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Correlation between inhibition of calcium-dependent apoptosis by cyclosporin A and calcium transportation in HL-60 cells[1]

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ABSTRACT

Both calcium ionophore A_{23187} and endoplasmic reticulum Ca^{2+}- ATPase inhibitor thapsigargin (Tg) could increase intracellular free calcium concentration and induce apoptosis in some cell lines. In the present study, we found that HL-60 cells treated with A_{23187} (1 μg/ml) for 4 h or with Tg (0.5 μg/ml) for 2 h showed typical characteristics of apoptosis. Pretreatment with nontoxic concentration of cyclosporin A (CsA) (1 μg/ml) could block these effects. Flow cytometric analysis of intracellular Ca^{2+} after staining with fluo-3 AM showed that CsA did not prevent the increase of intracellular calcium induced by A_{23187} or Tg, but it could maintain the high level of intracellular Ca^{2+} for a long time. These results suggest that CsA may prevent calcium- induced apoptosis by blocking the transportation of Ca^{2+} in HL-60 cells.

Key words: *Cyclosporin A, calcium ionophore A_{23187}, thapsigargin, apoptosis, intracellular calcium.*

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INTRODUCTION

Cyclosporin A (CsA), an immunosuppressive agent, could selectively inhibit T lymphocyte activation and proliferation, and could also prevent activation-driven apoptosis[1]. Recently, evidences that CsA could block apoptosis were found in other cell lines such as T-cell hybridomas and B-cell lines[1, 2].

Ca^{2+} plays an important role in apoptosis. Many types of apoptosis are dependent on Ca^{2+}. In apoptosis of glucocorticoid-treated thymocytes, it has been postulated that activation of Ca^{2+}/Mg^{2+}-dependent endonuclease may be responsible for internucleosomal DNA fragmentation. In that system, elevation of intracellular Ca^{2+} concentration appeared to serve as an early signal for initiation of apoptosis[3]. In other cell lines, increasing intracellular Ca^{2+} with calcium ionophore or Tg also leads to apoptosis[4, 5].

In this paper, questions whether A_{23187} or Tg could induce apoptosis in HL-60 cells, and whether the induced apoptosis could be inhibited by CsA were investigated. Then, the possible correlation between calcium-transportation and CsA was examined, in order to find out the way by which CsA blocks apoptosis.

MATERIALS AND METHODS

Materials

Cyclosporin A was purchased from Sino-Amercian Medicine of East China Ltd. Propidium iodide, A_{23187}, thapsigargin and Fluo-3 AM were purchased from Sigma. Hoechst 33342 was purchased from Molecular Probe.

Cell culture and drug dosage-reaction curves

HL-60 cells were grown at 37℃ in RPMI 1640 medium (GIBCO) containing 10% heat-inactivated fetal bovine serum in an atmosphere with 5% CO_2. Cells in log phase were planted to 24 well plate at the density of 20×10^4 cells/ml, and various concentration of CsA was added to the medium. Cell numbers were counted at 12, 24, 48 h respectively, and cell viability was assessed as follows.

Drug treatment and cell viability assessment

Exponentially growing HL-60 cells were exposed to drugs for the time as indicated. Cells after drug treatment were stained with Hoechst 33342 (10 μM) and propidium iodide (50 μg/ml) for 20 min, then washed with PBS and resuspended in PBS. Morphological and quantitative analysis of apoptosis was performed with fluorescence microscopy (Olympus) as described[6].

DNA extraction and electrophoresis

The pattern of DNA cleavage was analyzed by agrose gel electrophoresis as described[7]. Briefly, cells (1×10^6) were lysed with 200 μl lysis buffer (10 mM EDTA; 50 mM Tris-HCL, pH 8.0; 0.5%(W/v) N-lauroyl sarcosine; 0.5 mg/ml proteinase K) and incubated for 1 h at 50℃. Then RNase A was added to a final concentration of 0.5 mg/ml, and incubated for another hour at 50℃. After phenol extraction and ethanol precipitation, samples of 1.5 μg in each lane were subjected to electrophoresis on a 1.2 % agrose gel. DNA was stained with ethidium bromide.

Flow cytometry analysis

Flow cytometric analysis was also performed to identify apoptotic cells as descried[6]. Briefly,

cells were fixed in 70% ethanol overnight at 4℃, incubated in PBS containing 50 μg/m] RNase A at 37℃ for 1 h, stained with 65 μg/ml PI for 1 h at 4℃, and then analyzed by the use of a FACS 420 flow cytometer.

Determination of intracellular Ca^{2+} concentration

Ceils were loaded with 10 μM Fluo-3 AM for at least 30 min at 37℃, washed by centrifugation for 3 min at 800 ×g and resuspended in RPMI 1640 medium (37℃)[8]. Intracellular Ca^{2+} concentration was measured by flow cytometry.

RESULTS

Inhibitory effect of CsA on the HL-60 cell proliferation

As shown in Fig 1, the maximum concentration of CsA that did not obviously affect the growth of HL-60 cells was 3 μg/ml. Combined staining with PI and Hoechst 33342 revealed that cells treated with 0.5~3μg/ml CsA did not undergo apoptosis or necrosis (< 5%) within 24 h.

Apoptosis of HL-60 cells induced by A_{23187} or Tg

Exposure of HL-60 cells to A_{23187} (1μg/ml) for 4 h or Tg (0.5 μg/ml) for 2 h led to apoptosis (Fig 2). Combined staining with Hoechst 33342 and PI showed condensed nuclei in a large number of cells (Fig 3B, 3C), and the necrotic cells (PI positive) was below 5% (data not shown). Agarose gel electrophoresis of DNA revealed a "ladder" pattern (Fig 4 lane 2, 4). Apoptotic DNA peak could be seen in DNA histogram after flow cytometric analysis (Fig 5A).

CsA blocks apoptosis induced A_{23187} or Tg

Pretreatment of HL-60 cells with nontoxic CsA (0.5~3 μg/ml) for 4 h, then added A_{23187} to a final concentration of 1 μg/ml or Tg to 0.5 μg/ml. The amount of apoptotic cells were greatly decreased (Fig 2).

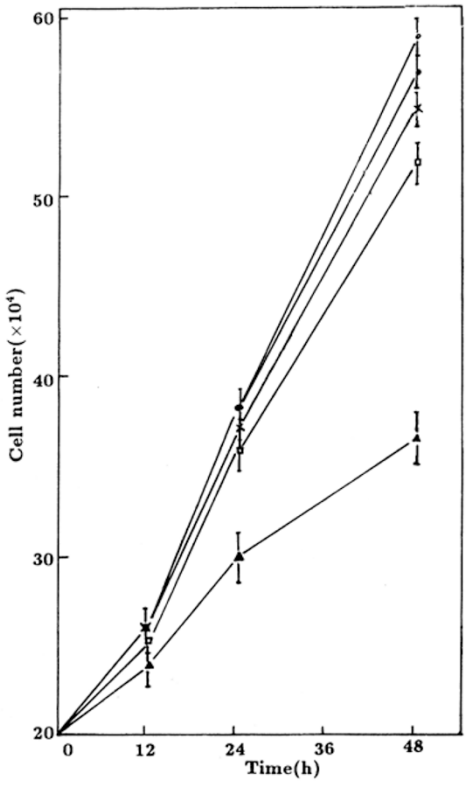

Fig 1. Growth curves of HL-60 cells treated with CsA. ○, control; ●, 0.5 μg/ml CsA; ×, 1 μg/ml CsA; □, 3 μg/ml CsA; ▲, 5 μg/ml CsA.

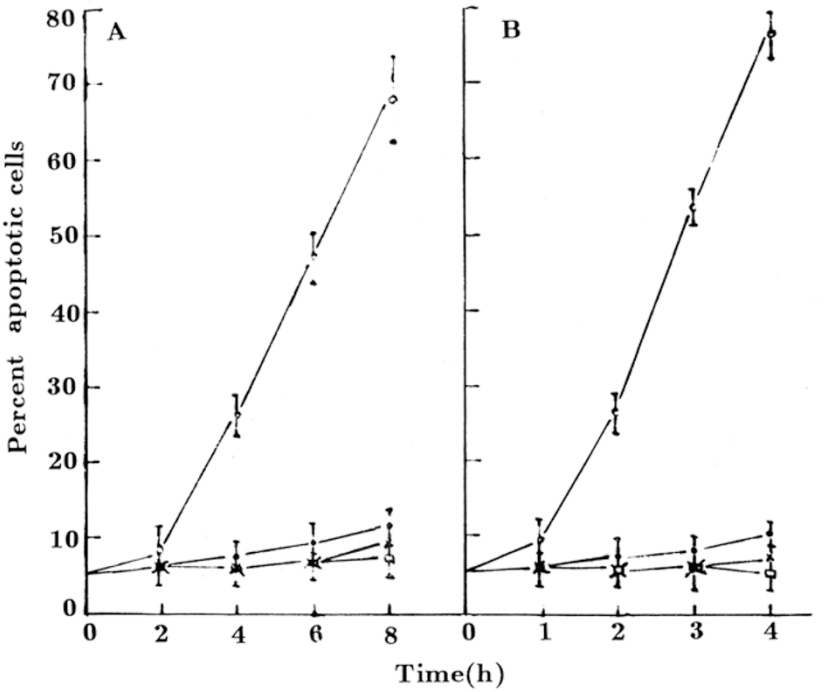

Fig 2. Percent of apoptotic cells in HL-60 cells preincubated with or without CsA for 4h, then treated with A_{23187} or Tg for various time.
A. ○, A_{23187} (1 μg/ml) only; •, CsA(3 μg/ml)+A_{23187}(1 μg/ml); ×, CsA(1 μg/ml)+A_{23187}(1 μg/ml); □, CsA(0.5 μg/ml)+A_{23187} (1 μg/ml).
B. ○, Tg(0.5 μg/ml) only; •, CsA(3 μg/ml)+Tg (0.5 μg/ml); ×, CsA(1 μg/ml)+Tg (0.5 μg/ml); □, CsA (0.5 μg/ml)+Tg(0.5 μg/ml).

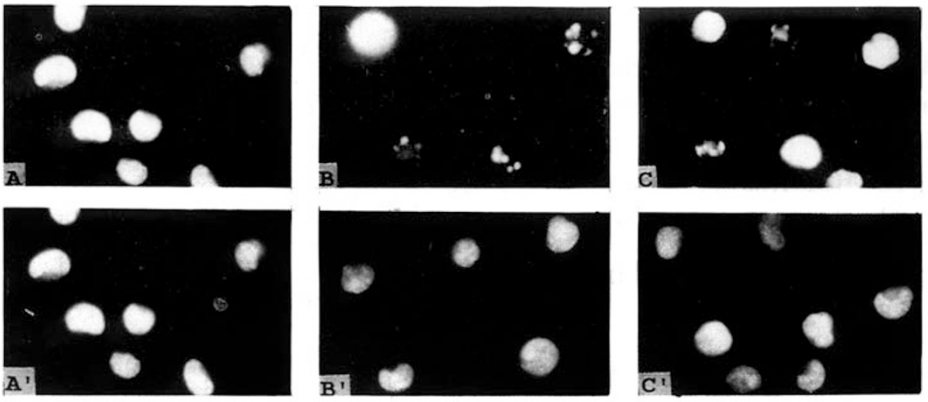

Fig 3. Morphological appearance of HL-60 cells stained with Hoechst 33342 and observed under fluorescence microscope. (A) and (A'), control. (B), A_{23187} 1 μg/ml for 4 h. (B'), CsA 1 μg/ml for 4 h, then A_{23187} 1 μg/ml for 4 h. (C), Tg 0.5 μg/ml for 2 h. (C'), CsA 1 μg/ml for 4 h, then Tg 0.5 μg/ml for 2 h. × 400.

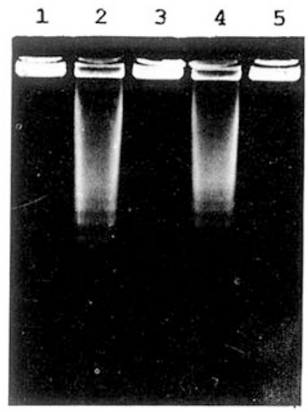

Fig 4. Agarose gel electrophoresis of DNA extracted from HL-60 cells. Cells were pretreated with (lane 3, 5) or without (lane 2, 4) CsA 1 μg/ml for 4 h, then treated with A_{23187} 1 μg/ml for 4 h (lane 2) or Tg 0.5 μg/ml for 2 h (lane 4). Lane 1, control.

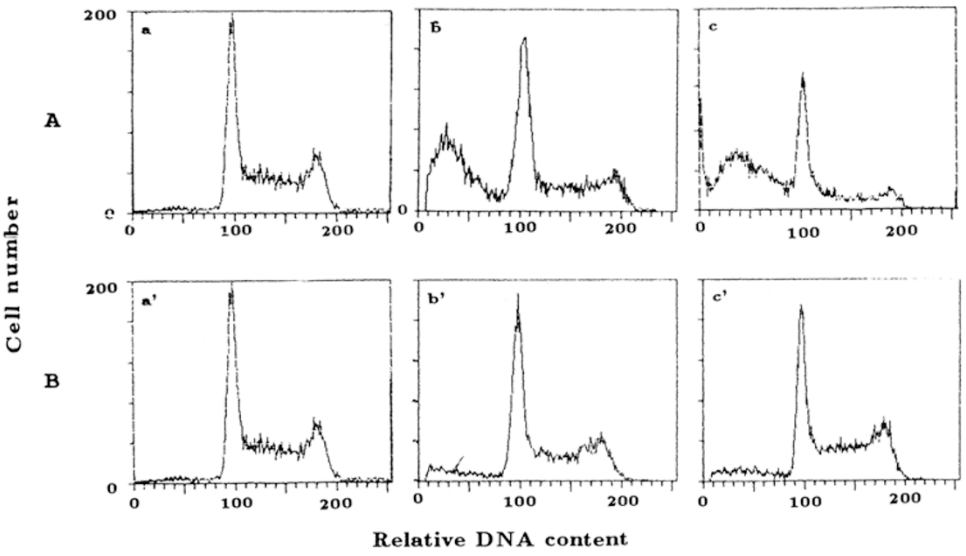

Fig 5. Flow cytometric studies of propidium iodide-stained HL-60 cells. Cells were pretreated with (B) or without (A) CsA 1 μg/ml for 4 h, then treated with A_{23187} 1 μg/ml for 4 h (b, b') or Tg 0.5 μg/ml for 2 h (c, c'). (a) and (a') control. Apoptotic cells can be recognized by their diminished stainability with propidium iodide and appearance of a "sub-Gl" peak.

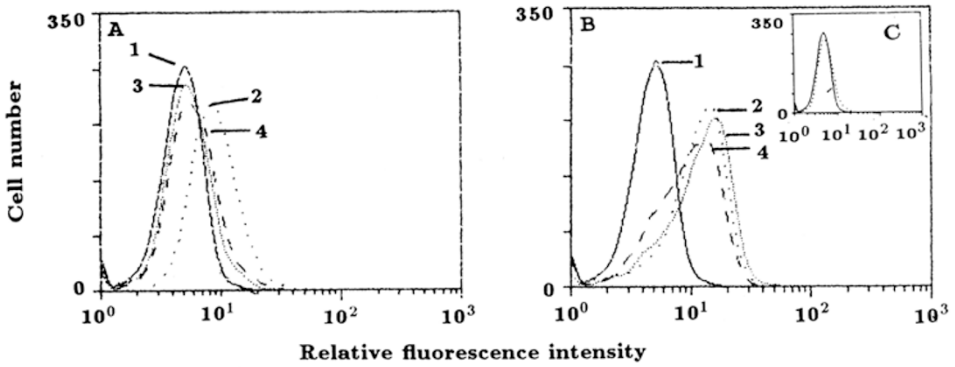

Fig 6. Intracellular Ca^{2+} levels in HL-60 cells. Histograms (log scale) of fluorescence intensity after staining with fluo-3 AM. Cells were preincubateed with (B) or without (A) CsA 1 μg/ml for 4 h, then treated with A_{23187} 1 μg/ml for various time. (1) Control, (2) 10 min, (3) 30 min, (4) 60 min. (C) control, CsA 1 μg/ml for 4 h.

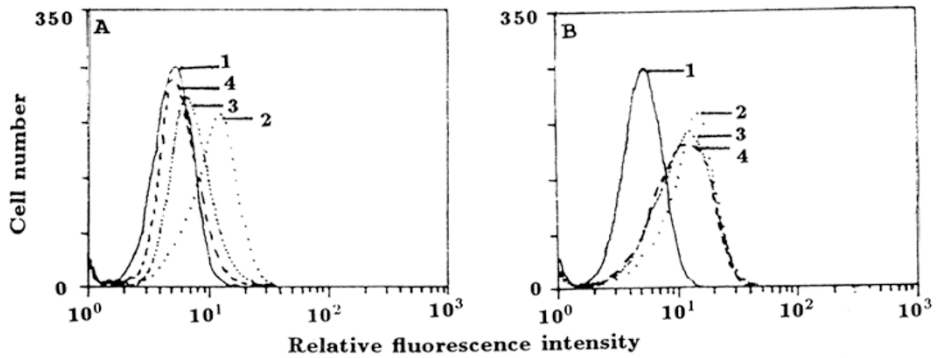

Fig 7. Intracellular Ca^{2+} levels in HL-60 cells. Histograms (log scale) of fluorescence intensity after staining with fluo-3 AM. Cells were preincubated with (B) or without (A) CsA 1 μg/ml for 4 h, then treated with Tg 0.5 μg/ml for various time. (1) Control, (2) 5 min, (3) 20 min, (4) 40 min.

When appropriate concentration of CsA (1 μg/ml) was used for further study, we could see those characteristics of apoptosis induced by A_{23187} or Tg disappeared (Fig 3B', 3C'; Fig 4 lane 3, 5; Fig 5B).

Alteration of intracellu]ar Ca^{2+} level

Measurement of intracellular Ca^{2+} showed that A_{23187} increased the intracellular

Ca^{2+}, but the high level of intracellular Ca^{2+} decreased quickly (Fig 6A). CsA did not prevent the increase of intracellular Ca^{2+} induced by A_{23187}, but it could maintain the high level of intracellular Ca^{2+} for much longer time (Fig 6B). However, CsA alone did not alter the intracellular Ca^{2+} concentration (Fig 6C). The same result was got with Tg (Fig 7).

DISCUSSION

Our results showed that both A_{23187} and Tg could induce apoptosis of HL-60 cells, which indicated that Ca^{2+} is an important factor during apoptosis of HL-60 cells. But high level of intracellular Ca^{2+} was not required to maintain for long time for the induction of apoptosis. So it seems that Ca^{2+} only initiates apoptosis, and other later events induced by Ca^{2+} such as activation of Ca^{2+}/Mg^{2+}-dependent endonuclease etc. finally lead to apoptosis, as observed in other system[3].

Studies on apoptosis VP-16 treated HL-60 cells indicated that no significant increase of intracellular Ca^{2+} was observed. Pretreatment with EGTA failed to prevent apoptosis induced by VP-16. On the contrary, BAPTA-AM, a chelator of intracellular Ca^{2+}, could inhibit it. These evidences suggested that in HL-60 cells, apoptosis may depend not on extracellular Ca^{2+}, but on intracellular Ca^{2+}. So it may be the redistribution of intracellular Ca^{2+} that plays an important role in apoptosis of HL-60 cells, especially the elevation in nuclear Ca^{2+} due to Ca^{2+} influx from cytosol fraction[9].

Our results showed that the high level of intracellular Ca^{2+} induced by A_{23187} or Tg maintained for a short time, and decreased quickly. Yet the increase is sufficent to initiate apoptosis. The reason may be that high level of intracellular Ca^{2+} activated Ca^{2+}- ATPase located in the membrane of intracellular Ca^{2+} pool and nuclear evelope[10], and thus induced the redistribution of intracellular Ca^{2+}, especially the transportation of Ca^{2+} into nucleus which finally leads to apoptosis. At the same time, Ca^{2+}- ATPase on plasma membrane was also activated, which pumped excessive Ca^{2+} out of the cell.

It is not clear how CsA blocks apoptosis. Since it is a specific inhibitor of protein phosphatase 2B (calcineurin), some people thought that CsA prevents apoptosis by inhibiting the activity of calcineurin[11]. Recently, CsA was found to affect some other factors involved in apoptosis, such as "tissue" transglutaminase (tTG)[12], and transcription factor Nur77[13]. According to our results, CsA prevented the decrease of intracellular Ca^{2+}, indicating that CsA may inhibit transportation of Ca^{2+}. Since CsA could inhibit the synthesis of ATP[14], so the subsequent loss of energy supply may lead to inactivation of Ca^{2+}-ATPase. As a result, Ca^{2+} could not be pumped out of the cell, intracellular Ca^{2+} may then be maintained at a high level. At the same time, Ca^{2+} could not flux into nucleus, and thus the low intranuclear Ca^{2+} concentration would be insufficient to activate the endonuclease, and apoptosis couldn't occur. However, this hypothesis needs much further study to verify.

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