Apoptosis Induced by Cadmium in Human Lymphoma U937 Cells through Ca\(^{2+}\)-calpain and Caspase-Mitochondria-dependent Pathways*

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Apoptosis induced by cadmium has been shown in many tissues in vivo and in cultured cells in vitro. However, its molecular mechanism is not fully understood. When the human histiocytic lymphoma cell line U937 was treated with cadmium for 12 h, evidence of apoptotic features, including change in nuclear morphology, DNA fragmentation, formation of DNA ladder in agarose gel electrophoresis, and phosphatidylserine externalization, were obtained. Moreover, loss of the mitochondrial membrane potential (ΔΨm) was observed in the cadmium-treated cells and was inhibited by a broad caspase inhibitor (Z-VAD-FMK). Caspase inhibitors suppressed the DNA fragmentation in the order of Z-VAD-FMK > caspase-8 inhibitor > caspase-3 inhibitor. Expression of Bcl-xL and Bid decreased significantly in the cadmium-treated cells, although no apparent change in Bcl-2 and Bax expression was found. Tetrakis-(2-pyridylmethyl) ethylenediamine, a cell-permeable heavy metal chelator, partially reversed the increase of fluorescence of Fluo-3 in the cadmium-treated cells. In addition, verapamil (70 μM), a voltage-dependent Ca\(^{2+}\) channel blocker, inhibited the DNA fragmentation induced by cadmium less than 100 μM and decreased the fluorescence of Fluo-3. Cadmium up-regulated the expression of type 1 inositol 1,4,5-trisphosphate receptor (IP\(_{3}\)R) but not type 2 or type 3 IP\(_{3}\)R. Calpain inhibitors I and II partially prevented DNA fragmentation. No effects of Z-VAD-FMK on the expression of type 1 IP\(_{3}\)R or of calpain inhibitors on the loss of ΔΨm were observed. These results suggest that cadmium possibly induced apoptosis in U937 cells through two independent pathways, the Ca\(^{2+}\)-calpain-dependent pathway and the caspase-mitochondria-dependent pathway.

Cadmium, a potent toxic metal, is very harmful to the environment and to human beings because of its long lifetime. The toxicity of cadmium as an industrial pollutant and a food contaminant, and as one of the major components in cigarette smoke is well established (1). Cadmium can cause a number of lesions in many organs, such as the kidney, the testis, the lung, the liver, the brain, the bone, the blood system, etc. (2). However, the mechanism of toxicity of cadmium is not yet clear. Recently, several reports have shown that cadmium can induce apoptosis of many tissues and cells both in vivo and in vitro, such as the cells of the respiratory system (3), the testis (4–6), the kidney (7, 8), the liver (9), and the immune system (10, 11), etc. This evidence indicates that apoptosis probably plays a very important role in acute and chronic intoxication with cadmium. Further research on this aspect of cadmium would have significance in the prevention and cure of the diseases induced by cadmium.

Apoptosis is a fundamental form of cell death, and it plays an essential role in the development and homeostasis of multicellular organisms. Apoptosis disorders are associated with many diseases, such as cancer, autoimmune disorders, neurodegenerative disease, toxin-induced disease, etc. (12). In the apoptotic process, caspases, a family of asparate proteases, lie in a pivotal position (13). Two independent pathways of apical caspases activation, receptor-intermediated caspase 8 and mitochondria-cytochrome c intermediated caspase 9 activation, converge on the activation of executing caspases, key substrate cleavage, and apoptotic death (14).

Intracellular Ca\(^{2+}\) homeostasis is very important in maintaining the normal function of the cell. Increases and decreases in calcium ion are possible causes of apoptosis (15, 16). The radius of Cd\(^{2+}\), a common form of free cadmium in the body, is very similar to that of Ca\(^{2+}\) (0.099 and 0.097 nm, respectively). Cd\(^{2+}\) not only competitively inhibits the influx of Ca\(^{2+}\) (17) but also causes the increase of [Ca\(^{2+}\)]\(_{i}\), through inhibiting Ca\(^{2+}\)-ATPase on the membrane of the depot of calcium (18). In addition, cadmium can activate or inhibit some calcium-related enzymes instead of Ca\(^{2+}\) (19). Thus, investigation on the role of interactions of calcium ions in cadmium-induced apoptosis is important for understanding the mechanism of toxicity of cadmium.

In this study, the molecular mechanism of apoptosis induced by cadmium in human histiocytic lymphoma U937 cells, which possess the Ca\(^{2+}\)/Mg\(^{2+}\)-dependent endonuclease (20), was investigated. We herein show that two independent pathways, the Ca\(^{2+}\)-calpain pathway and the caspase-mitochondria pathway, are probably involved in the regulation of cadmium-induced apoptosis.

MATERIALS AND METHODS

Reagent—Cadmium chloride, verapamil, 3,3'-dihexyloxacarbocyanine iodide (DiOC\(_{3}(3))\), dichlorodihydrofluorescein diacetate, and dihy-

1 The abbreviations used are: DiOC\(_{3}(3), 3,3’\)-dihexyloxacarbocyanine iodide; TPEN, tetrakis-(2-pyridylmethyl) ethylenediamine; Annexin

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Cadmium-induced Apoptosis in U937 Cells

RESULTS

Induction of Apoptosis by Cadmium—The results of examinations of DNA fragmentation, DNA ladder formation, observation of cell morphology, and PS externalization consistently revealed that cadmium induced apoptosis in U937 cells. Non-random DNA fragmentation has been regarded as one of the typical end points of apoptosis (26). Fig. 1A illustrates that DNA fragmentation induced by cadmium was concentration-dependent and time-dependent; DNA fragmentation increased with increasing concentrations of cadmium up to 100 μM cadmium and 12 h of treatment. Furthermore, DNA fragmentation culminated 12 h after treatment with cadmium. Thus, 100 μM cadmium and 12 h of treatment were often used in this study. After cadmium treatment for 12 h, observation of DNA ladder formation and morphological changes supported the evidence of DNA fragmentation (Figs. 1B and 2, C and D). The observation of cell morphology illustrated that cadmium-treated cells underwent prominent cytoplasmic aggregation, nucleic condensation, and fragmentation, which were typical signs of apoptotic features. Morphological change (including PI staining) showed

DNA fragmentation induced by cadmium was concentration-dependent and time-dependent; DNA fragmentation increased with increasing concentrations of cadmium up to 100 μM after 6, 12, and 24 h. Then DNA fragmentation assay was carried out according to the method of Sellins and Cohen (21). Data are presented as the means ± S.D. (n = 4). B, U937 cells were treated with 100 μM cadmium for 12 h, and DNA was extracted from control and cadmium-treated cells. Then the formation of DNA ladder was examined in 1.2% agarose gel electrophoresis.
that at concentrations of more than 100 μM, the number of necrotic cells apparently increased (data not shown).

PS externalization is an early symbol of apoptosis (27). Flow cytometry using Annexin V/FITC and PI double staining revealed that after exposure to cadmium, cells with externalized PS significantly increased depending on cadmium concentration and exposure time. At 6 h, early apoptotic cells (only Annexin V/FITC staining) were predominantly observed; as time progressed or as the concentration increased, secondary necrotic cells in a later stage of apoptosis (double Annexin/FTC V and PI staining) were in the main position. These results indicate that at later stages of apoptosis, the plasma membrane was damaged by cadmium (Fig. 2, A and B).

**Elevation of Ca²⁺/Cd²⁺ Concentration in Cells—**To eluci-
date the effect of the interaction between Ca\(^{2+}\) and Cd\(^{2+}\) in apoptosis induced by cadmium, we used Fura-2, which is derived from Fura-2/AM through esterase in cells, to determine the change in ion concentration of the cells. Fluorescence in cells treated for 6 h with cadmium became much stronger than that in control cells, and this change showed a dose-effect relationship. Unfortunately, the excitation response of Fura-2 to Cd\(^{2+}\) is almost an exact match to that of Fura-2 to Ca\(^{2+}\). Therefore, it is difficult to distinguish Ca\(^{2+}\) from Cd\(^{2+}\) (28).

Verapamil, a voltage-dependent Ca\(^{2+}\) channel inhibitor (10), reduced fluorescence of Fura-2 in cells; moreover, 70 \(\mu\)M verapamil delayed DNA fragmentation induced by cadmium. These results indicated that cadmium probably entered into U937 cells via voltage-dependent calcium channels, and verapamil inhibited this passage, resulting in delayed DNA fragmentation (Fig. 3). After combined treatment of cadmium and verapamil, the increased fluorescence (cells indicating a ratio higher than 1.6) appeared to indicate intracellular Ca\(^{2+}\) release induced by cadmium, because verapamil blocks both Ca\(^{2+}\) and Cd\(^{2+}\) entry into the cells.

To further determine the contribution of [Ca\(^{2+}\)]\(_i\), to the increase in ion concentration, we used a cell-permeable, specific Cd\(^{2+}\) chelator (TPEN, Cd\(^{2+}\) \(K_0 = 10^{16.33}\); Ca\(^{2+}\) \(K_0 = 10^{4.47}\)), and fluorescence in cells significantly decreased, but it did not completely reverse the fluorescence of Fura-2 in cells, even though the concentration of TPEN increased to 100 \(\mu\)M. These results suggest that in addition to augmentation of [Cd\(^{2+}\)]\(_i\), [Ca\(^{2+}\)]\(_i\) was probably elevated (Fig. 4A).

\(\text{IP}_3\)-R Expression and Effects of Calpain Inhibitor—Because cadmium competitively inhibits Ca\(^{2+}\) influx (18), the mechanism of [Ca\(^{2+}\)]\(_i\) increase in cells exposed to cadmium has not been elucidated. In some apoptotic systems, elevation of [Ca\(^{2+}\)]\(_i\) was mediated through \(\text{IP}_3\)-Rs (25, 29). In our study, therefore, the expression of \(\text{IP}_3\)-R subtypes was investigated at 12 h after cadmium treatment (Fig. 4B). The expression of \(\text{IP}_3\)-R1 was apparently enhanced in a dose-dependent manner, but \(\text{IP}_3\)-R2 and \(\text{IP}_3\)-R3 were not detected. These results demonstrated that, probably via the \(\text{IP}_3\)-R1 pathway, cadmium induced the release of calcium into the cytoplasm from its intracellular depot, such as endoplasmic reticulum. In addition, caspase inhibitor (Z-VAD-FMK) did not affect the expression of \(\text{IP}_3\)-R1.

Calcium ion can act on multiple targets to trigger apoptosis (14). Recently, calpain, calcium-dependent protease has been considered as a possible target through which elevated calcium triggers apoptosis (30). Calpain inhibitor I significantly reduced DNA fragmentation by 40% at low concentrations, and there was little difference between 20 and 50 \(\mu\)M. However, calpain inhibitor II was not effective until its concentration reached 50 \(\mu\)M. The results, therefore, indicate that calpain may take part in the Ca\(^{2+}\)-dependent pathway of apoptosis induced by cadmium (see Fig. 6A).

Loss of Mitochondrial Membrane Potential Induced by Cadmium—In many systems, apoptosis is associated with the loss of mitochondrial inner membrane potential (\(\Delta\psi_m\)), which may be regarded as a limiting factor in the apoptotic pathway (31). To observe the change in \(\Delta\psi_m\) in cells exposed to cadmium, DiOC\(_6\)(3), a mitochondria-specific and voltage-dependent dye, was employed. After U937 cells were exposed to cadmium for 12 h, \(\Delta\psi_m\) was significantly reduced in a dose-dependent manner (Fig. 5C). However, at 3 and 6 h, the loss of \(\Delta\psi_m\) was indiscernible. Although it has been reported that cadmium was able to cause the loss of \(\Delta\psi_m\) via oxidant injury (32, 33), no significant increase in intracellular oxidants, such as hydrogen peroxide or superoxide, in the cadmium-treated cells was observed by flow cytometry with dichlorodihydrofluorescein diacetate and dihydroethidium in our study (data not shown).

Inhibition of Caspase Inhibitors in Cadmium-induced Apoptosis—Because of the loss of mitochondrial membrane potential in the cadmium-treated cells, it was speculated that caspases may play an essential role in the process of cadmium-triggered apoptosis. First, Z-VAD-FMK (caspase inhibitor I) and Boc-D-FMK (caspase inhibitor III), two kinds of broad spectrum, irreversible caspase inhibitors, were used. Z-VAD-FMK almost completely inhibited cadmium-induced DNA fragmentation (Fig. 6B); Boc-D-FMK also showed a similar result (data not shown). Moreover, Z-VAD-FMK also inhibited the loss of \(\Delta\psi_m\). These results suggest that cadmium probably triggers apoptosis of U937 cells via a caspase-dependent pathway.

To determine which kinds of caspases take effect in apoptosis induced by cadmium, a series of inhibitors, caspase-1 inhibitor VI (Z-YVAD-FMK), caspase-2 inhibitor I (Z-VDVAD-FMK), caspase-3 inhibitor II (Z-DEVD-FMK), caspase-5 inhibitor
I (Z-WEHD-FMK), caspase-6 inhibitor I (Z-VEID-FMK), caspase-8 inhibitor II (Z-IETD-FMK), and caspase-9 inhibitor I (Z-LEHD-FMK), were employed for preventing DNA fragmentation in apoptosis. The results revealed that both caspase-8 inhibitor and caspase-3 inhibitor were able to prevent cadmium-induced DNA fragmentation. Moreover, caspase-8 inhibitor was more effective than caspase-3 inhibitor in this respect (Fig. 6).

Expression of Apoptosis-related Protein—Cleavage of Bid is important for the release of cytochrome c from mitochondria in CD95-induced apoptosis (34). Bid was present as a ~26-kDa protein in U937 cells (Fig. 7A). The proform of Bid slowly decreased depending on the cadmium concentration. At the same time, caspase inhibitor (Z-VAD-FMK) and caspase-8 inhibitor (Z-IETD-FMK) both inhibited the cleavage of Bid induced by 100 μM cadmium.

A member of the Bcl-2 family, Bcl-xL, significantly decreased in a concentration-dependent manner, and at the same time, Bcl-xS, which is the cleaving product of Bcl-xL, slowly appeared as Bcl-xL was reduced. It has been reported that Bcl-xL and Bcl-xS have completely contrary effects; the former inhibits but the latter promotes apoptosis (35) (Fig. 7B). This evidence is consistent with our results. Caspase inhibitor (Z-VAD-FMK) and caspase-8 inhibitor (Z-IETD-FMK) did not affect the expression of Bcl-xL. In contrast, no change in the expression of Bcl-2/Bax proteins was apparent in cadmium-induced apoptosis in U937 cells (Fig. 7C), although Bcl-2/Bax has been regarded as the regulator of the release of cytochrome c in apo-

**FIG. 5. Loss of Δψₘ induced by cadmium.** U937 cells were treated with 100 μM cadmium for 12 h, cells were stained with 40 nM DiOC₆(3) as described under “Materials and Methods,” and then Δψₘ was measured by flow cytometry. When caspase inhibitor (Z-VAD-FMK (100 μM)) and calpain inhibitors (calpain inhibitors I (20 μM) and II (50 μM)) were used, cells were preincubated for 2 h and then treated with 100 μM cadmium. A, control. B discorrn (100 μM). C, discorrn (100 μM) + Z-VAD-FMK (100 μM). E, discorrn (100 μM) + Z-VAD-FMK (100 μM) + Z-VAD-FMK (100 μM). C, after treatment with cadmium at indicated concentrations for 12 h, the percentage of cells with low Δψₘ was measured, as shown in Fig. 5A. The results were presented as the means ± S.D. (n = 5).
Cadmium-induced Apoptosis in U937 Cells

39707

The present study shows that cadmium is able to induce apoptosis in U937 cells, and in this process, there may be two different and independent pathways for inducing apoptosis by cadmium. One is the Ca$^{2+}$-calpain-dependent pathway, and the other is the caspase-dependent pathway.

**The Ca$^{2+}$-Calpain-dependent Pathway**—In recent years, the effect of IP$_3$R in apoptosis has been the focus of attention. The elevation of IP$_3$R subtype involved in apoptosis are due to many stimuli, such as anti-immunoglobulin M, dexamethasone (29), irradiation (25), B-cell antigen receptor (39), etc. On the other hand, cells with failure in Ca$^{2+}$ elevation owing to deficient IP$_3$R1 or IP$_3$R2 are resistant to apoptosis by dexamethasone, irradiation, or Fas ligand in U937 cells (40). Our study reveals that cadmium is able to up-regulate the expression of IP$_3$R1 but not IP$_3$R2 or IP$_3$R3 in U937 cells. It has been reported that a majority of IP$_3$R1 was distributed on the surface of endoplasmic reticulum (41). Hence, because of competitive inhibition of Ca$^{2+}$ influx via the calcium channel and the expression of IP$_3$R1 in U937 cells, the elevation of calcium ion caused by cadmium possibly derives from endoplasmic reticulum. Verapamil delayed cadmium-induced DNA fragmentation, probably because it inhibited the Cd$^{2+}$ influx via the Ca$^{2+}$ channel so that the IP$_3$R1 up-regulation was extended. Of course, the possibility should not be excluded that cadmium in cells may directly mobilize Ca$^{2+}$ or enhance Ca$^{2+}$ mobilization, because cadmium can inhibit all types of Ca$^{2+}$-ATPase (19, 42, 43).

Calpain is a family of Ca$^{2+}$-dependent cysteine proteases whose members are expressed ubiquitously (44). Calpain has been reported to be involved in several models of apoptosis and to take effect as a target of Ca$^{2+}$-dependent activation (45, 46). Presumably, calpain plays an essential role in apoptosis because of cadmium, because calpain inhibitors inhibited the DNA fragmentation induced by cadmium in our study. Furthermore, calpain inhibitor I was more potent than calpain inhibitor II. In addition, in addition to calpain, calcium ions are able to activate other targets to trigger apoptosis, such as Ca$^{2+}$/Mg$^{2+}$-dependent endonuclease, whose involvement has been shown in UV-induced apoptosis in U937 cells.

On the other hand, the other effects of cadmium in cells should be considered. Cd$^{2+}$ not only causes Ca$^{2+}$ elevation but also activates some calcium-related enzymes, for example, protein kinase C (47), mitogen-activated protein kinase (48), calmodulin-dependent kinase (49), etc. Hence, further research is necessary to determine whether cadmium can directly activate apoptotic proteases (calpain, calcium-dependent endonuclease, etc.) instead of calcium.

**The Caspase-dependent Pathway**—The caspase family is divided into two groups: one group, derived from procaspase with long predomains (caspase-2, -8, -9, and -10), is called “initiator” or “upstream” caspases, and the other, which is derived from precursor with short predomains is called “effector” or “downstream” caspases (caspase-3, -6, -7, and -14) (50). In our study, Z-VAD-FMK (broad spectrum inhibitor) inhibited DNA fragmentation induced by cadmium in our study. Furthermore, caspase-8 and -3 inhibitors also inhibit cadmium-induced DNA fragmentation, but caspase-2, -6, -7, and -9 inhibitors do not. Therefore, we hypothesize that probably caspase-8 is the most apical caspase in cadmium-induced apoptosis, and finally signal converges to caspase-3. According to previous reports, caspase-8 and -3 inhibitors also inhibit cadmium-induced DNA fragmentation, but caspase-2, -6, -7, and -9 inhibitors do not. Therefore, we hypothesize that probably caspase-8 is the most apical caspase in cadmium-induced apoptosis, and finally signal converges to caspase-3. According to previous reports, caspase-8 not only directly cleaves and activates caspase-3 (51) but also indirectly activates caspase-3 by inducing cytochrome c release (52). According to observations, the loss of $\Delta \psi_m$ because of cadmium seems to support this evidence.

Bid, a proapoptotic Bcl-2 family member containing BH$_3$ domain, can be cleaved by caspase-8, and the cleaved Bid, the carboxyl-terminal fragment, translocates to mitochondria to induce the release of cytochrome c, which is 500 times more numerous than Bid (53). In our study, the decrease in the proform of Bid means that it is cleaved in cadmium-induced apoptosis, and Z-VAD-FMK and caspase-8 inhibitor block this cleavage. This suggests that the cleavage of Bid is caspase-8-dependent.

Besides Bid, other Bcl-2 family members are associated with the release of cytochrome c. For example, Bax promotes cyto-
chrome c release, but Bcl-2 and Bcl-xL counteract the effect of Bax and inhibit the release of cytochrome c (37, 54). Moreover, Bcl-xL can itself bind to cytochrome c and Apaf-1 to prevent apoptosis (55, 56). In cadmium-induced apoptosis, the change in Bcl-2 and Bax is not apparent, but the level of Bcl-xL apparently decreases in a concentration-dependent manner, and Bcl-xS, which is the product of Bcl-xL cleavage and which promotes apoptosis (36), increases along with the decrease in Bcl-xL. Bad, another Bcl-2 family member that sequesters Bcl-xL, was not detected in U937 cells. Taken together, these results indicate that Bid and Bcl-xL are possibly involved in the caspase-dependent pathway of cadmium-induced apoptosis, resulting in cytochrome c release and enhancement of the apoptotic process.

Although no reports have been published showing the activation of caspase-8 induced by cadmium, it is speculated that cadmium may activate caspase-8 by elevating the expression of Fas ligand. This is not only because caspase-8 is a key component of the Fas/APO 1 death receptor-triggered apoptosis pathway (53), but also because many other apoptotic pathways initiated by distinct stimuli require Fas engagement. For instance, cell apoptosis triggered by anticancer drugs (57–59), irradiation (60) and ceramide (61) is mediated by up-regulation of Fas L and its interaction with Fas. Of course, we cannot exclude the possibility that cadmium activates caspase-8 via a pathway independent of Fas L, such as activated Lck (62). Further study is necessary to elucidate the mechanism.

In summary, possibly via two different pathways, cadmium induces apoptosis in U937 cells (Fig. 8). In addition, because caspase degrades calpastatin, which is an endogenous inhibitor of calpain (63), and promotes the Ca2⁺-calpain pathway and because the Bcl-2 family and Ca2⁺ act on each other (64), they may complement each other.

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