Induction of Apoptosis by Intracellular Ca^{2+} Inhibitors in Human Leukemic HL-60 Cells

Yong Soo Lee, Jin Hyen Baek[1], Jung-Ae Kim[2], Han Chul Son[3] Kyung Sool Kwon[3] and Kyu-Won Kim[1,3]

Department of Physiology, College of Medicine, Kwandong University,
Kangnung 210-701; [1]Department of Molecular Biology, Pusan National University,
Pusan 609-735; [2]College of Pharmacy, Yeungnam University, Kyongsan 712-749;
[3]Pusan Cancer Research Center, Pusan National University, Pusan 602-739, Korea

Apoptosis which plays an important role in tumorigenesis as well as embryo development has been known to be induced by the sustained rise in intracellular free Ca^{2+}. However, many studies have shown that apoptosis is not always related with increased intracellular free calcium concentration. In the present study, the effect of several intracellular Ca^{2+} inhibitors on cell death was investigated in order to evaluate the role of intracellular Ca^{2+} signal inhibition in apoptotic cell death using HL-60 human promyelocytic leukemia cells as a model cellular system. Plasma membrane Ca^{2+} channel blockers (verapamil, nifedipine and diltiazem), intracellular Ca^{2+} release blockers (dantrolene, TMB-8 and ruthenium red), an extracellular Ca^{2+} chelator (EGTA) and an intracellular Ca^{2+} chelator (BAPTA/AM) were used as intracellular Ca^{2+} inhibitors in the experiments. Treatment with these agents resulted in a concentration-dependent decreased cell viability assessed by MTT assay. These agents also induced genomic DNA fragmentation, a hallmark of apoptosis, indicating that the mechanism by which these agents induce cell death was through apoptosis. No effect of cycloheximide, a protein synthesis inhibitor, on the cell death was observed, implying that new protein synthesis may not be required for the apoptosis caused by these intracellular Ca^{2+} inhibitors. These results suggest that the inhibition of intracellular Ca^{2+} signals may be involved in the induction of apoptosis in HL-60 cells.

Key Words: HL-60 cell, Apoptosis, Cycloheximide, BATA/AM, Dantrolene, Diltiazem, EGTA, Nifedipine, Ruthenium red, Verapamil
Abbreviations: BAPTA/AM, bis-(o-aminophenoxy)-ethane- N,N,N',N'-tetraacetic acid/ acetoxymethyl ester; CHX, cycloheximide; Dant, dantrolene; Dilt, diltiazem; EGTA, ethylene glycol-bis(-aminoethyl ether)N,N,N,N-tetraacetic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyl tetrazolium bromide; Nife, nifedipine; RR, ruthenium red; TMB-8, 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester; Vera, verapamil.

system.

INTRODUCTION

Apoptosis, a naturally occurring form of cell death, has been reported to be involved in the various cellular processes.[1] The common feature of apoptosis, which is quite different from that of necrosis, has been described as morphologically and biochemically. The morphological events characterizing apoptosis are cytoplasmic and nuclear condensation, membrane blebbing and formation of apoptotic body.[2] The biochemical events underlying apoptosis are internucleosomal chromatin fragmentation which is a "hallmark" of apoptosis, and the activation of Ca^{2+}-dependent endonuclease and Ca^{2+}-dependent transglutaminase.[3]

Apoptosis has been demonstrated to play an important role in carcinogenesis.[4,5] Recently, many researchers have tried to take advantage of apoptosis as a tool for tumor therapy and prevention.[6] In fact, many of the presently used chemotherapeutic agents induce apoptosis.[7] Thus, in order to develop more effective and specific cancer therapies, the understanding of the regulatory mechanism of apoptosis is required. Moreover, there has been great interest in the intracellular events mediating apoptosis.

Prolonged increase in the level of intracellular free Ca^{2+} appears to be a common mediator of chemically induced cytotoxicity.[8] Increased intracellular Ca^{2+} concentration has also been implicated in the induction of apoptosis in a variety of cells.[9,10] However, the opposite phenomenon has been found that in neuronal cells decreased intracellular Ca^{2+} concentration by an intracellular Ca^{2+} chelator, can also induce apoptosis.[11]

Thus, in this study we investigated the role of inhibition of intracellular Ca^{2+} signals in the induction of cancer cell apoptosis using HL-60 human promyelocytic leukemia cells as a model cellular

MATERIALS AND METHODS

1) Materials

HL-60 human promyelocytic leukemia cell line was purchased from American Type Culture Collection (Rockville, MA). The powder for RPMI 1640 medium, fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). Verapamil (Vera), nifedipine (Nife), diltiazem (Dilt), dantrolene (Dant), 3,4,5-trimethoxybenzoic acid-8-(diethylamino)octyl ester (TMB-8), ruthenium red (RR), ethylene glycol-bis(-aminoethyl ether)N,N,N, N-tetraacetic acid (EGTA), cycloheximide (CHX) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), were obtained from Sigma Chemical CO. (St. Louis, MO). Bis-(o-aminophenoxy)-ethane-N,N,N',N'-tetraacetic acid/acetoxymethyl ester (BAPTA/AM) was from Molecular Probes, Inc. (Eugene, OR). Unless otherwise indicated, all other chemicals were the purest grade available and were obtained from Sigma. Vera, Nife, Dilt and TMB-8 were dissolved in ethanol. Dant and BAPTA/AM were dissolved in dimethylsulfoxide (DMSO), and other drugs were dissolved in distilled water. These stock solutions were sterilized by filtration through 0.2 μm disc filters (Gelman-Sciences: Ann Arbor, MI).

2) Cell culture

HL-60 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 200 IU/ml penicillin and 200 μg/ml of streptomycin at 37°C in a humidified incubator under 5% CO$_2$/95% air. The cells were split in a 1 : 5 ratio every two or three days.

3) Cell viability test (MTT staining)

Cell viability was assessed as described by Mosmann.[12] Briefly, the cells were incubated in 100 μl of media in 96-well plates with the indicated concentration of the compounds for 24 hr, at an initial cell density of 3×10^5 cells/ml. An appropriate volume of drug vehicle was added to untreated cells. After each period of incubation, 10 μl of MTT solution (5 mg MTT/ml in H$_2$O) were added and the cells were further incubated for 4 hr. One hundred μl of acid-isopropanol (0.04 .N HCl in isopropanol) were added to each culture and mixed by pipetting to dissolve the reduced MTT crystals. The relative cell viability was obtained by scanning with an ELISA reader (Molecular Devices Corp., USA) with a 570 nm filter.

4) DNA isolation and electrophoresis

Following incubation for 24 hr with the intracellular Ca^{2+} inhibitors, HL-60 cells were collected by centrifugation ($200 \times g$, 5 min), washed twice in phosphate-buffered saline, pH 7.4, and resuspended at the density of 2×10^6 cells/200 μl in lysis buffer containing 150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl, pH 8.0. To each 200 μl of cell suspension, 25 μl of proteinase K (10 μg/μl) and 25 μl of 10% sodium dodesyl sulfate (SDS) were added. Samples were incubated at 70°C for 90 min. Ten μl of RNAse A (100 units/200 μl) was added and the incubation continued for a further 60 min.

The total purified DNA from each sample was dissolved in 15 μl of water and mixed with 1.5 ml loading buffer (10 mM EDTA, 0.25% (w/v) bromophenol blue, 50% (w/v) glycerol), before application onto 1.5% agarose gels. Electrophoresis was carried out in TBE buffer (90 mM Tris, 90 mM sodium borate, 2 mM EDTA, pH 8.0), and the gels were run at 55 V for 4 hr. The pattern of DNA

fragmentation was visualized under UV light, after staining the gel with ethidium bromide (0.5 μg/ml in TBE buffer).

5) Data analysis

All experiments were performed four times. All data were displayed as % of control condition. All the control experiments were carried out in the same media containing drug-free vehicle. Data were expressed as mean±standard error of the mean (S.E.M.) and were analyzed using one way analysis of variance and Student-Newman-Keul's test for individual comparisons. P values less than 0.05 are considered to be statistically significant.

RESULTS

As shown in Fig. 1, Vera, Nife and Dilt, known L-type Ca^{2+} channel blockers which antagonize extracellular Ca^{2+} influx through the Ca^{2+} channels,[13] decreased cell viability in a dose-dependent manner in HL-60 cells. TMB-8 and Dant, inhibitors
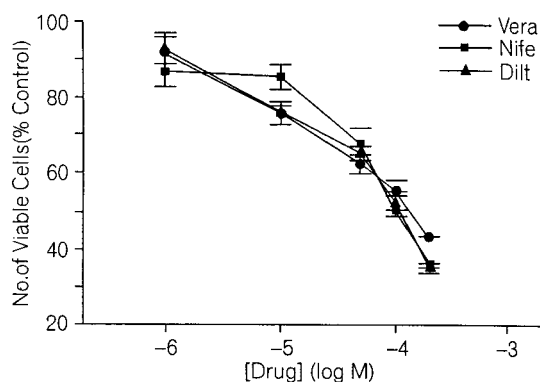

Fig. 1. Decreased cell viability by Ca^{2+} channel antagonists in HL-60 human promyelocytic leukemia cells. Cell viability was assessed by MTT staining. Results are expressed as the percent change of the control condition in which the cells were grown in the medium containing the drug-free vehicle. Data points represent the mean values of four replicates with the bars indicating S.E.M.
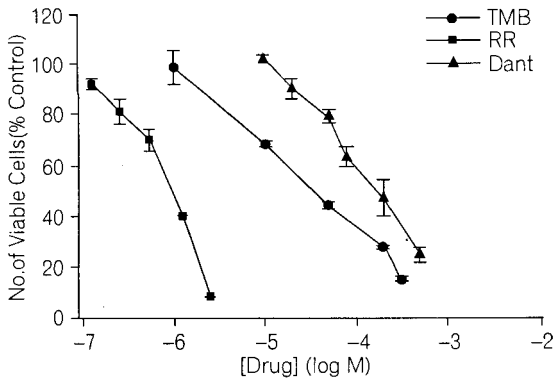
Fig. 2. Decreased cell viability by intracellular Ca^{2+} release blockers in HL-60 human promyelocytic leukemia cells. Cell viability was assessed by MTT staining. Results are expressed as the percent change of the control condition in which the cells were grown in the medium containing the drug-free vehicle. Data points represent the mean values of four replicates with the bars indicating S.E.M.
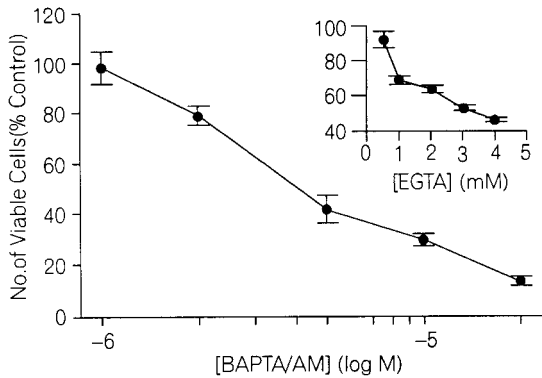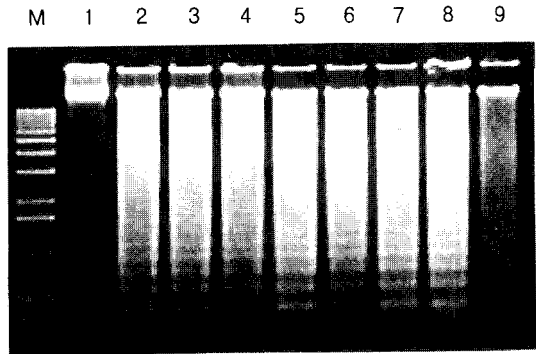

Fig. 4. Photograph of 1.5% agarose gel electrophoresis of DNA from HL-60 cells. The cells were treated for 24 hr without or with intracellular Ca^{2+} inhibitors. DNA was isolated from the cells and analyzed by agarose gel electrophoresis. *M*, DNA marker; *Lane 1*, Control; *Lane 2*, 50 μM Vera; *Lane 3*, 50 μM Nife; *Lane 4*, 50 μM Dlit; *Lane 5*, 10 μM TMB-8; *Lane 6*, 10 μM Dant; *Lane 7*, 10 μM RR; *Lane 8*, 1 μM BAPTA/AM; *Lane 9*, 2 mM EGTA.

Fig. 3. Decreased cell viability by Ca^{2+} chelators in HL-60 human promyelocytic leukemia cells. Cell viability was assessed by MTT staining. Results are expressed as the percent change of the control condition in which the cells were grown in the medium containing the drug-free vehicle. Data points represent the mean values of four replicates with the bars indicating S.E.M.

of intracellular Ca^{2+} release,[14,15] and RR, another inhibitor of intracellular Ca^{2+} release, specifically from the ryanodine-sensitive Ca^{2+} pools,[16] showed the similar results as depicted in Fig. 2. BAPTA/AM

and EGTA, an intracellular[10] and an extracellular Ca^{2+} chelator,[17] respectively, also reduced cell viability in a dose-related fashion as shown in Fig. 3.

In order to determine whether cell death induced by these agents, occurs through an apoptotic pathway, an assay for the genomic DNA fragmentation, a hallmark of apoptotic cell death,[2] was performed. As shown in Fig. 4, treatment with the intracellular Ca^{2+} inhibitors at concentrations associated with significant reduction of cell viability, produced DNA fragmentation, indicating that these agents induced apoptosis in the HL-60 cells.

Since new protein synthesis was reported to be required for the induction of apoptosis,[18] the effect of CHX, a protein synthesis inhibitor,[19] on the cell death induced by the intracellular Ca^{2+} inhibitors, was investigated. Fig. 5 revealed that CHX did not significantly alter the viability-reducing activities of these agents at two different concentrations. Three μM of CHX alone resulted in a significant re-
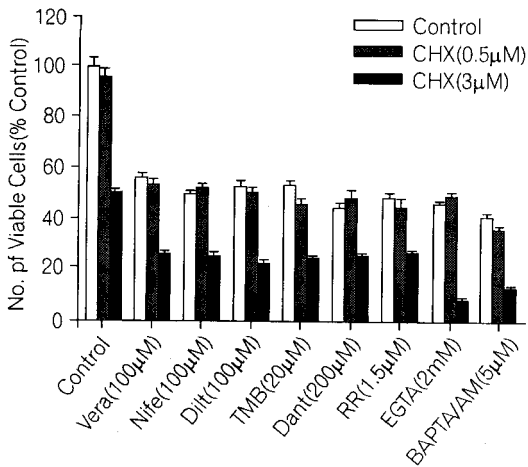
Fig. 5. Effect of CHX on the decreased viability induced by intracellular Ca^{2+} inhibitors in HL-60 human promyelocytic leukemia cells. CHX was added to the cells 4 hr before the treatment with intracellular Ca^{2+} inhibitors. Cell viability was assessed by MTT staining. Results are expressed as the percent change of the control condition in which the cells were grown in the medium containing the drug-free vehicle. The data represent the mean values of four replicates with the bars indicating S.E.M.

duction of cell viability.

DISCUSSION

Increased intracellular Ca^{2+} concentration is thought to play a regulatory function in apoptosis.[9,10] Although the precise mechanism by which intracellular Ca^{2+} promotes cell death is unclear, the endogenous Ca^{2+}/Mg^{2+}-dependent endonuclease which mediates the double-strand cleavage of DNA at nucleosome linker regions,[3,20] remains an attractive target for the effects of Ca^{2+}. This endogenous enzyme appears to be activated by mobilization of cytosolic Ca^{2+}.[20]

On the other hand, a number of studies have shown that apoptosis is not always associated with an increased intracellular Ca^{2+} concentration.[22,23] Moreover, in HL-60 cells the induction of apoptosis

by treatment with Ca^{2+} ionophore, was correlated with intracellular acidification rather than increased intracellular Ca^{2+} concentration.[24]

The results of the present study clearly showed that the inhibition of intracellular Ca^{2+} signals by the intracellular Ca^{2+} inhibitors induced apoptosis in the HL-60 cells (Fig. 1-4). The precise mechanism of the actions of these agents is unknown at present, and only can be speculated. Cell survival and cell death are regulated by multiple factors.[25] The disruption of these balanced factors can induce cell death.[26] Although these factors exert their functions through diverse signal transduction pathways, intracellular Ca^{2+} has been known as one of important signal transducers in these pathways.[27,28] Thus, the inhibition of intracellular Ca^{2+} signals may resemble the deprivation of cell survival factors, which leads to induction of cell death. Under these circumstances, even though cell survival factors exist, they will not function correctly. The functional inhibition of cell survival factors, therefore, can be one of the possible mechanisms of the apoptosis-inducing actions of intracellular Ca^{2+} inhibitors. Furthermore, no effect of CHX on the cell death induced by these agents (Fig. 5) imply that the intracellular Ca^{2+} inhibitors induce apoptosis not by synthesis of cell death proteins, but by modulation of activity of pre-existing cell survival factors.

Other reported data pointed that physiological elevation of intracellular Ca^{2+} concentration blocked apoptosis induced by the deprivation of nerve growth factor in rat sympathetic neurons[29] and in neutrophils.[30] In addition, the basal activity of protein kinase C (PKC) which is dependent upon intracellular Ca^{2+} concentration, prevents apoptosis in HL-60 cells.[31] These findings support the proposed hypothesis that down-regulation of intracellular Ca^{2+} signals may trigger apoptotic cell death.

Based on these results, we suggest that inhibition of intracellular Ca^{2+} signals can induce apoptosis in HL-60 cells. Further, the apoptosis induced by these intracellular Ca^{2+} inhibitors may be related with the down-regulation of the activities of intracellular Ca^{2+}-dependent cell survival factors. The induction of apoptosis by intracellular Ca^{2+} inhibitors may be a potential tool for the therapy-related study of human leukemia.

ACKNOWLEDGMENTS

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