Effect of Bcl-2 and caspase-3 on calcium distribution in apoptosis of HL-60 cell

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ABSTRACT

Apoptosis manifests in two major execution programs downstream of the death signal: the caspase pathway and organelle dysfunction. An important antiapoptosis factor, Bcl-2 protein, contributes in caspase pathway of apoptosis. Calcium, an important intracellular signal element in cells, is also observed to have changes during apoptosis, which maybe affected by Bcl-2 protein. We have previously reported that in Harringtonine (HT) induced apoptosis of HL-60 cells, there's a change of intracellular calcium distribution, moving from cytoplasm especially Golgi's apparatus to nucleus and accumulating there with the highest concentration. We report here that caspase-3 becomes activated in HT-induced apoptosis of HL-60 cells, which can be inhibited by overexpression of Bcl-2 protein. No sign of apoptosis or intracellular calcium movement from Golgi's apparatus to nucleus in HL-60 cells overexpressing Bcl-2 or treated with Ac-DEVD-CHO, a specific inhibitor of caspase-3. The results indicate that activated caspase-3 can promote the movement of intracellular calcium from Golgi's apparatus to nucleus, and the process is inhibited by Ac-DEVD-CHO (inhibitor of caspase-3), and that Bcl-2 can inhibit the movement and accumulation of intracellular calcium in nucleus through its inhibition on caspase-3. Calcium relocation in apoptosis seems to be irreversible, which is different from the intracellular calcium changes caused by growth factor.

Key words: *Apoptosis, calcium, caspase-3, bcl-2, laser scanning confocal microscopy (LSCM).*

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INTRODUCTION

Apoptosis is a kind of cell death that is executed through a suicide program[1], which is characterized by a variety of morphological and biochemical events, including chromatin condensation, fragmentation of DNA, blebbing of the plasma membrane, and PS externalization[2]. Genetic analysis of apoptosis in *Caenorhabditis elegans* started researches on the mechanism of apoptosis and found three most important genes are *ced-3*, *ced-4* and *ced-9*. *Ced-3* and *ced-4* accelerate the process of apoptosis, while *ced-9* prevents the course. A family of cysteine proteases with specificity for aspartic acid residues is reported to be mammalian homolog to *ced-3*, named as caspase. caspase-3, a member of caspase family, is activated in apoptosis of a multitude of cells, and always plays an important role in the signal pathway of apoptosis[3],[4]. A caspase-3-specific tetrapeptide inhibitor, Ac-DEVD-CHO, can abolish caspase-3-involved apoptosis[5]. Bcl-2 is a homolog of *ced-9* in mammal. As a key regulator of the pathway of apoptosis, it is an anti-apoptosis member of this family[6-8].

Calcium is one of the most important intracellular signal elements in cells. Apoptosis induced by different factors differs in the dependence of calcium. It was reported that intracellular calcium could be transferred from cytoplasm, especially from Golgi's apparatus to nucleus in HT-induced apoptosis of HL-60 cells[9]. But the connection between intracellular calcium changes and signal pathway of apoptosis is unknown.

We reported in this paper on the effect of Bcl-2 protein and caspase-3 on calcium distribution in apoptosis of HL-60 cells. We found that activated caspase-3 can promote the movement of intracellular calcium from Golgi's apparatus to nucleus, and the process is inhibited by Ac-DEVD-CHO (inhibitor of caspase-3). Bcl-2 protein inhibits the movement and accumulation of intracellular calcium in nucleus because of its inhibition on caspase-3. Calcium relocation taking place in apoptosis is irreversible, which is different from the intracellular calcium changes caused by growth factor.

MATERIALS AND METHODS

Reagents

HT was kindly provided by Professor Han Rui, Chinese Academy of Medical Sciences. Fluoro-Jade AM, Hoechst 33342 and EnzChek™ caspase-3 Assay kit#2 were purchased from Molecular Probes. ECL Western Blot analysis kit were purchased from Amersham. Monoclonal antibody against Bcl-2 protein from Santa Cruz and DNA extraction kit from Dingguo Corporation.

Cell culture

The transfected human promyelocytic leukemia HL-60 / neo and HL-60/ bcl-2 cell lines were kindly provided by Dr. Wang Xiaodong (University of Texas Southwestern Medical Center, USA). Human acute myeloid leukemia (HL-60) cells were transfected with a retroviral vector containing a neomycin resistance gene (neo cells), or the same vector containing a cDNA encoding human bcl-2 (bcl-2 cells). All cells were cultured with RPMI 1640 medium (Gibco) containing 10 % heat-inactivated fetal bovine serum in an atmosphere with 5 % CO₂.

Overexpression of Bcl-2 protein identified by Western Blot

Cells were collected and washed by cold PBS, resuspended in cell lysate (100M Tris HCl, 2.5 % SDS, 10 % β -mercaptoethanol, 1 mM PMSF, 10 % glycerol), and incubated on ice for 10min. After being broken by ultrasonic, cells were boiled for 2 min. Then the lysate were centrifuged at 4°C for 10 min (14, 000g), and the supernatants were subjected to 12 % SDS-PAGE and then transferred onto a nitrocellulose filter. The filter was probed by mouse monoclonal antibody against Bcl-2, and the antigen-antibody complexes were visualized by an ECL method.

DNA extraction and electrophoresis

DNA were extracted from treated cells and analyzed by agarose gel electrophoresis as described in the reference of DNA extraction kit.

Assay of caspase-3 activity

Cells were collected and analyzed by Enzc hekTM caspase-3 Assay kit#2, using HITACH F4010 fluorescence spectrophotometer (496nm/520nm). This kit provides the substrate of caspase-3 Z-DEVD-R110, which can be lysed by active caspase-3 and release R110, the fluorescence of which was analyzed by fluorescence spectro-photometer.

Staining HL-60 (neo)/(bcl-2) cells with Hoechst 33342 and Fluo-3 AM

Cells were collected and stained with 20 μ M Fluo-3 AM and 10 μ M Hoechst 33342 for 30 min at 37°C. After washed for more than 3 times, cell were resuspended by RPMI 1640 + 10 % FCS. Stained cells were observed by fluorescence microscope.

Intracellular calcium distribution observed by laser scanning confocal microscopy (LSCM)

Cells were observed by LEICA PCS-NT laser scanning confocal microscopy (LSCM) after being stained with Fluo-3 AM and treated with drugs. Excitation at 488nm, emission at 530 ± 30 nm.

RESULTS

The inhibition of HT-induced apoptosis in HL60 cells by Bcl-2 protein

When the expression of Bcl-2 protein was compared in two cell lines used in this experiment, it was found that HL-60 (bcl-2) cells contained about 20 fold more protein than HL-60 (neo) cells (Fig 1).

Now, if HT (1 μ g/ml) was applied to these cells, only HL-60(neo) cells, but not HL-60 (bcl-2) cells, can be induced to undergo apoptosis, which is characterized by chromatin condensation and DNA fragmentation. The results suggest that overexpressed Bcl-2 protein in HL60 (bcl-2) cells can inhibit HT-induced apoptosis (Fig 2).

At the same time, activity of caspase-3 was analyzed by Enzc hekTM caspase-3 Assay kit#2 as mentioned in Materials and methods. It was found that caspase-3 was activated during HT-induced apoptosis, and the elevation of caspase-3 activity in HL-60(neo) cells was totally inhibited in HL-60 (bcl-2) cells. Thus, Bcl-2 protein could inhibit the activation of caspase-3 (Fig 3).

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Fig 1. Expression of Bcl-2 protein in HL-60 (neo/ bcl-2) cells
1. HL-60 (neo) cells 2. HL-60 (bcl-2) cells

Fig 2. Bcl-2 protein inhibited HT-induced apoptosis A. Nuclear chromatin condensation × 400. (a) HL-60(neo) cells control; (b) HL-60(neo) cells treated with HT(1 μg/ml) for 2 h; (c) HL-60(bcl-2) cells control; (d) HL-60(bcl-2) cells treated with HT(1 μg/ml) for 2 h. B. DNA fragmentation induced by HT; 1. DNA marker, 1 kb marker; 2. HL-60(neo) cells control; 3. HL-60(neo) cells treated with HT(1 μg/ml) for 2 h; 4. HL-60(bcl-2) cells control; 5. HL-60(bcl-2)cells treated with HT(1 μg/ml) for 2 h.

► **Fig 4.** A-E. Redistribution of intracellular calcium during HT-induced apoptosis in HL-60 (neo/ bcl-2) cells; its inhibition by Bcl-2 protein and caspase-3 inhibitor Note that the highest $[Ca^{2+}]_i$ is found in the perinuclear region (Golgi apparatus, area 1). Cell nucleus (area 2) and the rest areas of the cytosol (area 3) showed a very low $[Ca^{2+}]_i$. A. HL-60 (neo) control cells; B. HL-60 (neo) control cells treated with HT (1mg/ml) for 2 h; C. HL-60 (bcl-2) control cells; D. HL-60 (bcl-2) cells treated with HT (1mg/ml) for 2 h; ,b. E. HL-60 (neo) cells pretreated with Ac-DEVD-CHO (100 m M for 1 h and then treated with HT(1mg/ml) for 2 h; a. stained with 10 μ M Hoechst 33342 × 400; b. stained with 20 μ M Fluo-3 AM × 400; c. stained with Fluo-3 AM and visualized by LSCM.

Fig 3. Bcl-2 protein inhibited the activation of caspase-3

The change of intracellular calcium distribution in HT-induced apoptosis and its relation to caspase-3

Intracellular calcium accumulated mostly in the peri-nuclear area (i.e. Golgi apparatus) in control HL-60 (neo) cells[10], while cell nucleus showed a distinctly lower $[Ca^{2+}]_i$ than peri-nuclear region and other areas of cytoplasm (Fig 4A). However, HL-60 (neo) cells treated with HT present a very different pattern of Fluo-3 AM staining. Labeling of

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intracellular Ca^{2+} and chromatin revealed the accumulation of calcium in the nuclear region (Fig 4B, compare with A). These results suggest that in apoptotic HL-60 (neo) cell intracellular calcium accumulated in the nucleus due to the release of calcium from the peri-nuclear area and relocalized in area of chromatin condensation (i.e. nuclear region).

But intracellular calcium relocalization was not observed in HL-60 (bcl-2) cells after their being treated with HT. In these cells, intracellular calcium showed predominantly higher calcium accumulation in the peri-nuclear area (Fig 4D, compare with C). These results indicated that overexpressed Bcl-2 protein could prevent this calcium redistribution induced by HT. (Fig 4C, D,)

However, the above intracellular calcium relocalization was not observed in Ac-DEVD-CHO treated HL-60 (bcl-2) cells after their being treated with HT, a fact which indicated that inhibitor of caspase-3 can prevent such calcium translocation (Fig 4E).

DISCUSSION

In spite of the recent progress in studies on the mechanism of apoptosis, it is still unclear the relationship between calcium and signal pathway of apoptosis. There are calcium changes during apoptosis: (1) Enhanced Ca^{2+} influx. McConkey showed that intracellular free calcium increased in glucocorticoid-stimulated apoptosis and EGTA inhibited Ca^{2+} increase and apoptosis[11]. (2) Intracellular calcium released from calcium store. For example, thapsigargin (TG), inhibitor of endoplasmic reticulum (ER) Ca^{2+} -ATPase, can induce apoptosis in neuroblastoma cells through the increase of $[\text{Ca}^{2+}]_i$ [12]. (3) $[\text{Ca}^{2+}]$ in mitochondria varies in apoptosis. When neural cell was induced by staurosporine, $[\text{Ca}^{2+}]$ increased in cytosol first, and then it was accompanied with $[\text{Ca}^{2+}]_m$ increase in mitochondria[13]. (4) Intracellular calcium relocalization. We have reported that free $[\text{Ca}^{2+}]_i$ do not change much in HT-induced apoptosis of HL-60 cells. However, intracellular calcium relocalized. Intracellular calcium transfers from cytoplasm, especially from Golgi's apparatus to nucleus in this kind of apoptosis[9], which was the first work to demonstrate relocalization of intracellular calcium at the final stage of apoptosis. Others also reported that Ca^{2+} pool in the endoplasmic reticulum was depleted in a lymphoid cell line in response to glucocorticoid treatment[14], suggesting that intracellular Ca^{2+} storage sites appeared to be affected.

In the above-mentioned cases of apoptosis, it seems clear that the source of calcium for its increase or change in distribution may be derived either from Ca^{2+} influx from outside or released from intracellular calcium store. However, both the increase of $[\text{Ca}^{2+}]_i$ and Ca^{2+} accumulation in nucleus can bring forth the increased activity of various protease[15] and nuclease[16]. Activation of Ca^{2+} -dependent protein kinases calpain, and/or phosphatases leading to alterations in gene transcription is one way which Ca^{2+} might regulate apoptosis. As an example, cyclosporin A, an immunosuppressant, can block Ca^{2+} -dependent apoptosis in lymphoid model systems[17], indicating that calcineurin activa-

tion may be required for these responses. However, researchers seldom establish relationship between Ca^{2+} storage sites and protease activation.

On the basis of our previous finding of calcium relocation in HT-induced apoptosis of HL-60 cells, the present results shows that the activity of caspase-3 is enhanced in HT-induced apoptosis in HL60 cells, and Bcl-2 protein can inhibit the apoptosis and the activation of caspase-3. Furthermore, Bcl-2 protein and Inhibitor of caspase-3 can prevent the relocation of intracellular calcium in HT-induced apoptosis. These results lead us to draw a conclusion that caspase-3, one of the most important caspases in signal pathway in apoptosis, can promote, at least in our present system, the movement of intracellular calcium from Golgi's apparatus to nucleus, and the process can be inhibited by Ac-DEVD-CHO, inhibitor of caspase-3. Increased $[\text{Ca}^{2+}]$ in nucleus is potentially related to the activation of Ca^{2+} dependent endonuclease, chromatin condensation and DNA fragmentation. Bcl-2 protein can inhibit the movement of intracellular calcium and its accumulation in nucleus via its inhibition on caspase-3.

Earlier in our study with human fibroblasts (2BS) cells, we have found that Ca^{2+} which accumulated in Golgi's apparatus as intracellular store in control cells, could be released and accumulated in nucleus following their treatment with PDGF, but became re-accumulates in Golgi's apparatus 3 minutes later[10]. The change of calcium location in HT-induced apoptosis of HL-60 cells differs from the above physiological event in its irreversibility of intracellular calcium releasing from Golgi apparatus and accumulating in the nucleus. Recent work from Kruman et al. also showed that: NGF can resume the concentration of $[\text{Ca}^{2+}]$ and inhibit apoptosis in PC12 cells[18]. Exposure of cultured PC12 cells to staurosporine resulted in a rapid and prolonged elevation of intracellular free calcium levels $[\text{Ca}^{2+}]$, with calcium level elevating in nucleus. Treatment of cells with nerve growth factor within 1-2 h of staurosporine exposure resulted in the recovery of $[\text{Ca}^{2+}]$ and nuclear calcium to normal levels, leading to the inhibition of apoptosis, thus supporting the contention that the irreversibility of Ca^{2+} translocation is an important event in apoptosis.

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