Communication

Cytochrome c-dependent and -independent Induction of Apoptosis in Multiple Myeloma Cells*

(Received for publication, August 27, 1997, and in revised form, October 7, 1997)

Dharminder Chaunhan‡, Pramod Pandey§, Atshushi Ogata‡, Gerrard Teoh¶, Nancy Krett¶, Robert Halgren¶, Steven Rosen¶, Donald Kufe‡, Surender Kharbanda‡, and Kenneth Anderson‡

From ‡Hematologic Malignancy and §Cancer Pharmacology, Department of Adult Oncology, Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115 and ¶Northwestern University Medical School, Robert H. Lurie Cancer Center, Chicago, Illinois 60611

Cytochrome c is a mitochondrial protein that induces apoptosis when accumulated in the cytosol in response to diverse stress inducers. This protein has also been shown to cause apoptosis when added to cell free extracts. In this report, we studied the role of cytochrome c (cyto-c) in dexamethasone (Dex), anti-Fas monoclonal antibody (mAb), and ionizing radiation-induced apoptosis in multiple myeloma cells. The results demonstrate that ionizing radiation-induced apoptosis is associated with an increase in cytosolic cyto-c levels, whereas apoptosis induced by Dex or anti-Fas mAb has no detectable effect on cyto-c release. By contrast, caspase-3 was activated in response to all of these agents. Thus, our findings suggest that Dex or anti-Fas mAb-induced apoptosis is not accompanied by cyto-c release and that there are at least two different pathways leading to activation of caspases and induction of apoptosis in multiple myeloma cells that can be distinguished by accumulation of cytosolic cyto-c.

The cellular response to diverse classes of stress inducers includes growth arrest and activation of apoptosis; however, the intracellular signals that control these events are unclear. 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 by various stimuli is associated with the activation of cysteine proteases (caspases) (2) and cleavage of poly(ADP-ribose) polymerase (3, 4), DNA-dependent protein kinase (5), protein kinase C δ (PKC-δ) (6), and other substrates. Activation of caspases (CPP32, interleukin 1-β converting enzyme, interleukin 1-β converting enzyme homologue-1, and mammalian ced-3 homologue-2) and DNA fragmentation are two well characterized biochemical markers of apoptosis.

Direct evidence for involvement of caspases in apoptosis is derived from studies with the baculovirus protein p35, which directly inhibits cysteine proteases and blocks activation of apoptosis (7, 8). Genetic and biochemical evidence has indicated that apoptosis proceeds by one or more ordered pathways. Studies with Caenorhabditis elegans in particular have demonstrated that ced-3 is necessary for cell death (9–11). Mammalian homologues of ced-3 include the family of caspases (2).

Recent studies have shown that mitochondria may play an important role in inducing apoptosis by releasing cyto-c (12–14). The addition of exogenous cyto-c to cytosolic preparations from growing cells activates caspases and also induces DNA fragmentation in isolated nuclei (12). Furthermore, it was shown recently that DNA fragmentation factor is also required to mediate apoptosis after activation of caspase 3 (CPP32) (15).

To determine the role of cyto-c in induction of apoptosis, we examined the effect of three distinct stress inducers, IR, Dex, and anti-Fas mAb, in MM.1S multiple myeloma (MM) cell line and patient MM cells. We and others have previously demonstrated that IR, Dex, and Fas trigger apoptosis in MM cells (16–19). The results of the present study demonstrate that IR, but not Dex and anti-Fas mAb, induces accumulation of cytosolic cyto-c; all of these agents caused apoptosis in these cells. Similar results were obtained when cells from MM patient were treated with these agents. Taken together, the results of the present study indicate that in MM cells, there are at least two different pathways that lead to apoptosis in MM cells: one correlated and the other not correlated with release of cyto-c from mitochondria.

MATERIALS AND METHODS

Cell Culture and Reagents—Human MM.1S multiple myeloma cells 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 (20, 21). Mononuclear cells were isolated from a patient with MM (patient PCL) by Ficol-Hypaque density gradient centrifugation and incubated with HB-7 (anti-CD38) mAb-biotin-streptavidin and 2H4 (anti-CD45RA) mAb-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 RPMI 1640 medium containing 10% fetal bovine serum and antibiotics. γ-Irradiation was performed at room temperature using a Gamma-cell 1000 (Atomic Energy of Canada, Ottawa, Canada) under aerobic conditions, with a 137Cs source emitting at a fixed dose rate of 0.76 Gy min−1 as determined by dosimetry. Cells were also treated with 10 μM dexamethasone (Sigma) or with 7C11 anti-Fas monochlonal antibody as described previously (16).

Flow Cytometric Analyses—1 × 10⁷ cells were washed twice with cold PBS and fixed by the addition of 40% cold ethanol at 4 °C for 30 min. Cells were then washed with PBS and treated with RNase (50 μg/ml) for 45 min at 37 °C. After treatment with RNase, cells were washed with PBS and resuspended in PBS containing 15 μg/ml propidium iodide. Flow cytometric analyses were done using FACScan.

Preparation of S-100 Cytosolic Fractions from MM.1S and MM Patient Cells—MM.1S or patient MM cells were washed twice with PBS, and the pellet was suspended in 5 ml of ice-cold buffer A (20 mM HEPES, pH 7.5, 1.5 mM MgCl₂, 10 mM KC1, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonfyl fluoride, 10 μg/ml leupeptin, and aprotinin and pepstatin A) containing 250 mM sucrose. The cells were homogenized three times in a Dounce homogenizer with a sandpaper-polished pestle. Cytosolic fractions were isolated as de-

* This work was supported by U. S. Public Health Service Grant CA 50947 awarded by the NCI, National Institutes of Health. 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.

† The abbreviations used are: PKC-δ, protein kinase C δ; IR, ionizing radiation; cyto-c, cytochrome c; MM, multiple myeloma; Dex, dexamethasone; mAb, monoclonal antibody; PBS, phosphate-buffered saline; PAG, polyacrylamide gel electrophoresis; Gy, gray; CPP32, cysteine protease protein with a molecular mass of 32 kDa.

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Results and Discussion

To determine induction of apoptosis in MM.1S MM cells by diverse inducers, the cells were treated with 20 Gy of IR, 10 μM Dex, or 5 μg/ml of anti-Fas mAb for 48 h and flow cytometric analyses using propidium iodide was performed (16, 17). Treatment of cells with IR results in 71 ± 2% (n = 3) apoptotic cells, whereas Dex and anti-Fas mAb treatment leads to 61 ± 1.6 and 66 ± 5% apoptotic cells (n = 3), respectively. Previous studies have shown that accumulation of cyto-c in the cytosol occurs in response to multiple apoptotic stimuli and plays an important role in inducing apoptosis (12–14). To examine whether the release of cyto-c from mitochondria is a universal event during apoptosis, MM.1S MM cells were exposed to IR, Dex, or anti-Fas mAb antibody, and their effects on accumulation of cyto-c in cytosol were analyzed. Cytosolic S-100 fractions were isolated and analyzed for levels of cyto-c. Cytosolic cyto-c levels were increased at 3 and 6 h after irradiation (Fig. 1A). In contrast to IR, cyto-c levels in the cytosol were unchanged during treatment of MM.1S cells with either Dex or anti-Fas mAb (Fig. 1B and 2A). The IR-induced increase in cytosolic cyto-c was specific, because there was no change in the protein levels of tubulin (Fig. 1B, lower panel). Similar results were obtained when patient MM (PCL) cells were exposed to IR, Dex, or anti-Fas mAb (Fig. 2B, right and left upper panels, and data not shown). The treatment of PCL patient MM cells did not affect the protein levels, as evidenced by the unaltered tubulin.
protein levels (Fig. 2B, right and left lower panels). As an additional control, the blots were reprobed with a specific mitochondrial protein, HSP-60. The results demonstrate undetectable levels of HSP-60 in the S-100 cytosolic fractions (data not shown). These results indicate that the release of cyto-c from mitochondria may be important for triggering apoptosis in response to certain agents, in particular, DNA damaging agents, and that this is not an obligatory event during Dex or anti-Fas mAb-induced apoptosis in MM cells.

Multiple lines of evidence indicate that apoptosis can be triggered by the activation of cysteine proteases, and the most intensively studied apoptotic caspase is CPP32 (caspase-3) (24). Furthermore, it was recently shown in a cell free system that the addition of purified cyto-c to the cyto-c-depleted extracts activates CPP32 and DNA fragmentation (15). Because treatment of MM.1S MM cells with either Dex or anti-Fas mAb activates DNA fragmentation in the absence of cytosolic accumulation of cyto-c, we asked whether CPP32 is cleaved and activated by these inducers. MM.1S MM cells were treated with Dex or anti-Fas mAb, and the lysates were subjected to immunoblotting either with CPP32 or its known substrate PKC-δ (6). The results demonstrate cleavage and activation of CPP32 (as shown by cleavage of PKC-δ) in response to Dex or anti-Fas mAb (Fig. 3, A and B). Similar results were obtained when MM.1S cells were treated with IR (Fig. 3, A and B). Moreover, in an in vitro cell free system, the addition of purified cyto-c to the cyto-c immunodepleted MM.1S cytosolic lysates (Fig. 3C, left panel) showed cleavage of PKC-δ and CPP32 (Fig. 3C, right panel and data not shown). The results further suggest that MM.1S cytosolic fractions trigger the apoptotic program when cyto-c was added exogenously. Taken together, the results strongly suggest that cleavage and activation of CPP32 and induction of apoptosis by anti-Fas mAb and Dex in MM.1S cells is not accompanied by the accumulation of cyto-c in the cytosol.

The results described above indicate that there are multiple pathways that can trigger the program that leads to apoptosis. The multiplicity and complexity of apoptotic signals do not suggest the same linear pathway. Release of cyto-c from mitochondria to cytosol has been shown to be a potential target for regulation of apoptosis (13, 14, 22). Our results clearly demonstrate that accumulation of cyto-c in cytosol, which is associated with IR-induced apoptosis, is not observed during either Dex- or Fas-induced apoptosis in MM cells. Further studies are needed to elucidate the mechanisms involved in cyto-c-dependent and -independent induction of apoptosis in MM cells.

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