

SMAC/Diablo-dependent apoptosis induced by nonsteroidal antiinflammatory drugs (NSAIDs) in colon cancer cells

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Edited by George F. Vande Woude, Van Andel Research Institute, Grand Rapids, MI, and approved October 22, 2004 (received for review May 17, 2004)

Nonsteroidal antiinflammatory drugs (NSAIDs) form a paradigm for the chemoprevention of cancer, preventing colonic tumor progression in both experimental animals and humans. However, the mechanisms underlying the antineoplastic effects of NSAIDs are currently unclear. We found that the mitochondrial second mitochondrial-derived activator of caspase (SMAC)/direct inhibitor of apoptosis protein-binding protein with low pI (Diablo) protein translocates into the cytosol during NSAID-induced apoptosis in colon cancer cells. When *SMAC/Diablo* is disrupted by homologous recombination and RNA interference in these cells, the NSAID-induced apoptosis is abrogated. Biochemical markers of apoptosis, such as caspase activation, cytosolic release of cytochrome *c* and apoptosis-inducing factor, and mitochondrial membrane potential change, are accordingly decreased. These results establish that *SMAC/Diablo* is essential for the apoptosis induced by NSAIDs in colon cancer cells.

Prevention of human cancers through the use of chemical agents or dietary manipulation is an attractive option to reduce morbidity and mortality from these diseases. Colorectal cancer not only is a prototype for identifying genetic alterations underlying tumorigenesis but also provides a paradigm for developing chemoprevention strategies (1). In particular, the widely used nonsteroidal antiinflammatory drugs (NSAIDs) suppress the formation of colorectal tumors in both humans and rodents (2).

NSAIDs have been shown to inhibit a variety of enzymes, but the most well documented of them is cyclooxygenase-2 (COX-2) (2). Growing evidence has indicated that the antineoplastic effects of NSAIDs are mediated by the induction of apoptosis secondary to the inhibition of COX-2 (3). In mammalian cells, apoptotic cell death is unleashed through a cascade of events, among which a key event is the translocation of at least two apoptogenic proteins from the mitochondria into the cytosol to trigger the activation of caspase proteases (4–7). Cytochrome *c* is released from the mitochondria into cytosol, where it binds to other proteins and forms an “apoptosome” (8). Formation of this complex leads to activation of the caspase cascade (8). Second mitochondrial-derived activator of caspase (SMAC), also called direct inhibitor of apoptosis protein (IAP)-binding protein with low pI (Diablo), is the second mitochondrial protein found to be released into cytosol during apoptosis (9, 10). SMAC/Diablo (hereafter referred to as SMAC) promotes caspase activation, presumably by antagonizing IAPs such as X-linked IAP (XIAP) (10). Expression of SMAC sensitizes human cancer cells to apoptosis induced by a variety of anticancer agents (11). However, *SMAC*-knockout (KO) mice were not found to have abnormalities in apoptosis *in vivo*, raising the question of whether SMAC is required for apoptotic initiation (12). In this study, we investigated the role of SMAC in apoptosis induced by NSAIDs in colon cancer cells using gene targeting and RNA interference (RNAi).

Materials and Methods

Targeting *SMAC* in HCT116 Cells. *SMAC*-KO constructs were generated by cloning a 1.57- and a 3.20-kb *SMAC* genomic DNA fragment flanking exons 1 and 2 of the *SMAC* gene, respectively, into the bipartite targeting vectors depicted in Fig. 2A, as described (13). HCT116 colon cancer cells with two intact alleles of *SMAC* were transfected with the KO constructs by Lipofectamine (Invitrogen) and selected in G418 (0.4 mg/ml) for 3 weeks. Neomycin-resistant clones were pooled and screened for targeting events by PCR by using a *SMAC* primer (5'-GAAACGAAACTGTTTCGCAC-3') and a neomycin-resistance gene primer (5'-TTGTGCCAGTCATAGCCG-3'). The targeting events were confirmed by PCR by using different primer pairs (sequences available upon request). To target the second allele of *SMAC*, the neomycin-resistance gene flanked by two Lox P sites was excised from a heterozygous clone by infection with an adenovirus (Ad) expressing the Cre recombinase (Ad-Cre). The same targeting constructs were used to target the second allele. Targeting at the *SMAC* locus was verified by PCR and Western blotting.

Cell Culture and Drug Treatment. All cell lines were maintained in McCoy's 5A media (Invitrogen) supplemented with 10% FBS (HyClone), 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO₂. To reconstitute SMAC expression in the *SMAC*-KO cells, we transfected a SMAC-expressing plasmid (a gift from Chunying Du, Stowers Institute for Medical Research, Kansas City, MO) into the *SMAC*-KO cells and identified stable clones expressing SMAC by Western blotting.

Cells were plated in 12-well plates at 20–30% density 24 h before treatment with anticancer agents. DMSO stock solutions of anticancer agents were diluted into appropriate concentrations by cell culture medium and added to the cells. The anticancer agents used in this experiment are: ALLN (Sigma), Camptothecin (Sigma), Cisplatin (Sigma), 5-Fluorouracil (5-FU, Sigma), Indomethacin (Sigma), Mitomycin C (Sigma), Staurosporine (Sigma), Sulindac Sulfide (Merck), Taxol (Sigma), tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (Alexis Biochemicals, San Diego) and Vinblastine (Sigma). In some cases, caspase inhibitors (R & D Systems), including the pan-caspase inhibitor Z-VAD-fmk, caspase-3 inhibitor Z-

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: NSAID, nonsteroidal antiinflammatory drug; SMAC, second mitochondrial-derived activator of caspase; IAP, inhibitor of apoptosis protein; Diablo, direct IAP-binding protein with low pI; XIAP, X-linked IAP; KO, knockout; PI, propidium iodide; RNAi, RNA interference; TRAIL, TNF-related apoptosis-inducing ligand; AIF, apoptosis-inducing factor.

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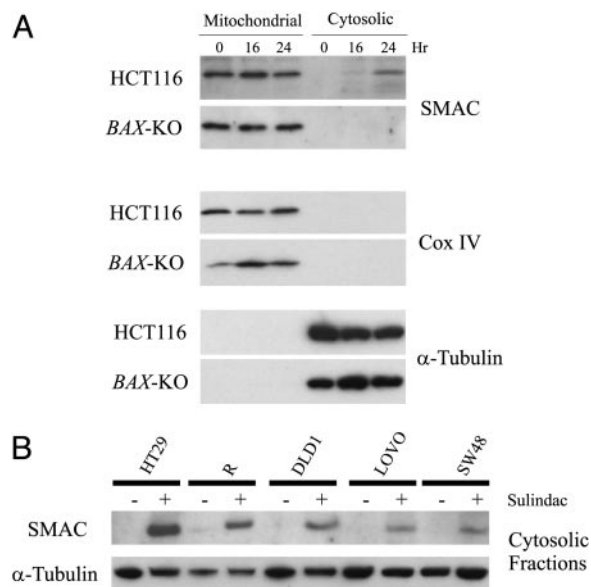


Fig. 1. Release of SMAC during sulindac-induced apoptosis in colon cancer cells. (A) Bax-dependent release of SMAC induced by sulindac in HCT116 cells. HCT116 and *BAX*-KO cells were treated with sulindac (120 μ M) for the indicated time and analyzed for mitochondrial and cytosolic SMAC expression by Western blotting after fractionation. Cytochrome oxidase subunit IV (Cox IV) and α -tubulin were used as controls for the mitochondrial and cytosolic fractionations, respectively. (B) SMAC release induced by sulindac in five other colon cancer cell lines. Colon cancer cell lines were treated with sulindac at different concentrations (HT29, 200 μ M; R, 200 μ M; DLD1, 180 μ M; LOVO, 120 μ M; and SW48, 120 μ M) for 48 h to induce apoptosis. Cytosolic fractions were isolated and examined for SMAC expression by Western blotting. α -Tubulin, which is exclusively expressed in cytosol, was used as a control for loading and for fractionation. Reduced α -tubulin levels in several cell lines after sulindac treatment reflect decreases in cell number.

DEVD-fmk, and caspase-9 inhibitor Z-LEHD-fmk, were used to treat cells before drug treatment.

Apoptosis Assays. After treatment, attached and floating cells were harvested at various time points. The fractions of apoptotic cells were evaluated by nuclear staining and annexin V/propidium iodide (PI) staining. For nuclear staining, cells were fixed in a solution containing final concentrations of 3.7% formaldehyde, 0.5% Nonidet P-40 and 10 μ g/ml DAPI in PBS, and assessed for apoptosis through microscopic visualization of condensed chromatin and micronucleation. For each measurement, at least three independent experiments and a minimum of 300 cells were analyzed. For annexin V/PI staining, cells were stained by using the Apo-Alert Annexin V kit (BD Biosciences), following the manufacturer's instruction, and then analyzed by flow cytometry. The long-term survival of cells after drug treatment was evaluated by colony formation assays. In brief, the harvested cells were plated in 25-cm² flasks at appropriate dilutions. Cells were allowed to grow for 10–14 days before staining with Crystal Violet (Sigma). All experiments were repeated at least three times. For detection of mitochondrial membrane potential change, harvested cells were stained by Mito Tracker Red CMXRos (Molecular Probes) for 15 min at room temperature and then analyzed by flow cytometry on a Beckman Coulter EPICS XL using the FL3 channel according to the manufacturer's instructions.

Western Blotting and Antibodies. Cell lysates were collected at various time points, and immunoblotting was performed as described (13). The antibodies used for Western blotting include

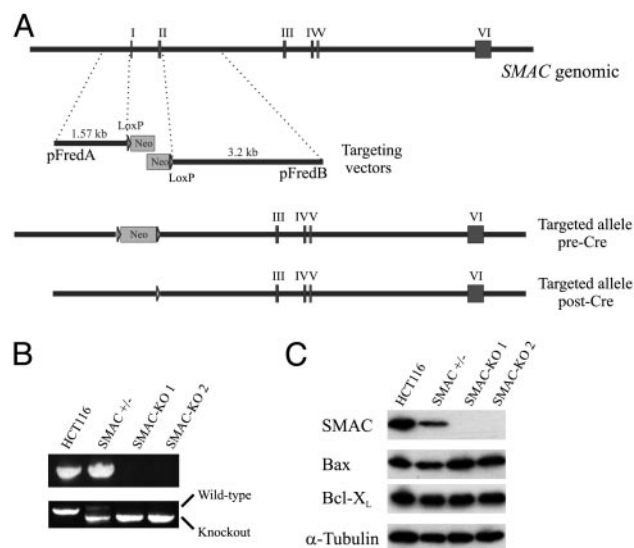


Fig. 2. Targeted deletion of *SMAC* in HCT116 colon cancer cells. (A) *SMAC* genomic locus and targeting constructs. Each targeting construct consists of a homologous arm (1.57 and 3.20 kb, respectively) and an overlapping fragment of the neomycin-resistance gene (*Neo*). Homologous recombination resulted in deletion of exons 1 and 2 of *SMAC*. The same constructs were used in the second round of gene targeting after the *Neo* was excised from the heterozygous cells by Cre recombinase. (B) *SMAC*-KO clones identified by genomic PCR. (Upper) PCR products amplified from cells with different *SMAC* genotypes using primers specific for the deleted region. (Lower) Larger PCR products represent the wild-type *SMAC* allele, whereas smaller products represent the recombinant allele, which was amplified from an endogenous PCR priming site and a PCR priming site introduced into the genomic locus by the KO construct. (C) The expression of *SMAC* protein in cells with different *SMAC* genotypes. Lysates from the cells with the indicated genotypes were analyzed by Western blotting using a *SMAC*-specific antibody. The same blot was stripped and reprobed for Bax and Bcl-X_L. α -Tubulin was used as a loading control.

rabbit polyclonal antibodies for *SMAC* (a generous gift from Eileen White, Rutgers University, Piscataway, NJ), caspase 9 (Cell Signaling Technology, Beverly, MA), Bax (N20, Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-X_L (BD Biosciences), active caspase 3 (BD Biosciences), and α -tubulin (Oncogene Science).

Cellular Fractionation. Two 75-cm² flasks of cells were harvested after a PBS wash at 4°C. Whole-cell pellets were resuspended in homogenization buffer (0.25 M sucrose/10 mM Hepes, pH 7.4/1 mM EGTA) and subjected to 40 strokes in a 2-ml Dounce homogenizer at 4°C. The homogenates were subjected to centrifugation at 1,000 \times g for 15 min at 4°C to pellet nuclei and unbroken cells. The supernatant was subsequently subjected to centrifugation at 10,000 \times g for 15 min at 4°C to obtain cytosolic fractions (supernatant) and mitochondrial fractions (pellet).

RNAi. Nucleotides 204–224 of the *SMAC* coding sequence (TAGTAGTGAAGCATTGATGAG) were chosen as the target for RNAi. Two 64-base oligonucleotides containing this sequence were designed based on criteria previously described (14). The oligonucleotides were annealed, and the duplex was cloned into the pSUPER vector (Oligoengine, Seattle) to direct the synthesis of small interference RNA (15). RNAi constructs were transfected into DLD1 colon cancer cells by Lipofectamine, and cells were plated in 96-well plates in the presence of puromycin (5 μ g/ml, Invitrogen). Puromycin-resistant clones were isolated. Western blotting was used to identify several clones with significant down-regulation of *SMAC*.

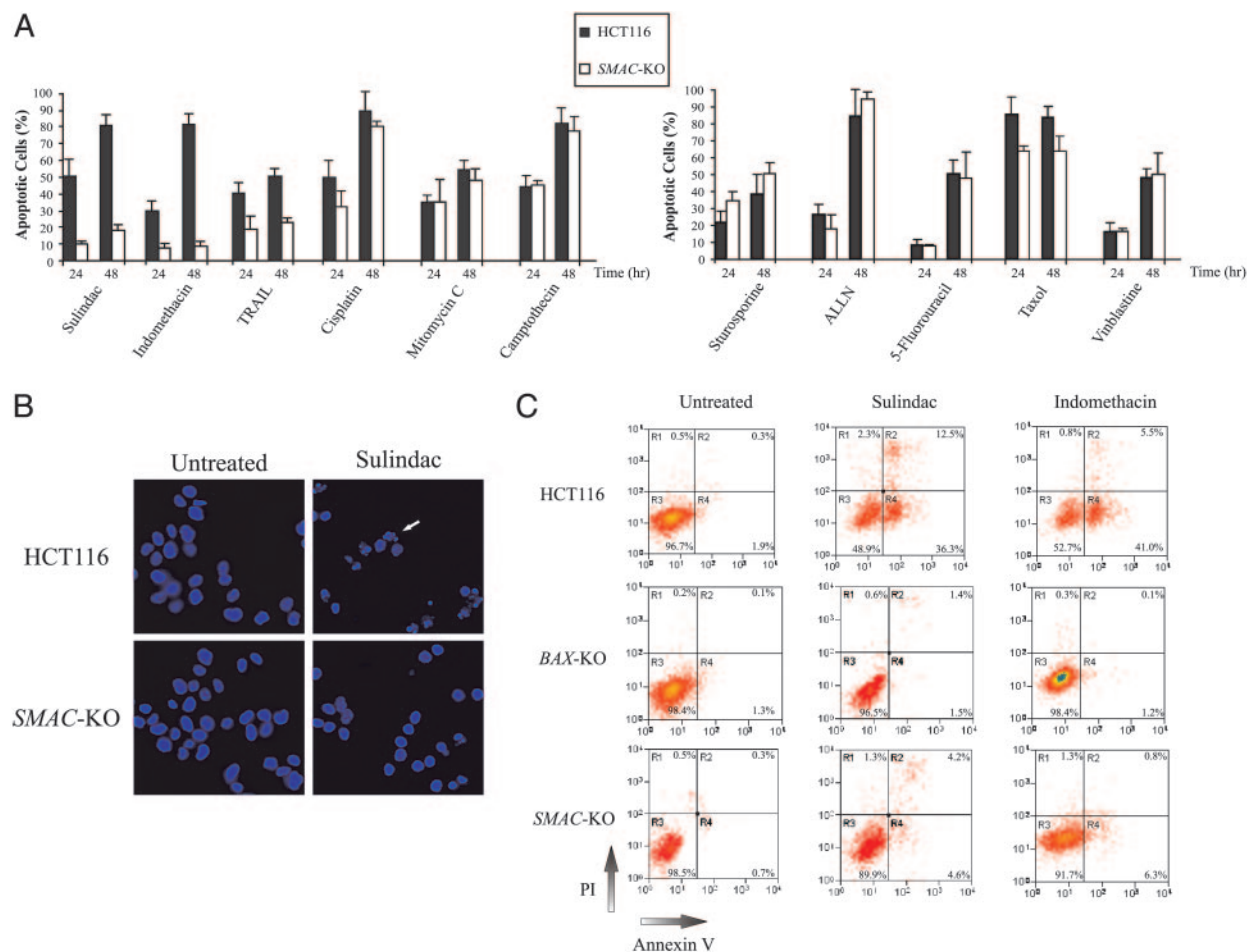


Fig. 3. Apoptosis induced by anticancer agents in the *SMAC*-KO cells. (A) Apoptosis induced by anticancer agents quantified by nuclear staining. Parental HCT116 cells and *SMAC*-KO cells were treated with the indicated anticancer agents for 24 and 48 h. Cells were fixed and analyzed by fluorescence microscopy after DAPI staining. At least three independent experiments were carried out for each condition, and a minimum of 300 cells were counted for each measurement. The anticancer agents include the NSAIDs sulindac sulfide (120 μ M) and indomethacin (500 μ M), the death receptor ligand TRAIL (50 ng/ml), the DNA crosslinking agents cisplatin (100 μ M) and mitomycin C (30 μ M), the topoisomerase inhibitor camptothecin (600 nM), the microtubule-targeting agents taxol (20 nM) and vinblastine (25 nM), the kinase inhibitor staurosporine (200 nM), the proteasome inhibitor ALLN (*N*-acetyl-leu-leu-norleu-al, 20 μ M), and the antimetabolite 5-fluorouracil (375 μ M). (B) Nuclear morphology of the cells undergoing apoptosis. Parental and *SMAC*-KO cells treated with sulindac (120 μ M) for 48 h were subject to fluorescence microscopy analysis after staining with DAPI. Cells containing fragmented and condensed nuclei (an example indicated by the arrow) are apoptotic cells. (C) Apoptosis quantified by annexin V/PI staining. Parental, *BAX*-KO, and *SMAC*-KO cells were treated with sulindac (120 μ M) and indomethacin (500 μ M) for 36 h. Cells were stained by annexin V and PI and then analyzed by flow cytometry. Four subpopulations and their quantities are indicated: nonapoptotic dead cells (R1), late apoptotic cells (R2), viable cells (R3), and early apoptotic cells (R4).

Results

NSAIDs Induce the Release of SMAC in Colon Cancer Cells. In previous studies, we found that NSAIDs induce a fulminant apoptosis in HCT116 colorectal cancer cells (16). The apoptosis in these cells is conventional, as indicated by alterations of Bcl-2 family proteins, disruption of mitochondrial membrane potential, release of apoptogenic mitochondrial proteins, and activation of the caspase cascade (13, 16–18). Moreover, isogenic *BAX*-KO HCT116 cells are completely resistant to sulindac, an NSAID and prototypic agent used for the prevention of colorectal cancers (19). To determine whether SMAC plays a role in this process, we analyzed HCT116 and *BAX*-KO cells. We found that SMAC was released into the cytosol of the parental HCT116 cells undergoing apoptosis induced by sulindac (120 μ M) (Fig. 1A), whereas SMAC was not found in the cytosol of the *BAX*-KO cells (Fig. 1A). These results indicate that sulindac induces a Bax-dependent release of SMAC. We then analyzed SMAC release in five other colon cancer cell lines. These cell lines have different genetic backgrounds, as assessed by mutational status

of *RAS*, *p53*, *APC*, *TGF- β RII*, and other genes. It is therefore not surprising that different concentrations of sulindac were required to induce apoptosis in the various lines. However, in each case, SMAC was found to be released into the cytosol after treatment of the cells with sulindac at the appropriate concentration (Fig. 1B). Similar results were obtained when the cells were treated with indomethacin, another NSAID (data not shown). These results suggest a consistent involvement of SMAC in NSAID-induced apoptosis in colorectal cancer cells.

Targeted Deletion of SMAC Abrogates NSAID-Induced Apoptosis. Is SMAC release important for NSAID-induced apoptosis or just a coincidental effect? To answer this question, we used homologous recombination to knock out the *SMAC* genomic locus in HCT116 cells. After two rounds of gene targeting (Fig. 2A), we isolated two *SMAC*-KO cell lines (*SMAC*-KO1 and -2) (Fig. 2B). The expression of SMAC protein was significantly decreased in the heterozygous KO cells (Fig. 2C), which is similar to what was reported for heterozygous mice (12). As expected, SMAC protein was completely absent in the homozygous KO cells (Fig.

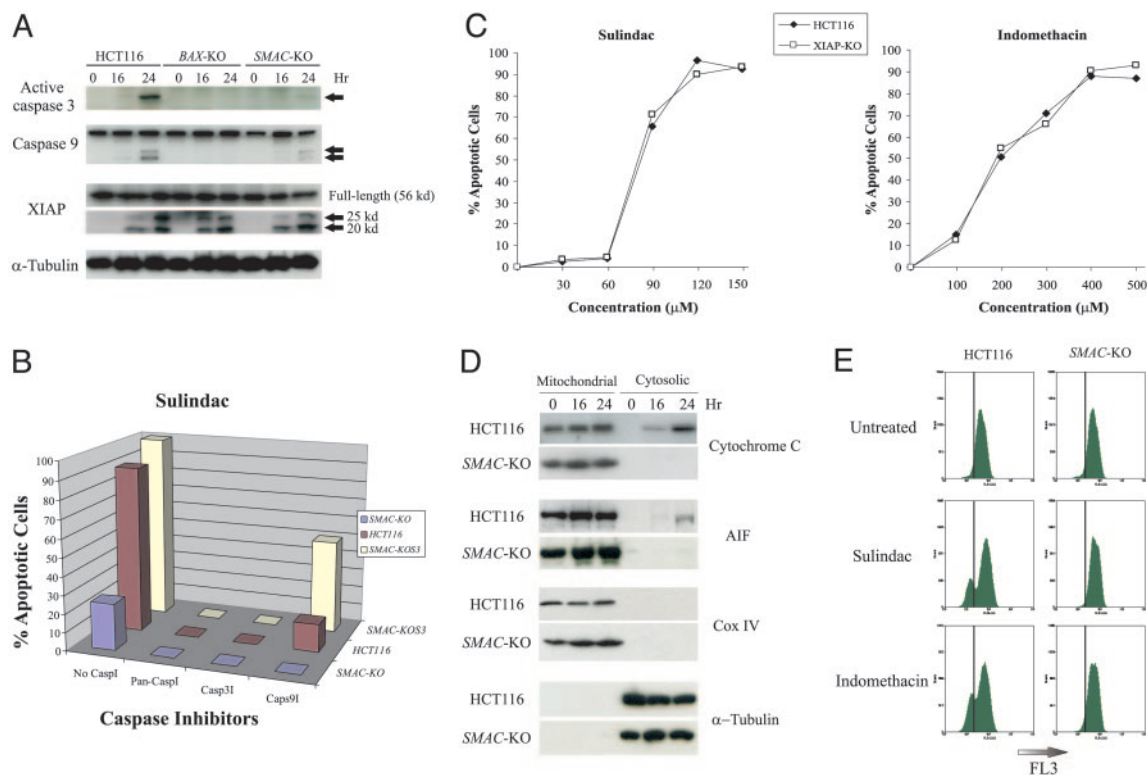


Fig. 6. Mechanisms of NSAID-induced apoptosis mediated by SMAC. (A) Cleavage of caspases and XIAP. Parental HCT116 cells, *BAX*-KO, and *SMAC*-KO cells were treated with 120 μ M sulindac. Cell lysates collected at the indicated time points were analyzed for active caspase 3, caspase 9, and XIAP by Western blotting. The arrows indicate the cleavage fragments of caspases and XIAP. α -Tubulin was used as a control for loading. (B) Effects of caspase inhibitors on apoptosis. Caspase inhibitors, including a pan-caspase inhibitor (pan-CaspI, Z-VAD-fmk), a caspase-3 inhibitor (Casp3I, Z-LEHD-fmk), and a caspase-9 inhibitor (Casp9I, Z-LEHD-fmk), were diluted to 20 μ M with cell culture medium and added to the cells 8 h before sulindac treatment. After cells were treated with sulindac (120 μ M) for 48 h, apoptosis was measured by nuclear staining. (C) NSAIDs induced apoptosis in parental and *XIAP*-KO cells. Cells with the indicated genotypes were treated with sulindac and indomethacin for 48 h, and apoptosis was analyzed by nuclear staining. A minimum of 300 cells were counted for each measurement, and the results are averages of three independent measurements. (D) Blockade of the release of cytochrome *c* and AIF in the *SMAC*-KO cells. Mitochondrial and cytosolic fractions from HCT116 and the *SMAC*-KO cells treated with sulindac (120 μ M) for the indicated time were subject to Western analysis for cytochrome *c* and AIF. Cytochrome oxidase subunit IV (Cox IV) and α -tubulin were used as controls for fractionation. (E) Lack of mitochondrial membrane potential collapse in the *SMAC*-KO cells. Parental HCT116 cells and *SMAC*-KO cells were treated with sulindac (120 μ M) and indomethacin (500 μ M) for 24 h. Cells were harvested, stained by MitoTracker Red CMXRos, and immediately analyzed by flow cytometry. The mitochondrial membrane potential was detected by FL3.

in XIAP, such as changes in its expression level or ability to bind caspases in *SMAC*-KO cells, despite the observation that XIAP was cleaved in sulindac-treated cells (Fig. 6A). To further investigate the role of XIAP in NSAID-induced apoptosis in colon cancer cells, we examined HCT116 cells in which the *XIAP* gene had been disrupted through targeted homologous recombination (21). It would be expected that *XIAP*-KO cells would be more sensitive to NSAIDs if SMAC functioned by inhibiting the antiapoptotic activity of XIAP. However, no differences were detected in the *XIAP*-KO cells compared with the parental cells with respect to NSAID-induced apoptosis, either with sulindac or indomethacin (Fig. 6C).

The major mechanism underlying SMAC function has heretofore been thought to be inhibition of XIAP. Our results suggest otherwise. It was not expected that disruption of SMAC alone would have significant effects on apoptosis and caspase activation, because several other mitochondrial apoptogenic proteins, including cytochrome *c*, endonuclease G, and AIF, are released into the cytosol during apoptosis (22–24), and the release of cytochrome *c* is thought to be the major caspase activation mechanism (8). To reconcile these observations, we analyzed the release of cytochrome *c* and AIF in cells treated with NSAIDs. Surprisingly, both cytochrome *c* and AIF release were reduced in the *SMAC*-KO cells relative to the parental cells (Fig. 6D). Further analysis showed that mitochondrial membrane potential change was significantly blocked in the *SMAC*-KO cells com-

pared with the parental cells (Fig. 6E). These results indicate that cytochrome *c* and AIF cannot be properly released into cytosol in the absence of SMAC, and the effects of SMAC may be mediated by the regulation of mitochondrial integrity.

Discussion

The results described above show that SMAC is essential for the apoptosis induced by NSAIDs in colon cancer cells. A number of biochemical studies have shown that SMAC and its homologues play an important role in programmed cell death in different organisms. Disruption of the SMAC homologues in *Drosophila* results in expected changes in apoptosis during fly development (25). However, targeted deletion of *SMAC* in mice did not show obvious alterations in apoptosis (12), raising questions about the importance of SMAC in apoptosis in mammalian cells. These observations indicate that the role of SMAC in apoptosis varies depending on specific organisms, cell types and apoptotic stimuli.

Although cytosolic release of mitochondrial proteins is known to be critical for apoptosis and caspase activation, the mechanism of this process is poorly understood. Previous studies suggested that full caspase activation requires release of both cytochrome *c* and SMAC from mitochondria to induce assembly of apoptosome and relieve the inhibition of caspase activation by IAPs (26). Cytochrome *c* release and SMAC release appear to be two

independent events that are mediated via different mechanisms (27, 28). However, our results suggest that these two events are related to each other and cytochrome *c* cannot properly translocate to the cytosol without SMAC. Cytochrome *c*-mediated caspase activation is therefore affected by the absence of SMAC. This explains why a majority of the apoptosis induced by NSAIDs is inhibited in the SMAC-KO cells with a concomitant decrease in caspase activation.

The concentrations of NSAIDs used in our study are much higher than those achieved in the serum of patients or animals treated with NSAIDs, raising important questions about the physiological relevance of our results. It has been suggested that intestinal epithelial cells are exposed to NSAIDs at much higher concentrations than those measured in serum assays because these drugs are taken orally and are metabolized in a unusual fashion (29). It has also been reported that sulindac, the most effective NSAID in tumor suppression, is metabolized to the active sulfide form by aerobic gut flora on the surface of the colon (30, 31), and that systemic sulindac sulfide concentrations are significantly reduced in patients who have undergone colectomy (32). This unusual mode of metabolism can create locally high concentration of NSAIDs, which has been shown to topically suppress tumor growth in rodents (33), and this may explain why it takes such high concentrations of drugs *in vitro* to induce apoptosis.

Although SMAC-KO cells are deficient in NSAID-induced apoptosis, the long-term viability of these cells was decreased after sulindac treatment, as measured by colony formation assays, suggesting that mechanisms other than apoptosis are involved in the growth-suppressing effect of NSAIDs. As the

apoptotic effects of NSAIDs have been described in a number of *in vivo* studies using animal models and familial adenomatous polyposis patients (3), our observations should stimulate further *in vivo* studies to clarify these issues.

Although the role of SMAC in apoptosis in mammalian cells has been less than definitive, substantial evidence has indicated that SMAC is important for the apoptosis induced by anticancer agents. SMAC overexpression and use of SMAC-derived peptides or small molecules can sensitize human cancer cells to apoptosis induced by TRAIL (34, 35). Knockdown of SMAC by RNAi inhibited apoptosis induced by tumor necrosis factor α (36). Our results demonstrate that SMAC is required for apoptosis induced by NSAIDs in colon cancer cells. Elucidating the role of SMAC in NSAID-induced apoptosis may have important implications for cancer prevention. SMAC and SMAC-derived reagents can potentially be used to enhance the antitumor effects of NSAIDs and other anticancer agents (11), and to overcome drug resistance caused by deficiencies in apoptosis due to genetic alterations in cancer cells (37).

We thank Dr. Eileen White for providing the SMAC antibody, Dr. Chunying Du for providing the SMAC expression plasmid, Dr. Fred Bunz (Johns Hopkins Oncology Center, Baltimore) for the XIAP-KO cell line, Drs. Robert W. Sobol, Jr. and Federica Marini for help with RNAi, Dr. Miaomiao Wang and Mrs. Sagarika Tiwari for technical assistance, and Drs. Daniel E. Johnson and Chunying Du for critical comments on the manuscript. This work is supported by the Edward Mallinckrodt, Jr., Foundation; the Elsa U. Pardee Foundation; the Clayton Fund; and National Institutes of Health Grants CA 043460 and CA106348. L.Z. is a scholar of the General Motors Cancer Research Foundation and the V Foundation for Cancer Research.

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