Magnolol induces apoptosis in human leukemia cells via cytochrome *c* release and caspase activation

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Magnolol, isolated from the stem bark of *Magnolia officnalis*, was found to inhibit proliferation of human HL-60 cells and Jurkat T leukemia cells via inducing apoptosis in a dose- and time-dependent manner. By contrast, magnolol did not cause apoptosis in neutrophils and peripheral blood mononuclear cells of healthy donors. Apoptosis was determined by detection of DNA fragmentation in gel electrophoresis, morphological alternations by flow cytometry, quantification of phosphatidylserine externalization by Annexin V labeling and oligonucleosomal DNA content by TUNEL labeling. Activation of caspase-9, -3 and -2, and the proteolytic cleavage of poly(ADP-ribose) polymerase were found during apoptosis induced by magnolol. In addition, both pan-caspase and selective caspase-9 inhibitor blocked magnolol-induced apoptosis. The apoptosis could also be partially attenuated by caspase-3 and -2 inhibitors. Magnolol induced the reduction of mitochondrial transmembrane potential and the release of cytochrome *c* into cytoplasm. In conclusion, our findings indicate that magnolol-induced apoptotic signaling is carried out through mitochondria alternations to caspase-9 and that then the downstream effector caspases are activated sequentially. Magnolol could be a potentially effective drug for leukemia with low toxicity to normal blood cells and it merits further investigation. *Anti-Cancer Drugs* 14:211–217 © 2003 Lippincott Williams & Wilkins.

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Introduction

Magnolol (5,5′-diallyl-2,2′-dihydroxybiphenyl), a hydroxylated biphenyl compound isolated from the stem bark of Chinese herb *Magnolia officnalis* (Magnoliaceae), is usually used to treat acute pain, cough, anxiety and gastrointestinal disorders in Eastern Asia. Various pharmacological effects are reported to result from magnolol, including an anti-inflammatory effect, an anti-microbial effect, inhibition of central neurons and scavenging of hydroxyl free radicals [1–4]. However, to our knowledge, possible anti-cancer or apoptosis-inducing effects of magnolol have not been investigated.

In the present study, we investigated the apoptotic effects of magnolol on HL-60 and Jurkat T leukemia cells, and show that the drug inhibits the growth of HL-60 and Jurkat cells by inducing apoptosis. We also elucidated the mechanism of magnolol-induced apoptosis.

Materials and methods

Magnolol

Magnolol was purchased from Pharmaceutical Industry Technology and Development Center (Taiwan). Anti-cytochrome *c* and anti-poly(ADP-ribose) polymerase (PARP) antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Caspase substrates and caspase inhibitors were obtained form Calbiochem (La Jolla, CA). Rhodamine 123 was purchased from Molecular Probes (Eugene, OR). Fetal bovine serum (FBS), RPMI 1640 medium, penicillin and streptomycin were obtained from Gibco/BRL (Grand Island, NY).

Cell culture

Human HL-60 cells and Jurkat T leukemia cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 20 mM HEPES (pH 7.4), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in a humidified atmosphere of 5% CO_2. They were subcultured twice a week and only those in the exponential growth phase were used in these experiments.

Cytotoxicity assay

For investigating the growth inhibitory effects of magnolol, HL-60 or Jurkat cells were incubated with different concentrations of magnolol for 12–72 h at an initial cell

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density of 2×10^5 cells/ml. After each indicated period of incubation, viable cell numbers were determined by hemocytometry using the Trypan blue dye exclusion method.

DNA fragmentation analysis with agarose gel electrophoresis

After treatment with magnolol, 2×10^6 cells at each indicated time point were harvested and suspended with lysis buffer containing 20 mM NaCl, 10 mM Tris–HCl (pH 8.0), 25 mM EDTA, 1% sodium dodecylsulfate and 1 mg/ml proteinase K for 24 h in a 55°C water bath. The standard phenol:chloroform:isoamyl alcohol method (25:24:1) was used to remove protein and extract nucleic acid. RNA was digested with RNase A (200 μg/ml) for 12 h at 37°C. DNA extracts were electrophoresed on a *2%* agarose gel at 50 V for 45 min and visualized with ethidium bromide staining under UV illumination.

Analysis of apoptotic cells by phosphatidylserine (PS) externalization

Cells (1×10^6) were suspended in 100 μl Annexin V-binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl and 5 mM $CaCl_2$). Thereafter, Annexin V–FITC (Boehringer Ingelheim, Vienna, Austria) and propidium iodide (PI) were added to a final concentration of 5 μg/ml. Cells were incubated for 15 min at room temperature in the dark and subsequently diluted with 300 μl binding buffer for flow cytometric analysis. Bivariate analysis was performed on a FACSCalibur (Becton Dickinson, Mountain View, CA) equipped with a 488 nm argon laser for excitation. Emission filters were BP 530/30 nm (FITC) and BP 585/42 nm (PI). Data analysis was performed using the standard CellQuest software (Becton Dickinson). Cells with positive Annexin V but negative PI fluorescence were defined as apoptotic.

Detection of apoptosis by morphological criteria

Apoptotic cells exhibit morphological alternations that are readily detected by flow cytometry according to their light scatter properties [5]. After treatment with magnolol, HL-60 cell shrinkage and increased granularity were analyzed following the FSC and SSC criteria, respectively.

TUNEL assay

HL-60 cells were fixed and stored in 70% ethanol at 4°C following PBS wash. The TUNEL labeling [6,7] of cells was performed using a commercially available kit (Boehringer Mannheim, Indianapolis, IN). After discarding the ethanol solution, cells were permeabilized with 500 μl of 0.1% sodium citrate and 0.1% Triton X-100 for 1 min. The permeabilized solution was then removed and the cell pellet was washed with PBS containing 1% BSA. Approximately 2×10^6 cells were resuspended in 50 μl of the manufacturer's labeling solution containing TdT enzyme and FITC–dUTP, and incubated for 60 min at 37°C. Subsequently, the cells were washed, resuspended in 500 μl PBS containing 1% BSA and subjected to flow cytometry.

Caspase activity

For studying the caspases activation profile in magnolol-induced apoptosis, we assayed caspase activity using a commercial assay kit (Promega, Madison, WI). Cells were harvested in lysis buffer [25 mM HEPES, 1 mM EGTA, 5 mM EDTA, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 0.01% 3-(3-cholamidopropyl)dimethyl-ammonio-1-propane sulfonate (CHAPS), 10 μgml pepstatin, 10 μgml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.4]. Cell lysates were clarified by centrifugation at 10 000 *g* for 5 min and clear lysates containing 50 μg of protein were incubated with 25 μM Ac-DEVD-AMC (caspase-3 substrate), Ac-YVAD-AMC (caspase-1 substrate), Ac-VDVAD-AMC (caspase-2 substrate), Ac-VEID-AMC (caspase-6 substrate), Ac-IETD-AMC (caspase-8 substrate) or Ac-LEHD-AMC (caspase-9 substrate), 25 μM each, at 37°C for 1 h. Levels of released 7-amino-4-methylcoumarin (AMC) were measured using a spectrofluorometer (F-4500; Hitachi, Tokyo, Japan) with excitation at 360 nm and emission at 460 nm.

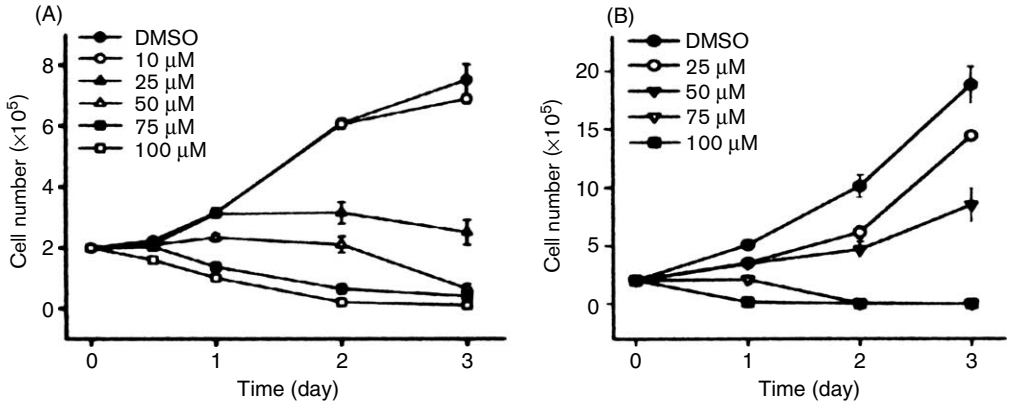
Cytochrome c release

Release of mitochondrial cytochrome *c* into the cytosol during magnolol-induced apoptosis was observed. HL-60 cells were incubated with magnolol for different periods, and then harvested and washed in cold PBS. Mitochondrial and cytosolic fractions were prepared by resuspending cells in ice-cold buffer A (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 17 mg/ml PMSF, 8 mg/ml aprotinin and 2 mg/ml leupeptin). Cells were passed through a needle 10 times. Debris and nuclei were pelleted by centrifugation for 10 min at 750 r.p.m. The supernatant was then centrifuged at 100 000 *g* for 15 min. The pellet was resuspended in buffer A and represents the mitochondrial fraction. The supernatant was again centrifuged at 100 000 *g* for 60 min. The supernatant from this final centrifugation step represents the cytosolic fraction that was electrophoresed on 10% SDS–polyacrylamide gels, and then transferred onto PVDF membranes and probed with an anti-cytochrome *c* antibody (#05-479; Upstate Biotechnology). The membrane was reacted with peroxidase-conjugated secondary antibody and then developed using enhanced chemiluminescence (ECL; Amersham, Little Chalfont, UK).

PARP cleavage

PARP (117 kDa) is cleaved to produce 85-kDa fragments during apoptosis [8]. In order to examine the cleavage of

Fig. 1



Inhibitory effect of magnolol on the growth of HL-60 (A) and Jurkat cells (B) assessed by the Trypan blue exclusion assay.

PARP in magnolol-induced apoptosis in HL-60 cells, the cells were lysed in 10 mM HEPES (pH 8.0), 150 mM NaCl, 0.1 mM EDTA, 1% Nonidet P-40, 1 mM DTT, 0.5 mM PMSF, 2.0 μg/ml leupeptin and 2.0 μg/ml aprotinin. The cells were then incubated on ice for 30 min followed by centrifugation for 10 min at 10 000 *g*. Protein concentrations were determined with Bio-Rad (Hercules, CA) reagents with standard BSA. The protein extracts from HL-60 cells treated with magnolol for various periods were electrophoresed and transferred as described above, and probed with anti-PARP monoclonal antibody (#06-557; Upstate Biotechnology).

Measurement of mitochondrial transmembrane potential

Alternations of the mitochondrial transmembrane potential [6] during the process of apoptosis were examined using rhodamine 123. HL-60 cells were incubated with magnolol for various periods and then stained with 10 μM rhodamine 123 for 30 min. The membrane potential-related fluorescence intensity was analyzed by flow cytometry (Becton Dickinson).

Results

Magnolol inhibits the proliferation of HL-60 and Jurkat leukemia cells

The inhibitory effect of magnolol on the growth of HL-60 and Jurkat cells was assessed by Trypan blue exclusion assay for 3 days (Fig. 1A and B). In parallel with this experiment, the effect of magnolol was also examined by the MTT assay. The results obtained with the two methods were essentially similar. Treatment with various concentrations of magnolol inhibited the proliferation of HL-60 cells in both a dose- and time-dependent manner. Treatment of HL-60 cells with magnolol at lower concentrations (less than 25 μM) for 3 days caused growth inhibition (IC_{50} = 13.8 μM, Table 1). However, higher concentrations (more than 25 μM) of magnolol caused cell death within 3 days. In addition, magnolol showed a considerable suppressive effect on growth in Jurkat cells, whereas it was relatively less effective against Jurkat cell growth (IC_{50} = 47.8 μM, Table 1). Similarly, higher doses cause significant cell number loss. Similar experiments were designed for human neutrophils or peripheral blood mononuclear cells (PBMCs), and each IC_{50} was estimated as 253.5 and 211.5 μM, respectively (Table 1). This result suggested that normal cells were more resistant to magnolol treatment than leukemia cells, *in vitro*, in our model.

Table 1 Cytotoxic effects of magnolol on human leukemic and normal white cells

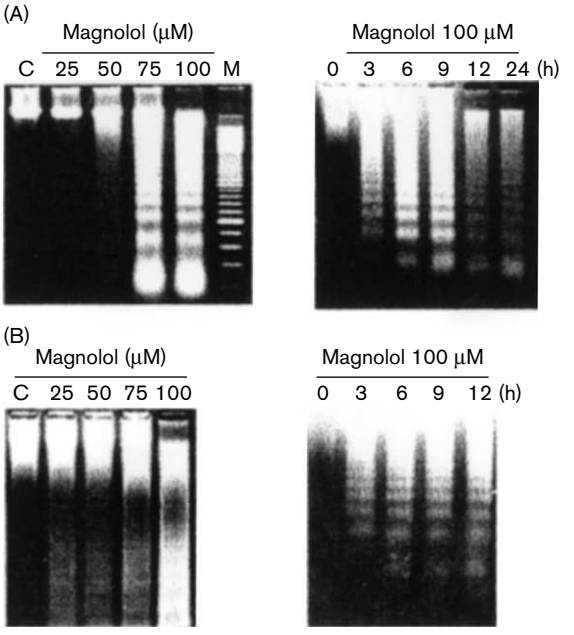
Cell type	Magnolol IC_{50}[b] (μM)
HL-60	13.8
Jurkat	47.8
Normal neutrophils	253.5
Normal PBMCs	211.5

[a] n = 4.
[b] Dose at which 50% of cells are not viable determined by Trypan blue exclusion. Treatment of HL-60 cells with magnolol at lower concentrations exhibited growth inhibition, but magnolol was relatively less effective against the growth of Jurkat cells; however, normal blood cells (neutrophils and PBMCs) were more resistant to magnolol treatment *in vitro*.

Magnolol induces apoptosis in HL-60 and Jurkat cells

To determine whether magnolol could induce apoptosis in HL-60 and Jurkat cells, we performed agarose gel electrophoresis with DNA extracts following magnolol treatment. DNA laddering was observed after incubation with magnolol at concentrations above 25 μM for 24 h in HL-60 cells (Fig. 2A). Treatment with magnolol at a concentration of 100 μM resulted in DNA fragmentation

Fig. 2



DNA laddering observed after incubation with magnolol in HL-60 cells (A) and Jurkat cells (B) in a time- and dose-dependent manner.

in 3 h. Furthermore, similar patterns of DNA laddering were observed in Jurkat cells incubated with magnolol (Fig. 2B). We used three flow cytometric methods for apoptosis quantification. First, the translocation of PS from the inner to the outer layer of the plasma membrane was detected with the FITC–Annexin V binding assays. In our model, Annexin V^+ and PI^- cell percentages as shown in Fig. 3(A–D) represent apoptosis of HL-60 cells following incubation with magnolol for 6 h in a dose-dependent manner. Secondly, HL-60 cells, which exhibited cell shrinkage and increased granularity to a slight extent, were important morphological criteria observed in processing of apoptotic cells. They could be analyzed following FSC and SSC criteria, respectively, in flow cytometry analysis. In the HL-60 cell model, both FSC and SSC were reduced during apoptosis induced by magnolol (Fig. 3E). Figure 4 shows the results of the TUNEL method to quantify the levels of fragmented DNA of HL-60 cells. Apoptotic ($TUNEL^+$) cells were obtained after the treatment of magnolol for 9 h. In addition, these results indicated that magnolol induced apoptosis in HL-60 and Jurkat cells in both a dose- and time-dependent manner.

Magnolol induces the activation of caspases and the cleavage of PARP proteins

Principally, proteolytic cleavage of caspase from the precursor form to the active form is necessary to activate the caspase cascade and downstream proteolytic effector function. In order to determine which caspases were activated during apoptosis induced by magnolol in HL-60 cells, we analyzed the enzymatic activity of each caspase *in vitro* using the following individual tetrapeptides as substrate: Ac-DEVE-AMC (caspase-3 substrate), Ac-YVAD-AMC (caspase-1 substrate), Ac-VDVAD-AMC (caspase-2 substrate), Ac-VEID-AMC (caspase-6 substrate), Ac-IETD-AMC (caspase-8 substrate) and Ac-LEHD-AMC (caspase-9 substrate). Our data revealed that magnolol (100 μM) activated caspase-3, -2 and -9 activities (Fig. 5A), but not caspase-1, -8 or -6 (data not shown for caspase-6) activities, in 6 h. In addition, cleavage of PARP, one of the caspase-3 substrates, suggested that DNA repair systems were destroyed [8–10]. As shown in Fig. 5(B), treatment of HL-60 cells with 100 μM magnolol resulted in progressive cleavage of PARP and production of the 84-kDa fragment in a time-dependent manner. These results confirmed that caspases were functionally activated during magnolol-induced apoptosis in HL-60 cells.

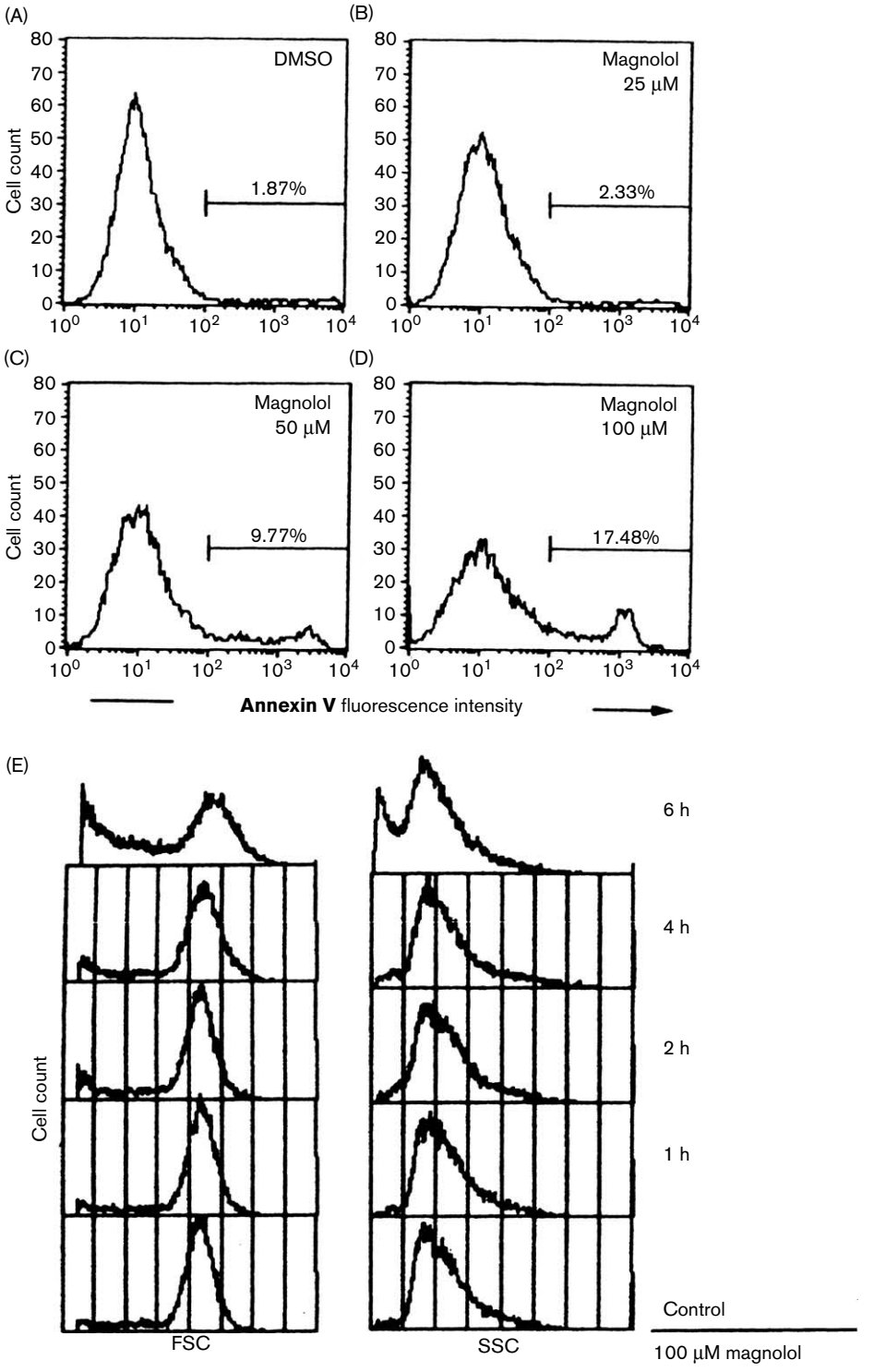
Magnolol induces the reduction of mitochondrial transmembrane potential and the release of cytochrome *c* into cytoplasm

Mitochondrial permeabilization has been shown to activate caspase-9 through the release of cytochrome *c* [11–13]. Magnolol (100 μM) triggered the disruption of the mitochondrial transmembrane potential, by an unknown mechanism, in 2 h (Fig. 6A). Furthermore, magnolol also elicited the release of cytochrome *c* into the cytoplasm from mitochondria in a time-dependent manner (Fig. 6B). These results indicated that the loss of mitochondrial transmembrane potential and release of cytochrome *c* occurred during the process of magnolol-induced apoptosis, suggesting that magnolol-induced apoptosis is through the pathway of caspase-9.

Attenuation of magnolol-induced DNA strand breaks in HL-60 cells by treatment with caspase inhibitors

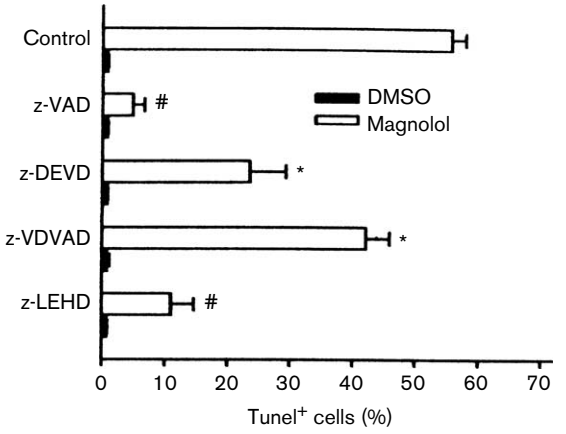
Representative results are presented in Figure 4. HL-60 cells were treated with magnolol for 9 h after pre-incubation with the pan-caspase inhibitor z-VAD-FMK (100 μM), caspase-3 inhibitor z-DEVD-FMK (100 μM), caspase-9 inhibitor z-LEHD-FMK (100 μM) or caspase-2 inhibitor z-VDVAD-FMK (100 μM). TUNEL analysis demonstrated that 58.1% of magnolol-treated HL-60 cells underwent apoptosis. Caspase-3 or -2 inhibitor treatment significantly reduced the apoptosis of HL-60 cells to 23.6 or 42.1%, respectively, while z-VAD-FMK reduced the magnolol-induced apoptosis process effectively to 4.8%. Moreover, the inhibitor of caspase-9 attenuated the apoptotic rate to 11%, which represents much more intensive inhibition of apoptosis in HL-60 cells treated with magnolol in 9 h. These results indicated that caspase-9 plays an important role in initiating the apoptotic process. However, other proteolytic effectors

Fig. 3



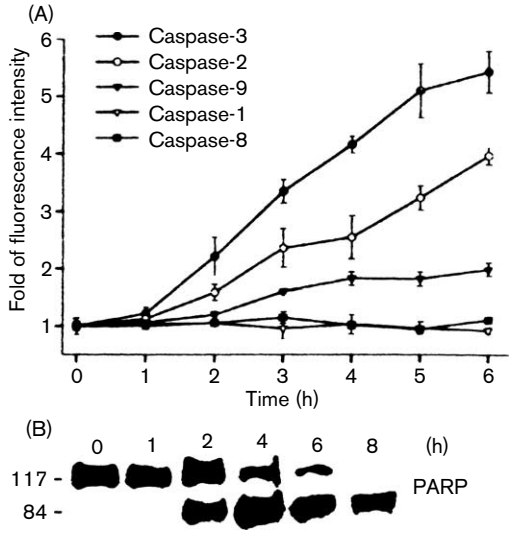
Apoptosis of HL-60 cells following incubation with magnolol for 6 h in a dose-dependent manner (A–D). The HL-60 cells exhibited cell shrinkage and increased granularity, and both FSC and SSC were reduced during apoptosis induced by magnolol (E).

Fig. 4



TUNEL analysis of HL-60 apoptotic cells after treatment with magnolol for 9 h.

Fig. 5



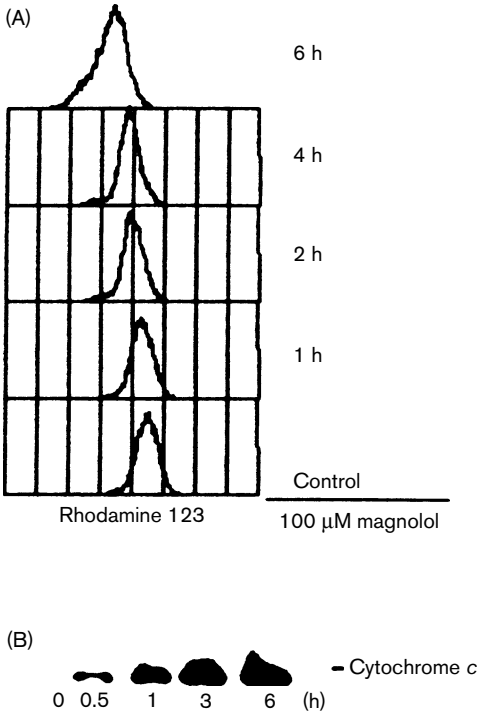
(A) Magnolol activates caspase -3, -2 and -9 activities, but not caspase -1 and -8 activities, in HL-60 cells. (B) Progressive cleavage of PARP and production of the 84-kDa fragment in a time-dependent manner following magnolol treatment.

with the exception of caspase-3 and -2 might be implicated in the late stage of apoptosis.

Discussion

We are the first to demonstrate that magnolol blocks proliferation of HL-60 and Jurkat T leukemia cells via apoptosis at concentrations of 25 μM or more, and in a dose- and time-dependent manner. No cytotoxicity on normal neutrophils and PBMCs from healthy humans was found at the same concentration. Meanwhile, magnolol

Fig. 6



(A) Disruption of mitochondrial transmembrane potential following magnolol treatment. (B) Magnolol elicited the release of cytochrome *c* to cytoplasm from mitochondria in a time-dependent manner.

also did not exhibit cytotoxicity in human gingival fibroblasts, smooth muscle cells and human umbilical vein endothelial cells (Lee *et al.*, unpublished data). The growth inhibitory effect on Jurkat cells was relatively less than that on HL-60 cells.

In our study, members of the caspase family, including caspase-9, -3 and -2, but not caspase-1, -6 and -8, were activated in the process of magnolol-induced apoptosis within 3 h. This result is consistent with the concept that multiple caspases are required for the execution of apoptosis [14]. LEHD (caspase-9 inhibitor) was effective in completely blocking magnolol-induced apoptosis in HL-60 cells, while apoptosis was partially blocked by either DEVD or VDVAD.

In addition, magnolol-induced apoptosis was sensitive to the pan-caspase inhibitor, z-VAD. We also observed that caspase-inhibitory proteases could delay, but not block, cell death resulting from the treatment of magnolol, suggesting the involvement of non-caspase proteases, such as cathepsins, calpains and granzymes [15]. On the other hand, the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol has been reported to be associated with the dissipation of

the mitochondrial membrane potential [16]. In our study, the appearance of cytosolic cytochrome *c* was observed 1 h after treatment of magnolol and the mitochondrial membrane potential deceased significantly at 2 h, indicating that the magnolol-induced release of cytochrome *c* is not closely related with the decline of the mitochondrial membrane potential.

Magnolol has been reported to be an effective scavenger of free radicals [17,18]. This effect has been observed in magnolol-induced apoptosis of HL-60 and Jurkat cells (Lu *et al.*, unpublished data). We suspect that the decrease of the intracellular hydrogen peroxide caused by magnolol may correlate with the induction of apoptosis, because it is well documented that reactive oxygen species are one of the proapoptotic mediators [19,20].

To conclude, we demonstrated that magnolol inhibits the proliferation of leukemic cells via the induction of apoptosis. The cytotoxic effect was noted on promyelocytic HL-60 cells and Jurkat T leukemia cells without destroying normal neutrophils and PBMCs. Magnolol could therefore be a candidate anti-cancer drug for leukemia with low toxicity to normal blood cells.

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