Thapsigargin increases apoptotic cell death in human hepatoma BEL-7404 cells¹

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ABSTRACT

Effects of thapsigargin, an inhibitor of Ca^{2+} -ATPase in surface of endoplasmic reticulum, on apoptotic cell death were studied in human hepatoma cells of BEL-7404 cell line by using both flow cytometry and electron microscopy. Propidium iodide staining and flow cytometry revealed that in the serum-free condition, thapsigargin increased the rate of apoptosis of BEL-7404 cells in a dose-dependent manner. Prolongation of the period of serum-free condition enhanced the apoptosis induced by thapsigargin treatment. Morphological observation with electron microscope further demonstrated that chromatin condensation and fragmentation, apoptotic bodies existed in TG-treated cells, supporting that thapsigargin is a potent activator of apoptosis in the cells.

Key words: *apoptosis, calcium, human hepatoma cells, thapsigargin, flow cytometry, electron microscope.*

INTRODUCTION

Apoptosis is a common form of cell death in eukaryocytes and play an important

1 Dedicated to Professor Yao Zhen's 80th Birthday.

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role in embryonic development and adult tissue regulation[i]. Like cell growth and differentiation, apoptosis is an active process which requires coordinated regulation of specific genes[2]. In addition, there is increasing evidence to suggest that apoptosis in most animal cells can be activated or suppressed by external signals[3]. As a member of second messengers, intracellular free calcium is considered to be important to cell growth, differentiation as well as apoptosis[4]. However, the precise mechanism by which Ca^{2+} signal regulates apoptosis is to be clarified.

Recently, we have found that thapsigargin (TG), an inhibitor of Ca-ATPase in surface of endoplasmic reticulum, produces a transient increase of intracellular Ca^{2+} in human hepatoma BEL-7404 cells and disturbs the response of intracellular Ca^{2+} following the stimulation with epidermal growth factor, due to the depletion of intracellular Ca^{2+} storage in endoplasmic reticulum[5]. Therefore, in the present study, the effects of thapsigargin on apoptotic cell death in BEL-7404 cells were investigated by using flow cytometry and morphological analysis.

MATERIALS AND METHODS

Cell culture

Human hepatoma cell line BEL-7404 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 13% fetal calf serum in 5% CO_2 incubator at 37°C and culture medium was changed every 48 h. After the cells grew into semi-confluent, the cells were kept in the serum-free medium, and treated with TG (Sigma) at indicated concentration for certain period before assay.

Transmission electron microscopy

BEL-7404 cells grown in monolayer culture were collected by trypsinizing. The cells were fixed in 5% glutaraldehyde, postfixed in 2% OsO_4 , and embedded with Epom812. Ultrathin sections were cut and stained with uranyl acetate and lead citrate and viewed in a JEM100B electron microscope.

Flow cytometry

After being trypsinized, the cells were centrifugated at $250 \times g$ for 5 min, and the cell pellet was resuspended in 1 ml hypotonic fluorochrome solution (propidium iodide $50 \mu\text{g/ml}$ in 0.1% sodium citrate plus 0.1% Triton X-100, Sigma). The sample was kept at 4°C in dark overnight. The propidium iodide fluorescence of individual cells was measured by using a FACStar Plus flow cytometry (Becton and Dickinson). The cell cycle distributions were analyzed, and the cells with lower DNA contents than those in G_1 phase cells were considered as apoptotic cells. Each value represented at least two separate experiments with more than 10,000 cells assayed.

RESULTS AND DISCUSSION

As shown in Fig 1, in the serum-free condition, TG (from $0.01 \mu\text{M}$ to $1 \mu\text{M}$) treatment for 48 h increased the rate of apoptotic cell death of BEL-7404 cells in a dose-dependent manner. Whereas in the presence of fetal calf serum (13%), treatment with TG, even at $1 \mu\text{M}$, did not produce the detectable increase in the rate of apoptosis. Furthermore, prolongation of the period of serum-free condition, from 24 h to 72 h, enhanced the rate of apoptosis induced by TG ($0.1 \mu\text{M}$ for 24 h) from

6.01% to 20.83% of total assayed cells (Fig 2). But serum-starvation itself demonstrated rather weak effect on apoptosis in BEL-7404 cells. These results suggest that serum-starvation and TG treatment have additive effects on apoptotic cell death.

Fig 1. Dose-dependence of TG-induced apoptosis in BEL-7404 cells.

In the presence (◆) or absence (◇) of fetal calf serum(13%) in the medium, the cells were treated with indicated concentrations of TG for 48 h and then stained with propidium iodide for flow cytometry analysis.

Fig 2. Effect of serum-starvation on TG-induced apoptosis in BEL-7404 Cells. The cells were treated with or without TG (0.1 μM) in the serum-free condition for 24 h after incubated in the serum-free medium for 0, 24, 48(h) respectively.

The ultrastructural features of the cells treated with TG were shown in Fig 3. In contrast to the control cells, the condensed and fragmented chromatin was observed in TG treated cells (Fig 3A and 3B), and the nuclear membrane as well as plasma membrane could keep intact for a certain period of time in TG-treated cells. In addition to the pycnotic nuclei, blebbing of apoptotic body containing organelles from the cell surface (Fig 3C) and appearance of apoptotic body containing fragmented chromatin in the cytoplasm can also be observed in TG treated cells (Fig 3D).

Cell death may occur by necrosis or through the specific process of apoptosis. Apoptosis, which is an active process triggered by internal or external signals, is morphologically characterized by condensation and fragmentation of chromatin, formation of apoptotic body; and existence of well-preserved plasma membranes. In TG-treated BEL-7404 hepatoma cells, typical morphological features of apoptosis

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Fig 2. Ultrastructure of BEL-7404 cells treated with or without TG for 48 h in serum-free medium.

(A) Control. ($\times 5,000$) (B) Treated with 0.1 μ M TG. ($\times 8,000$)

Fig 2. Ultrastructure of BEL-7404 cells treated with or without TG for 48 h in serum-free medium.

(C) Treated with 1 μ M TG. ($\times 5,000$) (D) Treated with 1 μ M TG. ($\times 5,000$)

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were observed by electron microscope and the increased rate of sub-G₁ cells, with lower DNA content than that in G₁ phase cells, was demonstrated by flow cytometric analysis. In addition, an assay of plasma membrane integrity showed more than 95% of cells excluded the charged dye, trypan blue, under the similar conditions mentioned above (data not shown). These findings indicate that TG induces apoptotic cell death in BEL-7404 cells.

DNA laddering in agarose gel electrophoresis has been considered as a marker of apoptosis in several cell systems. However, in our experiment, DNA ladder pattern was not detectable in TG-treated hepatoma BEL-7404 cells. It was reported that TGF β ₁-induced apoptosis occurred in rat liver tissue without DNA laddering[6]. On the other hand, the Morris hepatoma cells undergoing ischemic necrosis showed a distinct DNA ladder pattern without demonstrating the morphology of apoptosis[7]. Therefore, our data presented here support the notion that DNA laddering may not be a sensitive and specific indicator of apoptosis in hepatocytes.

Alteration in intracellular Ca²⁺ homeostasis can be critically involved in apoptosis, and sustained elevation of cytosolic Ca²⁺ is capable of inducing apoptosis[4], although the precise mechanism by which calcium promotes apoptosis is unclear. However, the endogenous Ca²⁺/Mg²⁺ dependent endonuclease appears to be activated by the mobilization of cytosolic Ca²⁺ [8]. Alternatively, Ca²⁺ might activate Ca²⁺/calmodulin-dependent kinase, leading to apoptosis[4]. TG, an inhibitor of Ca²⁺-ATPase in endoplasmic reticulum, produces an increase of intracellular Ca²⁺ in BEL-7404 cells[5]. Depletion of intracellular Ca²⁺ storage in endoplasmic reticulum results in the elevation of cytosolic Ca²⁺, and may cause the redistribution of Ca²⁺ in organelles, such as nucleus and mitochondria. TG-induced apoptosis occurred in serum-free condition, not in the presence of serum, suggesting that there are some factor(s) in serum, which act as the negative modulator(s) of apoptosis in TG-treated cells.

Previous focus of molecular oncology lay mainly in the control of cell proliferation and differentiation, however, there is now compelling evidence showing that the rate of cell death must be considered[9]. The induction of apoptosis is one of the major concerns in the recent development of therapeutic approach against cancer. TG, by inducing apoptosis of human cancer cells, may be considered as a novel potential agent in the treatment of cancer. Clarification of the biochemical pathways involved in the activation of apoptosis would lead to fundamental advances in cancer therapy.

ACKNOWLEDGEMENTS

The project is supported in part by Shanghai Joint Laboratory of Life Science Foundation.

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Received 12-4-1995. Revised 8-5-1995. Accepted 12-5-1995.