiates caspase-independent cell death by taxol as well as caspase-dependent cell death and that a novel IAP/Smac-regulated pathway could exist in taxol-induced caspase-independent cell death. Additional studies are needed to clarify the mechanism of this action.

ACKNOWLEDGMENTS

We thank Drs. Mikihiko Naito, Yoshikazu Sugimoto, Naoya Fujida, and Akihiro Tomida for their helpful comments and discussion, and Yukiko Muramatsu for technical advice.

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