A Fas-associated Death Domain Protein-dependent Mechanism Mediates the Apoptotic Action of Non-steroidal Anti-inflammatory Drugs in the Human Leukemic Jurkat Cell Line*

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Non-steroidal anti-inflammatory drugs (NSAIDs) are inhibitors of cyclooxygenase-1 and -2 and are useful for prevention and cure of cancers, especially colon and rectal cancers. The NSAIDs indomethacin and sulindac sulfide have been shown to induce apoptosis of colon epithelial cancer cells by a Bax-dependent mechanism that involves mitochondria-mediated activation of a caspase-9-dependent pathway. In this report, we demonstrate that indomethacin and sulindac sulfide induce apoptosis of human leukemic Jurkat cells by a mechanism that requires the Fas-associated Death Domain Protein-mediated activation of a caspase-8-dependent pathway. Therefore, NSAIDs induce apoptosis by different mechanisms depending on the cell type.

NSAIDs† are a group of chemicals of diverse structures that bind to and inhibit the activity of cyclooxygenase-1 and -2 (COX-1 and COX-2), which regulate a rate-limiting step in the production of prostaglandins (1, 2). Human epidemiological and animal studies have demonstrated that NSAID administration is beneficial in the treatment of cancers, especially those of the colon and rectum (3, 4). The mechanism by which NSAIDs exert this anti-cancer activity is, however, unclear and controversial. Originally, it was believed that NSAIDs inhibit cancer progression by inhibiting COX, especially COX-2, because (i) progression of colon cancer development was associated with an increase of prostaglandin E₂ production in cancer tissue (5), (ii) NSAIDs inhibited the activity of COX and thus the production of prostaglandins (5, 6), and (iii) NSAIDs inhibited the proliferation of normal and cancerous colon epithelial cells by inducing arrest of the cells at the G₀ and G₁ phases of the cell cycle (7, 8). COX-1 is constitutively expressed in many normal and cancerous cell types, whereas expression of COX-2 is stimulated by growth factors and tumor promoters and is often selectively expressed in tumor cells, including those of colon and rectal origins (9–11). Thus, COX-2 expression correlated with tumorigenicity. This correlation was supported by the finding that in a mutated adenomatous polyposis coli background (APC<sup>min</sup>/+,), which predisposes the animals to intestinal cancer, COX-2<sup>−/−</sup> mice developed fewer intestinal polyps than COX-2<sup>+/+</sup> mice (12). By extension, targeted inhibition of COX-2 by NSAIDs would logically be expected to suppress the development of cancers, at least those of colon origin. More recent data, however, suggested that the apoptotic activity of NSAIDs did not seem to be associated with their ability to inhibit COX-1 or COX-2, because (i) the concentration of NSAIDs required to induce apoptosis was several orders of magnitude higher than that required to inhibit the activity of COX (13) and (ii) NSAIDs such as indomethacin (IND) and sulindac sulfide (SuS) still induced apoptosis in COX-1<sup>−/−</sup>, COX-2<sup>−/−</sup>, and COX-1:Cox-2 double knock-out mouse embryonic fibroblast cells (14). Therefore, the apoptotic effect of NSAIDs appears to be mediated by other factors.

Execution of apoptosis requires activation of caspases, which are ubiquitously and constitutively expressed as inactive zymogens (pro-caspases) in the cytosol (15–17). Caspases can be divided into “initiator” caspases (e.g. caspases-2, -8, -9, and -10), which activate downstream “effector” caspases (e.g. caspases-3, -6, and -7), which, in turn, degrade certain cellular proteins and ultimately result in cell death (15–17). The initiator caspases can be activated by either mitochondrial-mediated or cell surface “death” receptor-dependent processes (15–17). The mitochondria-mediated process begins with the activation of the initiator caspase, caspase-9, which then activates downstream effector caspases, in particular caspase-3, that result in cell death (18–21). Activation of this caspase-9-dependent pathway can be induced by many anti-cancer drugs (22). In contrast, the death receptor-mediated process activates an apoptotic pathway that is dependent on the initiator caspase, caspase-8 (23). The best studied death receptor is Fas (24). Engagement of the extracelluar domain of Fas by the Fas ligand (Fas-L) results in Fas trimerization and intracellular recruitment of the adapter protein, FADD, to the receptor cluster (23, 25, 26). Recruitment is facilitated by homotypic

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† The abbreviations used are: NSAID, non-steroidal anti-inflammatory drug; COX, cyclooxygenase; IND, indomethacin; SuS, sulindac sulfide; PARP, poly(ADP-ribose) polymerase; DAF, 4,6-diamidino-2-phenylindole; FADD, Fas-associated death domain protein; TNFα, tumor necrosis factor α; TNFR, tumor necrosis factor receptor; DISC, death-inducing signaling complex; DN, dominant-negative; PBS, phosphate-buffered saline.
interactions between “death domains” located in Fas and FADD. Receptor-bound FADD oligomerizes, and then utilizes a second domain, the “death effector domain,” to recruit procaspase-8 (24–26). The complex of Fas-FADD-procaspase-8 is referred to as DISC (death-inducing signaling complex). DISC catalyzes proteolytic conversion of procaspase-8 into active caspase-8 (23, 24), which then activates the downstream effector caspases, caspases-3, -6, and -7, triggering cell death.

Recently, it was demonstrated that treatment of several human colon epithelial cancer cell lines, including HCT116, with NSAIDs such as IND and SuS induced (i) down-regulation of a key apoptotic suppressor, Bcl-xL; (ii) transduction of apoptotic signals to mitochondria by a Bax-dependent mechanism; and (iii) activation of the mitochondrial/caspase-9-dependent apoptotic pathway (27). The requirement for Bax and mitochondria-mediated apoptosis induction by NSAIDs was inferred from the observation that targeted knock-out of both alleles of the bax gene in HCT116 cells impaired their ability to respond to the apoptotic effect of IND and SuS (27). Interestingly, the HCT116/Bax−/− cells were still responsive to the apoptotic effect of several other anti-cancer drugs, indicating that Bax was uniquely required to mediate the apoptotic effect of NSAIDs.

In this report, we demonstrate that, similar to their effect on human colon cancer cells, treatment of human leukemia cell lines with IND and SuS induces apoptosis. We also demonstrate that the ability of NSAIDs to inhibit COX activity is insufficient to induce apoptosis. Surprisingly, however, our data show that the key step of apoptosis induction by IND and SuS involves a FADD-dependent, but not Bax-dependent, mechanism that activates the initiator caspase-8-dependent pathway. These results indicate that the apoptotic effect of NSAIDs on cells can be mediated by different mechanisms in cell type-specific fashion.

**EXPERIMENTAL PROCEDURES**

*Materials—*NSAIDs, DAPI, and a rabbit polyclonal TNFα neutralization antibody (T8300) were purchased from Sigma. Polyclonal rabbit antibodies to human caspase-3 and caspase-8 for Western blot analysis, mouse monoclonal Fas (clone DX2, IgG1) and Fas-L (clone NOK-1, IgG1) antibodies, and fluorescein isothiocyanate-conjugated rat monoclonal antibody to mouse IgG1 (clone A85-1) for flow cytometry analyses were purchased from PharMingen (San Diego, CA). NOK-1 Fas-L antibody was also used to neutralize Fas-L in bioassays (28). A polyclonal goat antibody to human COX-1, polyclonal rabbit antibodies to human COX-2, and a mouse monoclonal antibody to human β-actin were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and a mouse monoclonal antibody to human poly(ADP-ribose) polymerase (PARP) was purchased from BIOMOL (Plymouth Meeting, PA). ECL reagents were obtained from Amersham Pharmacia Biotech, and the pan-caspase inhibitor Z-VAD-FMK was purchased from Calbiochem-Novabiochem, Inc. (Boston, MA).

*Cell Stocks—*The Jurkat cell line was obtained from ATCC. A Jurkat cell line variant expressing a dominant-negative FADD (FADD-DN) cDNA transgene (FADD-DN Jurkat cells) has been described (29). An additional “wild-type” Jurkat cell line and two variants derived from it null for FADD or caspase-8 expression, respectively, were obtained from Dr. John Blenis and have been described elsewhere (30, 31). The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 units/ml penicillin, and 50 units/ml streptomycin. Cells were incubated at 37 °C in a humidified incubator with 5% CO2. To maintain a high expression level of FADD-DN in the FADD-DN Jurkat cell line before they were used for experimentation, the cells were treated for 72 h with 100 ng/ml of the CH-11 agonistic anti-Fas antibody, and the surviving cells (~75%) were expanded and subsequently used for experimentation.

**Determination of Apoptosis by DAPI Staining of Nuclear DNA—**Untreated (control) and NSAID-treated cells were collected by centrifugation, resuspended in fixing solution (2% paraformaldehyde, 0.1% Triton X-100, PBS), and incubated for 30 min at room temperature. The cells were then pelleted by centrifugation, washed once in PBS, and incubated in DAPI solution (500 ng/ml DAPI in PBS) for 10 min at room temperature. An aliquot of the stained cells was observed under a fluorescent microscope connected to a Power Macintosh computer, and the cell morphology was recorded using Adobe Photoshop software. Apoptotic cells were identified as those with densely stained granular nuclear bodies (32).

*Western Blot Analyses—*Cells were left untreated or treated with IND or SuS at the indicated doses. The cells were then harvested, washed three times in drug-free medium, resuspended in drug-free medium at a density of 50 cells/ml, and seeded into 96-well plates (200 μl/well) in triplicate. The cells were incubated for 2–4 weeks. Growth of cells in each well was inspected and recorded. The growth of untreated cells was designated as 100%, and the growth of the drug-treated cells was compared with that of untreated cells.

**Flow Cytometry Analysis of Cell Surface Fas and Fas-L Expression—**Cells were first incubated for 20 min with mouse monoclonal antibodies to Fas, Fas-L, or isotype-matched mouse IgG1 in PBS containing 1% bovine serum. The cells were then washed in PBS, and incubated with fluorescein isothiocyanate-conjugated rat anti-mouse IgG1 antibody for 20 min. The cells were washed in PBS containing 1% bovine serum three times and then subjected to analysis in a FACScan flow cytometer (Becton Dickinson).

**RESULTS**

Preliminary studies showed that IND induced various human leukemic cell lines to undergo apoptosis (data not shown). In a 48-h survival assay, the effective concentration range for IND to induce apoptosis of all cell lines was between 200 and 500 μM (Fig. 1A and data not shown). This concentration range was ~100-fold more than that required for IND to inhibit
COX-1 and COX-2 in cells (13). However, it was similar to the concentration required to induce Bax-dependent apoptosis of human colon cancer cell lines (27). To investigate in greater detail the mechanism mediating specifically the apoptotic effect of IND and other NSAIDs, we concentrated on the human leukemic Jurkat cell line.

Apoptotic Induction of Jurkat Cells by IND Is COX-2-independent—Treatment of Jurkat cells for 48 h with IND induced dose-dependent apoptosis with an IC$_{50}$ value of ~250 μM (Fig. 1A). Dying cells were identified by the appearance of granular nuclear apoptotic bodies containing condensed DNA (Fig. 1B). Jurkat cells did not express COX-2 protein at any detectable level, nor did IND induce its expression (Fig. 1C). Thus, the apoptotic effect of IND on Jurkat cells appeared to be independent of COX-2. COX-1 was expressed in Jurkat cells, and it was down-regulated by IND treatment in a fashion that paralleled apoptotic induction (Fig. 1C).

To pursue the correlation with COX-1, we investigated the effect of other NSAIDs, which are potent inhibitors of COX-1 (12, 34, 35), on Jurkat cells. Whereas SuS also induced apoptosis of Jurkat cells, the other NSAIDs had no apoptotic effect (Fig. 2). These results indicated that the ability of NSAIDs to inhibit the activity of COX-1 alone was insufficient to induce apoptosis, and that IND and SuS were unique in their ability to induce apoptosis among the NSAIDs tested.

IND Induces Activation of Caspase-8—Treatment of Jurkat cells with IND induced cleavage of the apoptotic targets PARP (36) and β-catenin (37), which was blocked by the irreversible pan-caspase inhibitor, Z-VAD-FMK (Fig. 3A). IND treatment of Jurkat cells progressively induced the activation of the initiator caspase, caspase-8, and the effector caspase, caspase-3, and both of these events paralleled the induction of PARP cleavage (Fig. 3B). Together, these results suggested that the exposure of Jurkat cells to IND resulted in the activation of caspase-8, which led to the activation of downstream effector caspases including caspase-3, which then cleaved cellular targets and caused apoptosis.

Inhibition of Caspase-8 Activation Blocks Apoptosis Induced by IND and SuS—To further assess whether caspase-8 activation was a critical event in the process of apoptotic induction, the ability of IND and SuS to induce apoptosis in a Jurkat cell line (29) expressing a dominant-negative version of FADD (FADD-DN) was investigated. The FADD-DN molecule expressed in these cells lacked the death effector domain, and it was therefore unable to interact with and induce the activation of caspase-8 and other caspases (23, 25, 26). The expression of FADD-DN dramatically blocked the ability of IND (Fig. 4A) and SuS (Fig. 4B) to induce apoptosis. Correspondingly, the FADD-DN cells exposed to IND or SuS failed to activate caspase-8 and caspase-3 and resulted in little, if any, cleavage of PARP (Fig. 4C).

After a 24-h treatment of wild-type Jurkat cells with IND (500 μM) or SuS (200 μM), and subsequent withdrawal of the drugs, there were virtually no viable cells left to resume proliferation (Fig. 4D). In contrast, Jurkat cells expressing the FADD-DN not only survived the 24-h treatment but were also able to proliferate after the withdrawal of the drugs (Fig. 4D). Taken together, these results indicated that IND and SuS induced apoptosis in Jurkat cells by a FADD-dependent mechanism that activated the initiator caspase-8-dependent pathway.

A Role for FADD and Caspase-8 in NSAIDs-induced Apoptosis—To confirm the role of FADD and caspase-8 in the process of apoptosis induction by NSAIDs, a set of three Jurkat cell lines that were wild-type (i.e., parental), caspase-8-null (30), or FADD-null (31) were treated with IND for 32 h. The caspase-8 and FADD-null cells were highly resistant to apoptosis induction in contrast to the wild-type cells (Fig. 5A). Correspondingly, the IND-treated FADD-null cells underwent little activation of caspase-8 (Fig. 5, B and C) and neither the FADD-null nor the caspase-8-null cells showed significant cleavage of either PARP (Fig. 5B) or β-catenin (Fig. 5C). These results further indicated that the IND-induced death of Jurkat cells occurred primarily through a FADD- and caspase-8-dependent mechanism.

Induction of Caspase-8 Activation by IND Is Independent of Death Receptor Ligands—Caspase-8 activation is normally associated with apoptotic induction caused by a death receptor ligand interacting with its cognate receptor (23). This interaction results in the recruitment of the adapter molecule FADD (23, 25, 26), which, in turn, recruits and activates caspase-8 (23, 24). Thus, having shown the requirement for FADD and caspase-8 in the process of apoptosis induction by IND, we investigated whether the known death receptor ligands Fas-L, TRAIL, and TNFα were involved in NSAID-induced apoptosis of Jurkat cells. The addition of the NOK-1 antibody, which

![Fig. 2. Apoptosis of Jurkat cells treated with various NSAIDs.](image-url)
potently neutralizes the activity of Fas-L and blocks its apoptotic effects (28), to cell culture medium did not alter the apoptotic activity of IND on Jurkat cells (Fig. 6A). Therefore, Fas-L was unlikely to be involved in IND-induced apoptosis. This was confirmed by the results from Western blot (data not shown) and flow cytometry analyses showing that, although Jurkat cells express high levels of Fas receptor (Fig. 6B, panel ii), they did not express detectable Fas-L either before (data not shown) or after (Fig. 6B, panel iii) IND treatment. Finally, IND-treated and untreated Jurkat cells were analyzed by immunoblotting for the expression status of TRAIL and TNFα. Neither ligand was expressed to a detectable level under either treatment condition (data not shown). From these experiments we concluded that IND-induced apoptosis was likely independent of the death receptor ligands Fas-L, TRAIL, and TNFα.

**DISCUSSION**

We have demonstrated that IND and SuS, members of the NSAIDs family, induce apoptosis in human leukemic Jurkat cells. Since Jurkat cells did not express COX-2 (Fig. 1), the inhibition of COX-2 by IND and SuS appears to be irrelevant to the mechanism mediating the apoptotic effect of these two chemicals. In contrast, COX-1 was expressed in Jurkat cells, and it was down-regulated in a comparable and parallel degree to the extent of apoptosis induced by IND (Fig. 1). However, it is unlikely that COX-1 was involved in apoptosis regulation in Jurkat cells, because the concentration of IND and SuS required to induce apoptosis was orders of magnitude higher than that required to inhibit COX-1 activity (13). In addition, proven potent COX-1 inhibitors, including acetaminophen, ibuprofen, fenoprofen, piroxicam, salicylic acid, and sulindac (13, 38), had no apoptotic effect on Jurkat cells (Fig. 2). Thus, the apoptotic effect of IND and SuS appeared to be mediated by factors other than COX-1 and COX-2.

A recent study demonstrated that IND- and SuS-induced apoptosis of human HCT116 colon epithelial cancer cells was mediated by a Bax-dependent mechanism (27). Thus, in colon cancer epithelial cells, the apoptotic signals generated by these two drugs were transduced through Bax to mitochondria, which induced cytochrome c efflux (Fig. 7A). Subsequently, this efflux presumably resulted in the activation of the initiator caspase, caspase-8, which then activated downstream effector caspases including caspase-3, finally culminating in apoptosis (39). However, we have demonstrated in this study that IND- and SuS-induced apoptosis in Jurkat cells was instead mediated by a FADD-dependent mechanism that activated the caspase-8-dependent pathway (Figs. 4, 5, and 7B). FADD was initially identified as a Fas-associated death domain protein (25, 26), which was required for activation of caspase-8 by death receptors such as Fas and TNFR1 (TNFα receptor type 1). Although we have not directly tested whether Bax is involved in the IND-induced death of Jurkat cells, other groups have demonstrated that FADD-de-
caspase-8-null Jurkat cells, which were treated for 0, 24, or 36 h with 500 μM IND and the percentage of apoptotic cells was determined by DAPI staining. B, determination by immunoblotting of the status of caspase-8 and PARP in FADD-null and apoptotic cells was determined by DAPI staining.

A plausible mechanism that would have explained the FADD-dependent apoptosis triggered by IND and SuS in Jurkat cells was an induction of expression of either Fas-L or TNFα. This hypothesis, however, seems unlikely since the apoptotic effect of the drugs on Jurkat cells was not decreased in the presence of antibodies (28) that could neutralize the activity of Fas-L (Fig. 6) and TNFα (data not shown). Moreover, IND- and SuS-treated Jurkat cells did not express either Fas-L or TNFα as determined by Western blot and FACS analysis (Fig. 6, and data not shown). These data support the conclusion that Fas-L or TNFα are not mechanistically relevant for NSAID-induced apoptosis of Jurkat cells.

Other death receptors, such as DR4 and DR5, can mediate caspase-8-dependent apoptosis after engagement of the TRAIL ligand (23, 42, 43). However, the role of FADD in this process remains controversial, with conflicting reports of FADD-independent (44), or FADD-dependent TRAIL-induced apoptosis (45, 46). Nevertheless, it is unlikely that TRAIL-mediated the apoptotic effect of IND and SuS on Jurkat cells, because TRAIL expression was also not detected in this cell line (data not shown). We cannot rule out the possibility that there is an unknown death ligand activated in Jurkat cells by IND and SuS that was responsible for causing apoptosis. Alternatively, however, we propose that IND and SuS induce apoptosis of Jurkat cells by a FADD-dependent, but death ligand-independent, mechanism. This mechanism has already been proposed to mediate the apoptotic effect of several other compounds, including cycloheximide (28) and several anti-cancer drugs (22, 47).

Although our data imply that IND and SuS apoptotic induction is death ligand-independent, they nonetheless implicate the involvement of death receptors. Thus, the FADD-DN construct we utilized is defective in its ability to interact with procaspases, whereas it is still competent to interact with death receptors, and it thus behaves as a dominant negative (29). Therefore, if IND and SuS could induce apoptosis without involvement of a death receptor but by activation, directly or indirectly, of FADD, then we would have expected to see no effect of the FADD-DN expression. Therefore, our finding that the FADD-DN-expressing cells were very resistant to apoptotic effect of the FADD-DN expression. Therefore, our finding that the FADD-DN expression. Therefore, our finding that the FADD-DN expressing cells were very resistant to apoptotic effect of the FADD-DN expression. Therefore, our finding that the FADD-DN expressing cells were very resistant to apoptotic effect of the FADD-DN expression. Therefore, our finding that the FADD-DN expressing cells were very resistant to the involvement of death receptors. Thus, the FADD-DN construct we utilized is defective in its ability to interact with procaspases, whereas it is still competent to interact with death receptors, and it thus behaves as a dominant negative (29). Therefore, if IND and SuS could induce apoptosis without involvement of a death receptor but by activation, directly or indirectly, of FADD, then we would have expected to see no effect of the FADD-DN expression. Therefore, our finding that the FADD-DN-expressing cells were very resistant to the involvement of death receptors. Thus, the FADD-DN construct we utilized is defective in its ability to interact with procaspases, whereas it is still competent to interact with death receptors, and it thus behaves as a dominant negative (29). Therefore, if IND and SuS could induce apoptosis without involvement of a death receptor but by activation, directly or indirectly, of FADD, then we would have expected to see no effect of the FADD-DN expression. Therefore, our finding that the FADD-DN-expressing cells were very resistant to the involvement of death receptors. Thus, the FADD-DN construct we utilized is defective in its ability to interact with procaspases, whereas it is still competent to interact with death receptors, and it thus behaves as a dominant negative (29). Therefore, if IND and SuS could induce apoptosis without involvement of a death receptor but by activation, directly or indirectly, of FADD, then we would have expected to see no effect of the FADD-DN expression. Therefore, our finding that the FADD-DN-expressing cells were very resistant to

It is also interesting to note that the phenotype of the FADD-
Induction of Apoptosis by Indomethacin

DN-expressing cells appeared more extreme than that of FADD- or caspase-8-null cells. FADD-DN cells underwent little apoptosis, even after prolonged IND or SuS treatment (Fig. 4C), and the majority of the cells were competent for continued proliferation upon removal of the apoptotic inducers (Fig. 4D). In contrast, the FADD- and caspase-8-null cells ultimately underwent apoptosis (Fig. 5 and data not shown) and generally succumbed even if NSAIDs were removed after 24 h (data not shown), although at a greatly reduced rate in comparison to wild-type cells. These observations suggest that the FADD- and caspase-8-dependant pathway is the preferred mode of apoptotic induction by NSAIDs in Jurkat cells. In the presence of the FADD-DN, most of the caspase-8 and other initiator caspases are apparently sequestered into nonproductive complexes such that little activation of caspase-8 nor the subsequent downstream steps occurs (Fig. 7C). In the absence of caspase-8, FADD is probably still capable of weakly activating other initiator caspases, such as caspase-2 or caspase-10 (15), and this results in an attenuated activation response (Fig. 7D). Similarly, in the absence of FADD, other adapter molecules with death domains and death effector domains (22) may be inefficiently utilized to activate caspase-8 (Fig. 7E). These molecules may have lower affinity for procaspase-8 than FADD and/or are not expressed at a sufficiently high level in Jurkat cells.

In summary, our data suggest that the ability of NSAIDs to inhibit the activity of COX alone was insufficient to induce apoptosis. Moreover, the apoptotic activity of IND and SuS was unique among the NSAIDs tested, and their effect on Jurkat cells was mediated by an apparent death ligand-independent, but FADD-dependant mechanism.

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Induction of Apoptosis by Indomethacin

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