XIAP Regulates DNA Damage-induced Apoptosis Downstream of Caspase-9 Cleavage

The IAP (inhibitor of apoptosis) family of anti-apoptotic proteins regulates programmed cell death. Of the six known human IAP-related proteins, XIAP is the most potent inhibitor. To study the mechanistic effects of XIAP on DNA damage-induced apoptosis, we prepared U-937 cells that stably overexpress XIAP. The results demonstrate that XIAP inhibits apoptosis induced by 1-[β-D-arabinofuranosyl]cytosine (ara-C) and other genotoxic agents. XIAP had no detectable effect on ara-C-induced release of mitochondrial cytochrome c and attenuated cleavage of procaspase-9. In addition, we show that ara-C induces the association of XIAP with the cleaved fragments of caspase-9 and thereby inhibition of caspase-9 activity. The results also demonstrate that ara-C induces cleavage of procaspase-3 by a caspase-8-dependent mechanism and that XIAP inhibits caspase-3 activity. These results demonstrate that XIAP functions downstream of procaspase-9 cleavage as an inhibitor of both proteolytically processed caspase-9 and -3 in the cellular response to genotoxic stress.

Apoptosis is essential for normal development and homeostasis in multicellular organisms and provides a defense against transformation and viral invasion (1). The caspases, a family of intracellular cysteine proteases, are the central executioners of apoptosis (2). Activation of the caspase cascade is associated with proteolytic cleavage of diverse structural and regulatory proteins that collectively contribute to the apoptotic phenotype (2).

The treatment of cells with 1-[β-D-arabinofuranosyl]cytosine (ara-C) and other DNA-damaging agents is associated with induction of apoptosis (3–5). Although the precise signals responsible for the induction of apoptosis by genotoxic agents remain unclear, studies have shown that DNA damage-induced lethality is mediated, at least in part, through the activation of caspase-3 (6, 7). Other work has shown that protein kinase C (PKC)-δ and PKCθ are proteolytically cleaved and activated by caspase-3 during DNA damage-induced apoptosis (7, 8). In addition, the finding that expression of the baculoviral p35 IAP (inhibitor of apoptosis) protein blocks the apoptotic response of mammalian cells to genotoxic stress (6, 7) has suggested that eukaryotic homologs may have similar functions.

Six human IAP-like proteins have been identified and designated as XIAP, cIAP1, cIAP2, NIAP, survivin, and BRUCE (9–13). XIAP is the most potent of the IAP-related proteins (14). The available evidence indicates that IAP proteins suppress apoptosis by direct inhibition of caspases (14, 15). For example, studies have shown that XIAP, cIAP1, and cIAP2 inhibit caspase-9 and thereby block the proteolytic cleavage of caspase-3, -6, and -7 (15). By contrast, IAP proteins are reported to have no apparent effect on caspase-8-induced activation of procaspase-3 (16).

In this study, human U-937 cells were stably transfected to overexpress XIAP. The results demonstrate that overexpression of XIAP is associated with inhibition of apoptosis induced by ara-C and other genotoxic agents. The results also demonstrate that XIAP attenuates procaspase-9 cleavage and, in addition, inhibits caspase-9 activity. Furthermore, our findings support a role for XIAP downstream of caspase-9 as an inhibitor of proteolytically processed caspase-3.

**MATERIALS AND METHODS**

**Cell Culture and Transfections—**Human U-937 myeloid leukemia cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μg/ml 1-glutamine. The full-length myc-tagged XIAP cDNA was removed from pcDNA3-Myc-XIAP (provided by Dr. John Reed, Burnham Institute, La Jolla, CA) by digesting with HindIII-XhoI. Blunt-ended Myc-XIAP cDNA was cloned into the peF1-neo vector (17). U-937 cells were transfected by electroporation (Gene Pulsar, Bio-Rad; 0.25 V, 960 microfarads) with either the pEF1-neo plasmid (U-937/neo) or pEF1-neo containing Myc-XIAP (U-937/XIAP). Transfectants were selected in the presence of 400 μg/ml Geneticin sulfate. Limiting dilutions were carried out to obtain single cell clones. The two highest XIAP-expressing clones were used in all experiments. Cells were treated with 10 μM ara-C (Sigma), 30 ng/ml TNF (BASF Bioresearch Corp., Worcester, MA), or 5 μg/ml mouse anti-Fas monoclonal antibody (18). Irradiation was performed with a γ-ray source (32P, Gamma Cell 1000, Atomic Energy of Canada, Ltd., Ontario, Canada) at a fixed dose rate of 15 grays/min.

**Immunoblot Analysis—**Lysates were prepared by suspending cells in lysis buffer (50 mM Tris (pH 7.6), 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium vanadate, 1 mM diethiothreitol, 10 mM sodium fluoride, 10 μM/ml each leupeptin and aprotinin, and 1% Nonidet P-40). The lysates were cleared by centrifugation and subjected to electrophoresis on SDS-polyacrylamide gels. Immunoprecipitations were performed with anti-Myc monoclonal (9E10, Santa Cruz Biotechnology, Santa Cruz, CA), anti-cytochrome c (monoclonal clone 6H2.B4, Pharmingen, San Diego, CA), or anti-mouse IgG (Santa Cruz Biotechnology) antibody. Immunoblot analysis was performed with anti-Myc (Santa Cruz Biotechnology), anti-caspase-9 (raised against recombinant full-length human caspase-9; Pharmingen), anti-caspase-3 (17), anti-PC6 (raised against amino acids 657–676 of human PKCδ; Santa Cruz Biotechnology), anti-cytochrome c (monoclonal clone 6H8.2C12, Pharmingen), or anti-XIAP (monoclonal clone 48, Transduction Laboratories, Lexington, KY) antibody. The release of mitochondrial cytochrome c was assessed in soluble cytosolic fractions as described (19) using anti-cytochrome c antibody (monoclonal clone 7H8.2C12). Anti-
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FIG. 1. Effects of XIAP overexpression on DNA damage-induced DNA fragmentation. A, cell lysates from U-937 cells transfected with empty vector (U-937.neo) or vector containing XIAP (U-937/XIAP) were subjected to immunoblot analysis with anti-Myc (upper panel) or anti-XIAP (lower panel) antibody. B, cells were exposed to 10 μM ara-C for 4 h or treated with 20 grays of IR and harvested at 4 h. C, cells were treated with 30 ng/ml TNF or 5 μg/ml anti-Fas antibody for 6 h. DNA was analyzed for fragmentation on agarose gels.

In Vitro Assays for Caspase-9 and -3 Cleavage and Activation—S-100 cytosolic fractions were immunodepleted of cytochrome c by incubation with anti-cytochrome c antibody (monoclonal clone 6H2.B4) and protein A-Sepharose beads for 2 h at 4 °C (20). The immunodepletion procedure was repeated twice with centrifugation to remove the beads. [35S]Met-thionine-labeled caspase-9 was synthesized by the coupled transcription and translation method (Promega, Madison, WI). Caspase-9 was incubated with 10 μl of cytochrome c-free lysate, 1 mM dATP, and 1 μg of cytochrome c (Sigma) at 30 °C for 1 h in a final volume of 25 μl of buffer containing 50 mM HEPEs (pH 7.5), 10% glycerol, 2.5 mM dithiothreitol, and 0.25 mM EDTA. The reaction products were analyzed by 15% SDS-polyacrylamide gel electrophoresis and then autoradiography. Lysates from U-937.neo and U-937/XIAP cells before and after cytochrome c depletion were immunoblotted with anti-Apaf-1 (a gift from Xiaodong Wang), anti-XIAP, or anti-cytochrome c antibody. Assays to measure the activities of caspase-9 and -3 using LEHD-pNA or DEVD-pNA as substrate were performed as described (caspase-9 and -3 colorimetric assay kit, BioVision, Palo Alto, CA).

RESULTS

Previous studies have used U-937 cells as a model to study the mechanisms of genotoxic stress-induced apoptosis (5, 6, 7, 17). To study the role of XIAP in apoptosis, we stably transfected U-937 cells with a Myc-tagged XIAP cDNA inserted in the pEF-neo expression plasmid (17). Immunoblot analysis of transfectedants with anti-Myc or anti-XIAP antibody confirmed overexpression of XIAP (Fig. 1A) and no detectable change in cell numbers (data not shown). U-937.neo cells treated with ara-C and harvested at 4 h exhibited a pattern of DNA fragmentation characteristic of apoptosis (Fig. 1B). Similar findings were observed after ionizing radiation (IR) exposure (Fig. 1B). By contrast, overexpression of XIAP was associated with inhibition of ara-C- and IR-induced DNA cleavage (Fig. 1B). XIAP also inhibited apoptosis induced by TNF or anti-Fas antibody (Fig. 1C). Apoptosis was also monitored by analyzing cells for sub-G1 DNA content. The results confirmed that XIAP inhibits apoptosis induced by ara-C and IR (Table 1). Similar results were obtained for TNF- and anti-Fas antibody-induced apoptosis (Table 1). These findings demonstrate that, like treatment with TNF or anti-Fas antibody, overexpression of XIAP inhibits apoptosis induced by genotoxic agents.

Genotoxic agents induce apoptosis by signaling the release of mitochondrial cytochrome c (21, 22). To determine whether XIAP affects cytochrome c release, we treated cells with ara-C and subjected cytoplasmic lysates to immunoblotting with anti-cytochrome c antibody. The results demonstrate that overexpression of XIAP had little, if any, effect on ara-C-induced release of cytochrome c (Fig. 2A). Similar findings were obtained in IR-treated cells (Fig. 2A). In the cytoplasm, cytochrome c and dATP bind to Apaf-1 and promote autoproteolytic activation of procaspase-9 (23). Moreover, recent studies have demonstrated that XIAP blocks the processing of procaspase-9 in vitro (14). In this context, comparison of proenzyme levels in ara-C-treated U-937.neo and U-937/XIAP cells indicated that XIAP expression attenuated, in part, the processing of procaspase-9. Moreover, treatment of U-937.neo and U-937/XIAP cells with ara-C resulted in proteolytic processing of procaspase-9 with increases in the 35-kDa cleaved fragment (Fig. 2A). Similar results were obtained in IR-treated cells (Fig. 2A). Processing of procaspase-9 in XIAP-overexpressing cells was therefore analyzed at different times of ara-C exposure. The results demonstrate that procaspase-9 was cleaved to a 35-kDa fragment in both ara-C-treated U-937.neo and U-937/XIAP cells (Fig. 2B). However, XIAP expression blocked ara-C-induced apoptosis, even after prolonged exposure to this agent (Fig. 2 legand and Table 1). To provide further evidence that procaspase-9 is cleaved in XIAP-expressing cells, we performed an in vitro assay using cytochrome c-immunodepleted S-100 extracts from U-937.neo and U-937/XIAP cells. The cell extracts were incubated with in vitro translated, [35S]-labeled procaspase-9 in the presence or absence of dATP and cytochrome c. Lysates from the XIAP-overexpressing cells failed to inhibit cytochrome c/dATP-induced proteolytic processing of procaspase-9 to the 35- and 37-kDa fragments (Fig. 2C). Since levels of both XIAP and Apaf-1 can affect the processing of procaspase-9, cytochrome c-immunodepleted lysates were assayed for XIAP and Apaf-1 levels. Immunoblot analysis with anti-XIAP antibody demonstrated that XIAP was not immunodepleted by cytochrome c (Fig. 2D). Similar results were obtained for Apaf-1 (Fig. 2D). These findings demonstrate that overexpression of XIAP attenuates, but does not completely inhibit, the proteolytic processing of procaspase-9.

To determine whether XIAP inhibits caspase-9 activity, we

TABLE I

| Cell type   | Control | ara-C | IR | TNF | Anti-Fas Ab |
|-------------|---------|-------|----|-----|-------------|
| U-937.neo   | 6.9 ± 1.1 | 59.4 ± 4.5 | 40.3 ± 1.1 | 24.2 ± 3.5 | 20.8 ± 2.2 |
| U-937/XIAP | 5.2 ± 2.4 | 8.2 ± 1.9 | 11.6 ± 0.8 | 12.4 ± 1.8 | 11.3 ± 2.1 |
assayed for cleavage of the conjugated substrate LEHD-pNA. Incubation of cytochrome c-immunodepleted S-100 extracts from U-937/neo cells with cytochrome c and dATP was associated with induction of caspase-9 activity, whereas this activity was significantly inhibited in U-937/XIAP extracts (Fig. 3A). Since procaspase-9 can be activated in the absence of processing by binding to Apaf-1 (24, 25), we assayed for caspase-9 activity in ara-C-treated U-937/neo and U-937/XIAP cells. Lysates from U-937/neo cells exhibited an increase in LEHD-pNA cleavage activity at 4 and 6 h of ara-C exposure (Fig. 3B). By contrast, there was little, if any, induction of LEHD-pNA in ara-C-treated U-937/XIAP cells (Fig. 3B). These results demonstrate that overexpression of XIAP inhibits caspase-9 activity.

Previous studies have shown that caspase-9 proteolytically cleaves and activates procaspase-3 (23). Cleavage of procaspase-3 by caspase-9 generates p24 and p12 fragments (23, 26). The p24 large subunit is then further processed to p20 or p17 subunits (26). U-937/neo cells responded to ara-C exposure with proteolytic activation of procaspase-3 to p20 and p17 subunits and cleavage of the caspase-3 substrate PKCδ (Fig. 4A). By contrast, there was little, if any, cleavage of procaspase-3 to p20/p17 subunits or PKCδ in ara-C-treated U-937/XIAP cells (Fig. 4A). Similar effects were observed in IR-treated U-937/neo and U-937/XIAP cells (Fig. 4A). However, overexpression of XIAP failed to inhibit the processing of procaspase-3 to the p24 subunit (Fig. 4A). Since XIAP inhibits caspase-9 activity, other caspases with large prodomains such as caspase-1, -2, -8, and/or -10 might substitute for caspase-9. To assess the role of XIAP in preventing caspase-3 activation and apoptosis after longer exposures to genotoxic stress, we treated U-937 and U-937/XIAP cells with ara-C for up to 18 h. Immunoblot analysis of lysates from U-937/XIAP cells with anti-caspase-3 antibody revealed that cleavage of procaspase-3 to p24, p20, and p17 subunits was detectable at 8 h and maximal at 18 h (Fig. 4B). Caspase-3-mediated cleavage of PKCδ, however, was not observed in U-937/XIAP cells treated with ara-C (Fig. 4C). Analysis of cells with sub-G1 DNA content after longer exposures to ara-C confirmed that XIAP inhibited apoptosis (Fig. 4 legend and Table I). To further define the effect of XIAP on caspase-3 activity, we assayed lysates for their ability to cleave DEVD-pNA. U-937/neo cells responded to ara-C with induction of DEVD-pNA-cleaving activity (Fig. 4D). By contrast, there was little, if any, increase in DEVD-pNA cleavage in ara-C-treated U-937/XIAP cells (Fig. 4D). These in vivo and in vitro findings are in concert with the demonstration that XIAP inhibits the activity of processed caspase-3 fragments in vitro (15). In addition, our results demonstrate that, despite XIAP overexpression, exposure to genotoxic stress nonetheless results in delayed and attenuated processing of procaspase-3. However, these findings do not exclude the possibility that even higher levels of XIAP overexpression might completely block caspase-3 activity.

To explore the mechanisms responsible for caspase-3 processing in U-937/XIAP cells, we asked whether the association between XIAP and processed caspase-9 is reversible during exposure to genotoxic agents. Cell lysates from ara-C-treated U-937/XIAP cells were subjected to immunoprecipitation with anti-Myc antibody, and the precipitates were analyzed by immunoblotting with anti-caspase-9 antibody. The results demonstrate that association of XIAP with the cleaved fragment of

Fig. 2. Effects of XIAP on proteolytic cleavage of caspase-9. A, cells were treated with ara-C or IR as described in the legend to Fig. 1. Soluble cytosolic fractions were separated by SDS-polyacrylamide gel electrophoresis and analyzed by immunoblotting with anti-cytochrome c (Cyt C, upper panel), anti-caspase-9 (middle panel), or anti-actin (lower panel) antibody. B, U-937/XIAP cells were treated with 10 μM ara-C for the indicated times. Lysates were subjected to immunoblot analysis with anti-caspase-9 or anti-actin antibody. Cells were assessed for DNA content at the indicated times. The percentages of cells with sub-G1 DNA content were as follows: control, 6.2%; 6 h, 11.9%; 8 h, 13.8%; and 14 h, 19.6%. C, [35S]methionine-labeled procaspase-9 was incubated with the indicated cytochrome c-free cell lysates in the absence and presence of cytochrome c and dATP. The proteins were separated by SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography. D, lysates from U-937/neo and U-937/XIAP cells before and after cytochrome c depletion were immunoblotted with anti-Apaf-1, anti-XIAP, or anti-cytochrome c antibody.

Fig. 3. Effect of XIAP on activation of caspase-9. A, [35S]methionine-labeled procaspase-9 incubated with the indicated cytochrome c-free cell lysates in the absence and presence of cytochrome c and dATP was assayed for activity of endogenous and in vitro translated caspase-9 using conjugated LEHD-pNA as substrate. B, U-937/neo and U-937/XIAP cells were treated with 10 μM ara-C for the indicated times. Lysates were assayed for protease activity using LEHD-pNA as substrate.
caspase-9, but not procaspase-9, was detectable at 8 and 14 h of ara-C exposure (Fig. 5A). These findings, although in contrast to published results (14), further indicate that overexpression of XIAP fails to inhibit processing of procaspase-9 in vivo in the response to genotoxic stress. Immunoblot analysis of lysates before and after immunoprecipitation with anti-Myc antibody demonstrated the presence of both Myc-XIAP and the cleaved caspase-9 fragment (Fig. 5A). Nonetheless, the low level of caspase-9 activity in ara-C-treated U-937/XIAP cells (Fig. 4D) supports the presence of sufficient Myc-XIAP to bind and inhibit the cleaved subunits of caspase-9. The association of XIAP and the apoptosome complex (cytochrome c/Apaf-1/procaspase-9) was further analyzed by subjecting lysates from untreated and ara-C-treated U-937/XIAP cells to immunoprecipitation with anti-cytochrome c or anti-Myc antibody. Immunoblot analysis of the immunoprecipitates with anti-cytochrome c or anti-Myc antibody demonstrated no detectable coprecipitation of Myc-XIAP and cytochrome c (Fig. 5B). Taken together, these results indicate that XIAP binds to processed caspase-9, and not to the apoptosome complex.

Considerable evidence indicates that caspase-9 is a major, but not obligatory, activator of caspase-3. In this regard, anti-Fas or anti-CD3 antibody-induced apoptosis in caspase-9-deficient T-cells is mediated through caspase-3 (27). Also, caspase-8 proteolytically cleaves and activates procaspase-3 in the absence of mitochondrial signals (28). Recent studies have demonstrated that DNA damage-induced apoptosis can be mediated through expression of Fas ligand and activation of caspase-8 (29). Direct substrates of caspase-8 include Bid and procaspase-3 (30, 31). To assess activation of caspase-8, we subjected lysates from ara-C-treated U-937/neo and U-937/XIAP cells to immunoblotting with anti-Bid antibody. U-937/neo cells responded to ara-C with the cleavage of Bid at 4 h, and this effect was delayed in U-937/XIAP cells (Fig. 6, upper panel). Cleavage of Bid in U-937/XIAP cells was observed at 8 h after ara-C exposure and coincided with the proteolytic cleavage of procaspase-3 (Fig. 6, lower panel). Since Bid can also be cleaved by caspase-3, Bid cleavage in ara-C-treated U-937/neo cells can be attributed to the activities of both caspase-3 and -8.

Thus, given the absence of caspase-3 activity in U-937/XIAP cells (Fig. 4), these results support the involvement of Apaf-1-bound procaspase-9, caspase-8, or a caspase-8-like protease in the proteolytic processing of procaspase-3 in response to genotoxic stress.

DISCUSSION

This study thus demonstrates that XIAP functions as an inhibitor of apoptosis induced by diverse genotoxic agents. ara-C incorporates into DNA and functions as a relative chain
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**Fig. 6. Effects of XIAP on proteolytic cleavage of Bid.** U-937/neo and U-937/XIAP cells were treated with ara-C for the indicated times. Immunoblot analysis of the lysates was performed with anti-Bid (upper panel) or anti-caspase-3 (lower panel) antibody. Lysates from anti-Fas antibody-treated Jurkat cells were used as a positive control.

**Fig. 7. Model for regulation of DNA damage-induced caspase cascade by XIAP.** FasL, Fas ligand.

The results indicate that, in the presence of both types of DNA damage, XIAP has no detectable effect on cytochrome c release, but attenuates, in part, procaspase-9 cleavage. Indeed, other studies have demonstrated that XIAP inhibits procaspase-9 cleavage in vitro (14) and may exhibit cell type-specific effects. Although other studies have demonstrated that activation of procaspase-9 may not require cleavage, binding to Apaf-1 is essential (24, 25). Additional proteins in the complex could thus conceivably interfere with the ability of unprocessed procaspase-9 to assume an active configuration (as found with cleaved caspase-9) that is necessary for the XIAP interaction. In this context, XIAP blocked induction of caspase-9 activity found in the response of cells to DNA damage. In addition, the results demonstrate that DNA damage-induced caspase-3 activity is blocked in the XIAP-overexpressing cells.

This study also demonstrates that DNA damage-induced apoptosis is mediated by at least two interrelated pathways (Fig. 6). Treatment of U-937 cells with ara-C or IR was associated with the release of cytochrome c, cleavage of procaspase-9, and activation of caspase-3. In addition, both genotoxic agents induced, as later events, the activation of caspase-3. The observation that cytochrome c is released in the absence of Bid cleavage in ara-C-treated U-937/XIAP cells supports involvement of a Bid-independent mechanism in DNA damage-induced cytochrome c release. In this regard, recent studies have supported the involvement of a cytosolic factor other than Bid in caspase-6 and -7-induced cytochrome c release (34, 35). The finding that benzylxoycarbonyl-VAD-fluoromethyl ketone, a broad inhibitor of caspases, blocks etoposide-induced cleavage of Bid, but not release of cytochrome c, further supports the involvement of a Bid-independent mechanism in DNA damage-induced apoptosis (36). Overexpression of CrmA in U-937 cells has no effect on cytochrome c release, activation of caspase-3, or induction of apoptosis (6) supports a caspase-8-independent pathway that involves recognition of DNA lesions and transduction of signals to mitochondria (Fig. 7). Although the precise signals of the caspase-8-independent pathway are unclear, previous work has demonstrated that DNA damage activates JNK/SAPK (38), and recent studies have shown that SAPK translocates to mitochondria and induces the release of cytochrome c (39, 40).

**Acknowledgments**—We thank John Reed for providing pCNA3-Myc-XIAP cDNA, Xiaodong Wang for anti-Bid and anti-Apaf-1 antibodies, and Emad Alnemri for anti-caspase-3 antibody.

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