Apaf-1/Cytochrome c-independent and Smac-dependent Induction of Apoptosis in Multiple Myeloma (MM) Cells*

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Smac, a second mitochondria-derived activator of caspases, promotes caspase activation in the cytochrome c (cyto-c)/Apaf-1/caspase-9 pathway. Here, we show that treatment of multiple myeloma (MM) cells with dexamethasone (Dex) triggers the release of Smac from mitochondria to cytosol and activates caspase-9 without concurrent release of cyto-c and Apaf-1 oligomerization. Smac binds to XIAP (an inhibitor of apoptotic protein) and thereby, at least in part, eliminates its inhibitory effect on caspase-9. Interleukin-6, a growth factor for MM, blocks Dex-induced apoptosis and prevents release of Smac. Taken together, these findings demonstrate that Smac plays a functional role in mediating Dex-induced caspase-9 activation and apoptosis in MM cells.

The cellular response to diverse classes of stress inducers includes growth arrest and activation of apoptosis. Apoptosis is triggered through a controlled program that is associated with distinctive morphological changes, including membrane blebbing, cytoplasmic and nuclear condensation, chromatin aggregation, and formation of apoptotic bodies (1). The induction of apoptosis involves a cascade of initiator and effector caspases that are activated sequentially (2, 3). Caspases, a family of cysteine proteases with aspartate substrate specificity, are present in cells as catalytically inactive zymogens (2). Effector caspases, such as caspase-3, are activated by initiator caspases, such as caspase-9. Once activated, the effector caspases induce proteolytic cleavage of various cellular targets, inducing poly-ADP-ribose polymerase (4, 5), DNA-dependent protein kinase, protein kinase C-δ, and other substrates (6), ultimately leading to cell death.

Recent studies have shown that the inhibitor of apoptosis (IAP) family of proteins suppresses apoptosis by directly binding to and inhibiting caspases (7, 8). For example, XIAP, c-IAP-1, and c-IAP-2 bind to procaspase-9 and prevent its activation (9), thereby blocking the downstream apoptosis-related events such as proteolytic cleavage of caspase-3, -6, and -7 (10).

One of the major caspase cascades is triggered by the release of mitochondrial apoptogenic protein, cytochrome c (cyto-c) (11–13). Cytosolic cyto-c binds to the CED-4 homolog Apaf-1 and induces caspase-9-dependent activation of caspase-3 (14–17). Recent studies have identified another important regulator of apoptosis, Smac (second mitochondria-derived activator of caspase) or DIABLO, which is released from mitochondria into the cytosol during apoptosis (18–20) and functions by eliminating inhibitory effects of IAPs on caspases (20, 21).

Our prior study demonstrated that dexamethasone (Dex)-induced apoptosis is independent of cyto-c release and associated with caspase-3 activation (22, 23). In the present study, we examined the upstream signaling leading to caspase-3 activation. The results demonstrate that Dex-induced apoptosis in MM cells is mediated by Smac, which activates caspase-9 by binding to and inhibiting XIAP. Interleukin-6 (IL-6), a growth factor for MM, blocks Dex-induced release of Smac and apoptosis. Taken together, this study provides evidence for an Apaf-1-cyto-c-independent pathway mediating caspase-9 activation via Smac. Moreover, these findings also demonstrate a functional role of Smac in IL-6-mediated block during Dex-induced apoptosis.

MATERIALS AND METHODS

Cell Culture and Reagents—Human MM.1S (Dex-sensitive) and MM.1R (Dex-resistant) multiple myeloma cells (22, 24) were grown in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Mononuclear cells were isolated from a patient with MM (PCL cells) by Ficoll-Hypaque density gradient centrifugation and incubated with HB-7 (anti-CD38) mAb-biotin-streptavidin and 2H4 (anti-CD45RA) were isolated using an Epics C cell sorter (Coulter Electronics, Hialeah, FL), washed, and resuspended in regular growth media. Cells were treated with 10 μM Dex (Sigma) in the presence or absence of 100 ng/ml of IL-6. γ-Radiation (IR) was performed as described previously (22). Cells were also treated with anti-Fas as described previously (22).

Preparation of Cytosolic and Mitochondrial Extracts from MM.1S, MM.1R, and MM Patient Cells—MM.1S or PCL cells were washed twice with PBS, and the pellet was suspended in 3 volumes of ice-cold buffer A (20 mM HEPES, pH 7.5, 1.5 mM MgCl2, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and aprotinin and pepstatin A) containing 250 mM sucrose. The cells were homogenized using a Dounce homogenizer, and cytosolic or mitochondrial extracts were isolated as described previously (12, 25).

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The abbreviations used are: IAP, inhibitor of apoptosis; Smac, second mitochondria-derived activator of caspases; Apaf-1, apoptotic protease-activating factor-1; IR, ionizing radiation; cyto-c, cytochrome c; MM, multiple myeloma; Dex, dexamethasone; IL-6, interleukin 6; PAG, polyacrylamide gel electrophoresis; Ab, antibody; mAb, monoclonal antibody; HO, Hoechst 33342; PI, propidium iodide; IB, immunoblotting; FMK, fluoromethylketone; pNA, p-nitroanilide.
Western Blot Analysis—Proteins were separated from cell lysates by SDS-PAGE, transferred to nitrocellulose, and probed with anti-cyto-c (22), anti-Smac (kindly provided by Dr. Xiaodong Wang), anti-tubulin (Sigma), anti-Hsp60 (Stressgen, Victoria, British Columbia, Canada), anti-XIAP (Transduction Laboratories), as well as anti-caspase-9, anti-caspase-8, and anti-caspase-3 (PharMingen) Abs. The blots were developed by enhanced chemiluminescence (ECL) using the manufacturer’s protocol (Amersham Pharmacia Biotech).

Transient Transfections—MM.1S cells were transiently cotransfected with FLAG-Apaf-1 and T7-Apaf-1 using Superfect™ (Qiagen, Santa Clarita, CA) and treated with 10 μM Dex for 24 h. Lysates from transfectants were then incubated with dATP and subjected to immunoprecipitation with anti-FLAG M2 (Eastman Kodak Co.). The immunoprecipitates were then analyzed by immunoblotting with anti-T7 (Novegen, Madison, WI) or anti-FLAG. MM.1S cells were also transiently transfected with pcDNA3-Myc-XIAP vector (26). Lysates were subjected to immunoprecipitation with anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), and the immunoprecipitates were then analyzed by immunoblotting with anti-caspase-9, anti-Smac, or anti-XIAP antibodies.

Caspase Activity Assays—Caspase-9 activation was performed using LEHD-pNA as a substrate, as per the manufacturer’s instructions (colorimetric assay kit, Biovision, Palo Alto, CA). MM.1S MM cells were also treated with Dex (10 μM) in the presence or absence of caspase-9 inhibitor LEHD-FMK (5 μM) for 24 h and then analyzed for apoptosis.

Quantification of Apoptosis—Flow cytometric analyses: dual fluorescent staining with DNA-binding fluorochromes Hoechst 33342 (HO) and propidium iodide (PI) was used to quantitate the percentage of apoptotic (HO+PI-) cells using flow cytometry (The Vantage, Becton Dickinson), as described previously (24). DNA fragmentation assays were also performed as described previously (24).

RESULTS AND DISCUSSION

To determine whether Dex-induced apoptosis in MM cells is associated with the release of Smac, MM.1S MM cells were treated with Dex for various times, and cytosolic and mitochondrial extracts were analyzed for the levels of Smac. The results demonstrate that Dex treatment is associated with an increase in Smac levels in the cytosol at 24 and 48 h, with a concomitant decrease in mitochondrial Smac levels (Fig. 1A). Dex-induced increase in cytosolic Smac and a corresponding decrease in mitochondrial Smac levels were specific, because no change was observed in the levels of tubulin protein and mitochondrial matrix protein, Hsp60, respectively (Fig. 1A). Similar results were obtained when patient MM cells were exposed to Dex (Fig. 1B). These results suggest that Dex-induced apoptosis is accompanied by accumulation of Smac in the cytosol.

Smac is known to promote caspase activation in the cyto-c/Apaf-1/caspase-9 pathway; therefore, we next examined the release of cyto-c triggered by Dex or IR in MM.1S MM cells. The cytosolic extracts from Dex- or IR-treated cells were subjected to immunoblot analyses with anti-cyto-c and anti-Smac. As in our previous findings (22), treatment of MM.1S MM cells with Dex did not induce release of cyto-c in the cytosol (Fig. 1C, upper panel); in contrast, γ-radiation (IR) stimulated the release of cyto-c (Fig. 1C, upper panel), demonstrating that the release of cyto-c is functional in these cells. To assay for Smac release these immunoblots were then stripped and reprobed with anti-Smac. As seen in Fig. 1C, both Dex and IR induced release of Smac in the cytosol. Furthermore, low to undetectable cytosolic Smac or cyto-c levels were observed in the untreated cells. Reprobing the immunoblots with anti-tubulin confirms equal protein loading (Fig. 1C).

Since Dex-induced apoptosis is associated with Smac release, but not cyto-c release, we next determined whether Apaf-1 oligomerization is required for Smac-related signaling. For these experiments, we utilized the same MM.1S MM cells model (22) to determine whether IR or Dex induce Apaf-1 oligomerization. Cells were transiently cotransfected with FLAG-Apaf-1 and T7-Apaf-1 or empty vector and treated with Dex or IR. Cell lysates were incubated with dATP. As shown in Fig. 1D, IR (lane 3), but not Dex (lane 2), induces Apaf-1 oligomerization in MM.1S MM cells (Fig. 1D, lane 3). The finding that IR induces Apaf-1 oligomerization in MM.1S MM cells indicates that the Apaf-1 oligomerization system is functionally intact and served as a positive control. Taken together, these findings suggest that Dex-induced apoptosis in MM cells is mediated by Smac and is independent of cyto-c/Apaf-1 mechanism.

To examine whether Dex-induced Smac release and apoptosis are associated with processing of caspase-9, the cytosolic extracts from Dex-treated cells were subjected to immunoblot analysis with anti-caspase-9. The results demonstrate that treatment of MM.1S cells with Dex induces proteolytic cleavage of procaspase-9 into 37- and 35-kDa fragments (Fig. 2A, upper panel). Reprobing the immunoblot with anti-tubulin confirms equal protein loading (Fig. 2A, lower panel). We next assayed for catalytic activity of caspase-9 using LEHD-pNA conjugated substrate in a colorimetric protease assays (26). Incubation of cytosolic extracts from Dex-treated MM.1S cells with LEHD-pNA was associated with efficient cleavage of LEHD-pNA (Fig. 2B). Although LEHD-pNA may be cleaved by caspasases other than caspase-9, our study indicates that activated caspase-8 in MM.1S MM cells does not cleave this substrate (Fig. 2B), further supporting its specificity for caspase-9. Taken together, these findings demonstrate that treatment of MM.1S cells with Dex is associated with activation of caspase-9.

We next asked whether caspase-9 activation is an obligatory event during Dex-induced apoptosis. MM.1S MM cells were

Fig. 1. Dex induces the release of Smac in the cytosol. MM.1S (A) or MM patient cells (PCL) (B) were treated with 10 μM Dex and harvested at the indicated times. Cytosolic and mitochondrial extracts were separated by 12.5% SDS-PAGE and analyzed by immunoblotting with anti-Smac (cytosolic Smac, first panels; mitochondrial Smac, third panels). As a control, filters were also analyzed by immunoblotting (IB) with anti-tubulin (second panels) or with anti-Hsp60 (fourth panels). C, MM.1S cells were treated with Dex or IR and harvested at the indicated times. Cytosolic extracts were separated by 12.5% SDS-PAGE and analyzed by IB with anti-cyto-c (upper panel), anti-Smac (middle panel), or anti-tubulin (lower panel). D, effects of Dex or IR on Apaf-1 oligomerization. MM.1S cells were transiently transfected with FLAG-Apaf-1 (lanes 2 and 3) and T7-Apaf-1 (lanes 1 and 3) and treated with Dex (lane 2) or IR (lane 3). Cytosolic extracts were subjected to immunoprecipitation with anti-FLAG. The immunoprecipitates were then analyzed by IB with anti-T7 (upper panel) or anti-FLAG (lower panel).
cultured with Dex in the presence or absence of caspase-9 tetrapeptide inhibitor LEHD-FMK for 24 h and then assayed for proteolytic cleavage of caspase-9 and caspase-3. LEHD-FMK abrogates Dex-induced cleavage of both caspase-9 and caspase-3 (Fig. 2C, left and right panel). In contrast, LEHD-FMK did not inhibit anti-Fas-induced caspase-8 or caspase-8-mediated caspase-3 cleavage in MM.1S MM cells (data not shown), further indicating the selectivity of LEHD-FMK for caspase-9. We next determined whether blocking caspase-9 activation affects Dex-induced apoptosis. MM.1S MM cells were cultured with Dex in the presence or absence of caspase-9 inhibitor LEHD-FMK for 24 h and then assayed for apoptosis using flow cytometric analysis with PI and HO dual staining to determine the percentage for PI- and HO+ apoptotic cells. Dex-induced apoptosis (51 ± 3% apoptotic cells (n = 3)) was significantly inhibited in cells pretreated with caspase-9 inhibitor (27 ± 2% apoptotic cells (n = 3)) (Fig. 2D). Other studies have demonstrated that caspase-9 proteolytically cleaves and activates procaspase-3 (14). In that context, our previous studies have shown that Dex triggers caspase-3 activation in MM.1S MM cells (22, 23). Taken together, these results suggest that Dex induces sequential activation of Smac → caspase-9 → caspase-3 and is independent of cyto-c/Apaf-1 apoptosome complex formation.

We next determined the mechanism of Smac-mediated Dex-induced caspase-9 activation. Two potential mechanisms for caspase-9 activation have been suggested (18). First, the release of cyto-c leads to Apaf-1 oligomerization, which then activates caspase-9; second, Smac interacts with IAPs (inhibitors of apoptosis proteins), such as X-chromosome-linked IAP (XIAP), and eliminates the inhibitory effects of IAPs on caspase-9 (27). Since Dex-induced apoptosis is not associated with cyto-c release or Apaf-1 multimerization, we asked whether XIAP interacts with Smac during Dex-induced apoptosis. MM.1S MM cells were transiently transfected with Myc-XIAP and treated with Dex for 24 h. Cytosolic extracts were subjected to immunoprecipitation with anti-Myc and immunoblotting with anti-caspase-9, anti-Smac, or anti-XIAP. As

**Fig. 2.** Dex induces activation of caspase-9. A, MM.1S cells were treated with 10 μM Dex and harvested at 24 and 48 h. Cytosolic extracts were separated by 12.5% SDS-PAGE and analyzed by IB with anti-caspase-9 (Cas-9) (upper panel) or anti-tubulin (lower panel). B, MM.1S cells were treated with 10 μM Dex and harvested at the indicated times. Cytosolic extracts were assayed for protease activity using LEHD-pNA as substrate. As a control for LEHD-pNA, cytotoxic extracts from anti-Fas-treated MM.1S MM cells containing activated caspase-8 were also subjected to protease assays. Results are representative of three independent experiments (mean ± S.D., n = 3). C, MM.1S cells were treated with 10 μM Dex in the presence or absence of LEHD-FMK for 24 h. Cytosolic extracts were separated by 12.5% SDS-PAGE and analyzed by IB with anti-caspase-9 (left panel) or anti-caspase-8 (Cas-3) (right panel). D, MM.1S cells were treated with 10 μM Dex in the absence or presence of LEHD-FMK for 24 h, and the percent of apoptotic cells was obtained using flow cytometry. Results are representative of three independent experiments (mean ± S.D., n = 3). E, MM.1S cells were transiently transfected with Myc-XIAP and treated with Dex for 24 h. Cytosolic extracts were subjected to immunoprecipitation with anti-Myc and IB with anti-caspase-9 (upper panel), anti-Smac (middle panel), or anti-XIAP (lower panel).
shown in Fig. 2E, Dex treatment induces an interaction between XIAP and Smac. Importantly, Dex treatment also leads to dissociation of XIAP from caspase-9 (Fig. 2E). Equivalent levels of transfected XIAP protein were confirmed by reprobing the filters with anti-XIAP (Fig. 2E). These findings are in concert with other studies demonstrating that Smac promotes caspase activity of initiator caspase-9 by binding to and inhibiting IAPs (7, 20, 21).

To explore the functional significance of Smac release during Dex-induced apoptosis, we utilized IL-6, a known inhibitor of Dex-triggered apoptosis in MM cells. IL-6 abrogates Dex-induced apoptosis in MM cells and inhibits Smac release, thereby conferring Dex-resistance. Furthermore, IL-6 blocks Dex-induced apoptosis in MM cells and inhibits Smac release, thereby conferring Dex-resistance. Taken together, these findings provide evidence for a Smac-dependent activation of caspase-9 and apoptosis, independent of Apaf-1/cyto-c (Fig. 3D), and suggest therapeutic strategies based upon targeting both Smac and XIAP.

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