JNK-dependent Release of Mitochondrial Protein, Smac, during Apoptosis in Multiple Myeloma (MM) Cells*

Received for publication, February 17, 2003, and in revised form, March 18, 2003
Published, JBC Papers in Press, March 28, 2003,
DOI 10.1074/jbc.C300076200

Dharminder Chauhan, Guilan Li, Teru Hideshima, Klaus Podar, Constantine Mitsiades, Nicholas Mitsiades, Nikhil Munshi, Surender Kharbanda‡, and Kenneth C. Anderson§
From The Jerome Lipper Multiple Myeloma Center, Department of Medical Oncology, Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

Smac, second mitochondria-derived activator of caspases, promotes apoptosis via activation of caspases. Previous studies have shown that c-Jun NH2-terminal kinase (JNK) is involved in regulating another mitochondrial protein, cytochrome c during apoptosis; however, the role of JNK in the release of mitochondrial Smac is unknown. Here we show that induction of apoptosis in multiple myeloma (MM) cells is associated with activation of JNK, translocation of JNK from cytosol to mitochondria, and release of Smac from mitochondria to cytosol. Blocking JNK either by dominant-negative mutant (DN-JNK) or cotreatment with a specific JNK inhibitor, SP600125, abrogates both stress-induced release of Smac and induction of apoptosis. These findings demonstrate that activation of JNK is an obligatory event for the release of Smac during stress-induced apoptosis in MM cells.

The cellular response to diverse classes of stress inducers includes growth arrest and induction of apoptosis. Apoptosis is triggered through a controlled program that is associated with distinct 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 aspartic substrate specificity, are present in cells as catalytically inactive zymogens (2). Once activated, the effector caspases induce proteolytic cleavage of various cellular targets, including poly(ADP-ribose) polymerase (PARP) (4), DNA-dependent protein kinase, protein kinase C-δ (2), and other substrates, ultimately leading to cell death.

One of the major caspase cascades is triggered by the release of mitochondrial apoptotic protein, cytochrome c (cyto-c) (5, 6). Cytosolic cyto-c binds to the CED-4 homolog Apaf-1 and induces caspase-9-dependent activation of caspase-3 (7). 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 (8, 9) and functions by eliminating inhibitory effects of inhibitor of apoptosis proteins on caspase-9 (9, 10). Our prior studies showed that 2ME2-induced apoptosis in MM cells involves release of both cyto-c and Smac (11). However, the upstream signal that triggers the 2ME2-induced mitochondrial apoptotic pathways in MM cells is unclear.

A recent study has shown that stress-activated protein kinase (SAPK) or c-Jun NH2-terminal Kinase (JNK) mediates the release of cyto-c during apoptosis (12). JNK has been linked to apoptosis (13–15). Specifically, two serine residues (Ser62 and Ser73) in the amino-terminal transactivation domain of c-Jun are substrates for JNK (13, 14), and previous studies have shown that stress stimuli (e.g. irradiation, tumor necrosis factor, sphingomyelinase, and UV light) activate JNK (13–15). In the present study, we show that 2ME2-induced apoptosis in MM cells is, at least in part, mediated by JNK activation, and JNK-dependent release of Smac from mitochondria to cytosol. Our findings provide the first evidence for the requirement of JNK in triggering Smac-mediated apoptosis.

EXPERIMENTAL PROCEDURES

Cell Culture and Reagents—Human MM.1S MM cells (11) were grown in RPMI 1640 medium 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) MoAb-biotin-streptavidin and HB-2H4 (anti-CD45RA) MoAb-fluorescein isothiocyanate on ice. Tumor cells (96 ± 2% CD38 + 45RA-) were isolated using an Epics C cell sorter (Coulter Electronics, Hialeah, FL), washed, and resuspended in regular growth medium. Cells were treated with 3 μM of 2ME2, or 10 nM proteasome inhibitor PS-341, as described previously (11, 16). Cells were also treated with 3 μM 2ME2 in the presence or absence of JNK-specific inhibitor SP600125 (17).

Apoptotic Assays—Dual fluorescence staining with DNA-binding fluorochrome Hoechst 33342 and propidium iodide was used to quantitate the percentage of apoptotic (propidium iodide-Hoechst 33342+) cells by flow cytometry (The Vantage, BD Biosciences), as described previously (11).

In Vitro Immune Complex Kinase Assays—In vitro immune complex c-Jun or ATF2 kinase assays were performed as described previously (18).

Preparation of Cytosolic and Mitochondrial Extracts from MM.1S and MM Patient Cells—MM.1S or patient MM cells were washed twice with phosphate-buffered saline, 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, and 10 μg/ml leupeptin, aprotinin, and pepstatin A) containing 250 nM sucrose. The cells were homogenized with a Dounce homogenizer, and cytosolic or mitochondrial extracts

* This work was supported by National Institutes of Health Grants 50947 and CA 78375, a Doris Duke Distinguished Clinical Research Scientist Award (to K. C. A.), The Myeloma Research Fund, and The Cure Myeloma Fund. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
‡ Present address: ILEX Oncology Inc., Boston, MA 02215.
§ To whom correspondence and reprint requests should be addressed: Dana-Farber Cancer Inst., 44 Binney St., Boston, MA 02215. Tel.: 617-632-2144; Fax: 617-632-2140; E-mail: kenneth_anderson@dfci.harvard.edu.

1 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; Smac, second mitochondria-derived activator of caspases; cyto-c, cytochrome c; MM, multiple myeloma; 2ME2, 2-methoxyestradiol; PS-341, proteasome inhibitor-341; SAPK, stress-activated protein kinase; JNK, c-Jun NH2-terminal kinase; Ab, antibody; MoAb, monoclonal Ab; DN, dominant-negative; GFP, green fluorescence protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, GST, glutathione S-transferase.

This paper is available on line at http://www.jbc.org

17593
Western Blot Analysis—Proteins were separated from cell lysates by SDS-PAGE, transferred to nitrocellulose, and probed with anti-Smac (kindly provided by Dr. Xiaodong Wang, University of Texas Southwestern Medical Center, Dallas), anti-cyto-c (Sigma), anti-Hsp60 (Stressgen), anti-phospho-specific JNK (New England Biolabs), as well as anti-PARP (Pharmingen), or anti-JNK and anti-SHP2 (Santa Cruz Biotechnology, Santa Cruz, CA) Abs. The blots were developed by enhanced chemiluminescence (ECL), using the manufacturer’s protocol (Amersham Biosciences).

Transient Transfections—MM.1S cells were transiently transfected using “cell line Nucleofect™ kit V,” according to manufacturer’s instructions (Amaxa Biosystems), with vector alone or DN-JNK and cotransfected with vector containing green fluorescence protein (GFP) alone. Following transfections, GFP-positive cells were selected by flow cytometry, treated with 2ME2 (3 μM) and analyzed for cell viability as described above. Additionally, cytosolic extracts from these cells were also analyzed for the accumulation of Smac by Western blotting as described previously.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide) Assays—Cell viability was assessed by MTT (Chemicon International Inc., Temecula, CA) assay (Roche Molecular Biochemicals), and trypan blue exclusion, as described previously (11).

Analysis of Mitochondrial Membrane Potential (ΔΨm) Dissipation—MM.1S cells were treated with 2ME2 in the presence or absence of JNK inhibitor (SP600125) for 12 h, harvested, and analyzed for mitochondrial membrane potential (ΔΨm). 2ME2 or dexamethasone-treated MM cells were stained with lipophilic cationic dye CMXRos (MitoTracker Red) (Molecular Probes, Eugene, OR) in phosphate-buffered saline for 20 min at 37 °C and analyzed by flow cytometry to assay for alterations in ΔΨm.

RESULTS AND DISCUSSION

To examine whether 2ME2 induces apoptosis in the MM.1S MM cells, we performed flow cytometric analysis using propidium iodide and Hoechst staining. Treatment of cells with 3 μM 2ME2 for 24 h significantly increased the percentage of apoptotic cells (51% ± 3.6%, p < 0.005, n = 3), as in our prior studies (11). Previous studies have shown that activation of JNK plays an important role in apoptosis (14, 15). To examine whether 2ME2 induces activation of JNK, MM.1S MM cells were exposed to 2ME2 for various time intervals, and JNK activity was analyzed by in vitro immune complex kinase assays using GST-Jun as a substrate. Analysis of anti-JNK immunoprecipitates demonstrated significant (8–10-fold) increase in GST-Jun phosphorylation, indicating the activation of JNK (Fig. 1A, upper left panel). Moreover, cotreatment of MM.1S cells with SP600125, a specific inhibitor of JNK (17), blocks 2ME2-induced JNK activity (Fig. 1A, upper left panel). This activation of JNK in response to 2ME2 does not alter JNK protein levels (Fig. 1A, lower left panel). Increase in JNK activity was detectable as early as 1 h after 2ME2 treatment and peaked at 12 h/24 h (Fig. 1A, upper right panel). Our findings are in concert with those of Verheij et al. (15), which demonstrate a role of JNK in ceramide- and tumor necrosis factor-α-induced apoptosis. Moreover, coordinate regulation of JNKs, p38, and extracellular signal-regulated kinase (ERK) kinases facilitates apoptosis induced by withdrawal of growth factor, suggesting that activation of JNK may be essential for apoptosis (20). We next examined the mechanism whereby JNK mediates its apoptotic effects during 2ME2-induced apoptosis. Translocation of JNK from cytosol to the nucleus has been shown in response to osmotic stress (21). Other studies showed that JNK translocates to mitochondria after genotoxic stress and inhibits the anti-apoptotic function of proteins belonging to Bcl2 family members (22, 23), thereby allowing the release of mitochondrial apoptogenic proteins to cytosol and subsequent activation of caspase cascades. We next therefore determined whether 2ME2 induces translocation of JNK to mitochondria or nucleus in MM cells. Cytosolic, mitochondrial, and nuclear extracts from untreated and 2ME2-treated MM.1S cells were assayed for the JNK protein levels. As seen in Fig. 1B (upper panel), 2ME2 significantly increases JNK protein level in mitochondrial
fraction (4–5-fold, as assessed by densitometry). Purity of mitochondria was confirmed by reploting the blots with an antibody against mitochondrial-specific Hsp60 protein (Fig. 1B, lower panel). No increases in JNK protein levels in the nuclear fractions were detected in 2ME2-treated cells (data not shown). Together, these findings demonstrate that 2ME2-induced apoptosis is associated with both activation of JNK and its translocation to mitochondria. These results suggest a potential involvement of mitochondria during 2ME2-induced apoptosis in MM cells.

Mitochondria harbor two key modulators of apoptosis, cyto-c and Smac (second mitochondria-derived activator of caspase) or DIABLO, which are released from mitochondria into the cytosol during apoptosis (5, 6, 8, 9, 24). The upstream signal(s) that triggers the release of these proteins from mitochondria to cytosol is unclear. A recent study showed that activated JNK is required for the cyto-c release and associated apoptosis (12); however, the influence of JNK signaling on the release of Smac is presently undefined. Given that 2ME2 activates JNK in MM cells and JNK translocates to mitochondria, we next assessed whether 2ME2 induces the release of Smac and whether 2ME2-activated JNK mediates the release of Smac. MM.1S cells were treated with 2ME2, in the presence of absence of JNK inhibitor SP600125; cytosolic extracts were prepared and subjected to immunoblot analyses with anti-Smac Ab. As seen in Fig. 1C (upper panel), 2ME2 induces the release of Smac, as in our previous study (11). Importantly, cotreatment with SP600125 significantly inhibits the release of Smac (Fig. 1C, upper panel). Repploting the immunoblots with anti-SHP2 (as control) confirms equal protein loading (Fig. 1C, lower panel). These findings demonstrate that 2ME2-induced JNK activation is required for the mitochondrial release of Smac. Examination of the effects of 2ME2 on the release of cyto-c, as well as requirement of JNK for the release of cyto-c, demonstrated similar results (Fig. 1D). To further address this issue, we transiently transfected MM.1S cells with either dominant-negative mutant of JNK (pEBG-DN-JNK) or control vector; following treatment with 2ME2, cytosolic extracts were analyzed for accumulation of Smac in the cytosol. As seen in Fig. 1E (first panel), overexpression of DN-JNK abrogates the 2ME2-induced release of Smac from mitochondria to cytosol. To confirm the expression of endogenous JNK and exogenous DN-JNK (GST-DN-JNK), filters were reprobed with anti-JNK antibody (Fig. 1E, second panel). Immunoblotting with phospho-specific JNK antibody showed a marked decrease in 2ME2-induced JNK activity in cells transfected with DN-JNK compared with empty vector (Fig. 1E, third panel), which confirmed the function of DN-JNK. Immunoblotting with anti-GFP and anti-SHP2 Abs demonstrated equal transfection efficiency and protein load-
ing, respectively (Fig. 1E, fourth and fifth panels). To further confirm the specificity of DN-JNK, we examined lysates from DN-JNK transfected cells for both p38 MAPK and JNK activity using GST-ATF2 and GST-Jun as substrates, in an in vitro immune complex kinase assay. DN-JNK blocked 2ME2-induced JNK, but not p38 MAPK activity (data not shown). Taken together, these results demonstrate that JNK is required for the release of Smac during 2ME2-induced apoptosis in MM cells.

We next determined the functional significance of 2ME2-induced JNK activation. MM.1S cells were transiently transfected with either DN-JNK or empty vector and treated with 2ME2. As seen in Fig. 2A, after treatment with 2ME2, MM.1S DN-JNK transfectants survived significantly longer than cells transfected with vector alone: median viability was 48% (24 h) and 24% (48 h) after 2ME2 treatment of empty vector transfected cells versus 73% (24 h) and 45% (48 h) after 2ME2 treatment of DN-JNK transfected MM.1S cells (p = 0.05, as determined by one-sided Wilcoxon rank-sum test). Similar results were obtained in MM.1S cells treated with 2ME2 in the presence or absence of JNK inhibitor (Fig. 2B). As a control for specificity of a JNK inhibitor SP600125, we also treated cells with 2ME2 in the presence or absence of p38 MAPK inhibitor. As seen in Fig. 2B, in contrast to SP600125, p38 MAPK inhibitor does not attenuate 2ME2-induced cytotoxicity. Moreover, blockade of JNK also inhibits 2ME2-induced PARP cleavage, a signature event during apoptosis (Fig. 2C). Other studies have reported that a decrease in mitochondrial membrane potential (ΔΨm) causes release of Smac and cyto-c (5, 9), and we therefore asked whether 2ME2-induced apoptosis correlates with changes in ΔΨm, and whether inhibition of JNK affects the ΔΨm. As seen in Fig. 2D, 2ME2 triggers a significant decrease in ΔΨm, and conversely, cotreatment with JNK inhibitor SP600125 prevents 2ME2-induced reduction in ΔΨm. Together, these data suggest that blockade of JNK decreased 2ME2-induced apoptosis. Our prior study demonstrated that 2ME2-induced apoptosis is associated with activation of caspase-8 and -9 (11), and both are known to activate downstream mitochondrial proteins during apoptosis (23); however, not all apoptotic agents trigger a sequential activation of JNK and its requirement for the release of mitochondrial proteins is still unknown.

The observation that 2ME2 induces JNK activation in MM.1S cells suggest that JNK signaling is not defective in these cells. This is in concert with other studies demonstrating that anti-Fas-induced apoptosis is associated with the release of cyto-c in JNK-deficient mouse embryonic fibroblast cells, whereas UV-induced apoptosis in these same cells requires JNK activation for release of cyto-c (12). These findings indicate that both activation of JNK and its requirement for the release of mitochondrial proteins are stimuli-specific.

Collectively, our present study shows that two distinct apoptotic agents in MM cells induce JNK, which upon activation translocates to mitochondria and triggers the release of Smac. Inhibition of JNK activation by either DN-JNK or a specific inhibitor of JNK disables JNK-mediated Smac release and associated apoptosis. Finally, our findings have important biologic and therapeutic implications. Given that synthetic Smac peptides enhance the apoptotic activity of chemotherapeutic agents (27), coupled with our present results showing that anti-MM drugs induce apoptosis via release of mitochondrial Smac, suggest that Smac agonists or active Smac peptides may sensitize MM cells to 2ME2 or PS-341-induced cell death.

Acknowledgments—We thank Dr. Xiaodong Wang for providing Smac-related reagents and helpful suggestions, as well as J. Kyriakis and L. Zon for SAPK cDNA constructs.

REFERENCES

JNK-dependent Release of Mitochondrial Protein, Smac, during Apoptosis in Multiple Myeloma (MM) Cells
Dharminder Chauhan, Guilan Li, Teru Hideshima, Klaus Podar, Constantine Mitsiades, Nicholas Mitsiades, Nikhil Munshi, Surender Kharbanda and Kenneth C. Anderson

doi: 10.1074/jbc.C300076200 originally published online March 28, 2003

Access the most updated version of this article at doi: 10.1074/jbc.C300076200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 27 references, 16 of which can be accessed free at http://www.jbc.org/content/278/20/17593.full.html#ref-list-1