Magnolol Has the Ability to Induce Apoptosis in Tumor Cells

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We previously found that multiple intraperitoneal administration of magnolol from *Magnolia obovata* inhibited tumor metastasis and growth *in vivo*, and that the anti-metastatic effect of magnolol was due to the inhibition of the tumor cell invasion. The purpose of this study was to clarify the inhibitory mechanism of magnolol on the growth of tumor cells, and we expect that magnolol may have the ability to induce apoptosis in tumor cells. In an *in vitro* proliferation assay, 100 μM of magnolol inhibited the proliferation of B16-BL6, THP-1, BAE and HT-1080 cells, but 30 μM of magnolol did not affected cells proliferation. In addition, 100 μM of magnolol induced apoptotic cell death within 24 h in three tumor cell lines, B16-BL6, THP-1 and HT-1080, not BAE cells, and then up-regulated the activity of caspase-3 and caspase-8. The up-regulation of caspases activity by 100 μM of magnolol was suppressed by the inhibitor of all caspases, z-VAD-fmk. These data suggest that magnolol possesses ability to inhibit tumor growth, and the ability is due to the induction of apoptosis with the activation of caspases.

Key words magnolol; apoptosis; antitumor effect; caspase; mouse

Apoptosis is a programmed cell death in which tumor cells commit suicide, resulting in structural changes of the plasma membrane and DNA fragmentation in nuclei.¹⁾ Cysteine proteases called 'caspases' play important roles in apoptosis. A major event in the apoptotic process is the activation of caspases. Caspase family members are expressed as proenzymes, which are cleaved to be activated during the apoptotic process. The activation is caused by proteolysis of death substrates such as poly ADP-ribose polymerase (PARP), DNA-dependent protein kinase and caspases themselves.²⁾ Apoptosis can be induced by physiological stimuli, and by artificial stress such as the exposure to anti-cancer drugs.

Magnolia obovata THUNB. bark has been used in Chinese medicine, the components of which have been well investigated, and magnolol, honokiol and obovatol are well known. Magnolol has been known to have many biological activities such as anti-platelet aggregation,³⁾ hydroxyl radical scavenging,⁴⁾ Ca^{2+} -channel blocking⁵⁾ and anti-inflammatory action.⁶⁾ Our previous studies demonstrated anti-tumor effects of magnolol, particularly the inhibition of tumor cell invasion,⁷⁾ metastasis⁸⁾ and angiogenesis.⁹⁾ In addition, in a spontaneous lung metastasis model using B16-BL6 melanoma, multiple intraperitoneal administration of 10 mg/kg of magnolol after tumor inoculation significantly suppressed lung metastasis and primary tumor growth.⁸⁾ We previously reported that daily intraperitoneal injections of magnolol suppressed tumor growth by the subcutaneous inoculation of B16-BL6, and then inhibited the neovascularization. We have tried to evaluate the inhibitory mechanism of tumor growth caused by the administration of magnolol. In the present study, we investigated the ability of magnolol to induce apoptosis in the tumor cells treated, and its relation to the activation of caspase-3 and caspase-8.

MATERIALS AND METHODS

Cells and Culture Highly metastatic subline of murine B16 melanoma, B16-BL6 was kindly provided by Dr. I. J. Fidler, M. D. Anderson Cancer Center, Houston, Texas, through Dr. I. Saiki, Toyama Medical and Pharmaceutical

University. Human fibrosarcoma HT-1080 and human acute monocytic leukemia THP-1 cells were kindly provided by the Japanese Cancer Research Resources Bank. HT-1080 fibrosarcoma and B16-BL6 melanoma cells were maintained as monolayer cultures in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS), vitamin solution, 5% sodium pyruvate, nonessential amino acids, and L-glutamine. Human acute monocytic leukemia THP-1 were maintained in RPMI-1640 supplemented with 10% FBS. Bovine aorta endothelial (BAE) cells were kindly provided by Dr. T. Adachi, Gifu Pharmaceutical University. BAE cells were maintained as monolayer cultures in DMEM supplemented with 10% FBS, vitamin solution, 5% sodium pyruvate, nonessential amino acids, and L-glutamine.

Chemical Reagents Magnolol was kindly provided by Dr. T. Asao and Dr. T. Terada of Taiho Pharmaceutical Co., Ltd. (Hanno, Japan). The substrate for caspase-3, acetyl-Asp-Glu-Val-Asp-4-methyl-coumaryl-7-amide (DEVD-MCA), the substrate for cleaving enzyme of procaspase 3 (Caspase-8/-6 and granzyme B), acetyl-Ile-Glu-Thr-Asp-4-Methyl-coumaryl-7-amide (IETD-MCA) and Val-Ala-Asp(Ome)-CH₂F (z-VAD-fmk) as a inhibitor for all caspases were purchased from Peptide Institute (Osaka, Japan).

Cell Proliferation Assay Various concentrations of magnolol were added to 1×10^4 HT-1080 fibrosarcoma, B16-BL6 melanoma, THP-1 leukemia or BAE cells suspended in the culture medium supplemented with 10% FBS, in 9 cm dish. The culture was incubated at 37 °C for various time in a

Fig. 1. Structure of Magnolol

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Fig. 2. Inhibitory Effect of Magnolol on Growth of B16-BL6 (A), THP-1 (B), HT-1080 (C) and BAE (D)

Cells were incubated in the presence of various concentrations of magnolol for 24, 48, 72 or 96 h and measured by trypan blue dye exclusion assay. 0 μM (□), 1 μM (◇), 10 μM (○), 30 μM (▽), 100 μM (△) of magnolol.

5% CO₂ atmosphere. Number of viable cells was counted by trypan-blue dye exclusion.

Analysis of DNA Fragmentation Cells (1×10⁶) were collected and washed with cold phosphate-buffered saline (PBS) after they were treated with magnolol for 24 h. The cell were resuspended with PBS, and lysed in a lysis buffer (10% polyethylene glycol mono-*p*-isooctylphenyl ether, 0.5 M ethylenediamine tetraacetic acid (EDTA) and 1 M Tris-HCl buffer, pH 7.4) at 4 °C for 10 min. The lysate was centrifuged at 15000 *g* for 20 min, and the supernatant was incubated with RNase, and then with proteinase K, both at 37 °C for 60 min. Then, DNA was extracted with isopropanol-5 M NaCl (6:1, vol:vol). The purified DNA was dissolved in TE buffer (1 M Tris-HCl buffer, pH 7.4 and 0.5 M EDTA) and was separated electrophoretically on 2% agarose gel at 75 V for 100 min and stained with ethidium bromide. The bands of DNA fragments were visualized under UV light and photographed.

Caspase Fluorogenic Activity Assay Caspases activities were assayed according to the method of Mizukami *et al.*¹⁰⁾ Human fibrosarcoma HT-1080 (1×10⁶) were treated with magnolol for 24 h, then collected and washed with PBS. The cells were lysed (25 mM Hepes, 5 mM MgCl₂, 5 mM EDTA, 1 mM ethylene glycol bis-(2-aminoethylether) *N,N,N',N'*-tetraacetic acid, containing 1 mM phenylmethylsulfonyl fluoride and 5 mM dithiothreitol) and centrifuged at 60000 *g* for 20 min. The protein concentration of the supernatant was determined using Bio-rad reagent (Bio-rad, California, U.S.A.). This lysate was incubated at 37 °C with DEVD-MCA or IETD-MCA. The total volume of the reaction mixture was 50 μl. After incubation for 1 h, 2 ml of 1 M acetic acid was added to the reaction mixture and the fluorescence intensity was measured. Excitation and emission wavelengths are 380 nm and 460 nm, respectively.

Statistical Analysis The statistical significance of differences between groups was calculated by student's two-tailed *t*-test. *p* < 0.05 was considered significant.

RESULTS

Cytotoxic Activity of Magnolol towards Various Cell Lines To determine the cytotoxic activity of magnolol by trypan-blue dye exclusion assay, we used mouse melanoma B16-BL6, human fibrosarcoma HT-1080, human acute monocytic leukemia THP-1 and BAE cells. Magnolol at 100 μM inhibited the growth of the four cell lines, which were not affected up to 10 μM of magnolol for 24 h (Fig. 2). Magnolol at 30 μM did not affected the growth of THP-1 and BAE cells (Fig. 2).

Induction of Apoptosis by Magnolol in Various Cell Lines To investigate whether the inhibitory effects on tumor growth *in vivo* and *in vitro* are caused by apoptosis, cell morphology of magnolol-treated or non-treated human fibrosarcoma HT-1080 was observed under a microscope at a magnification of ×200. HT-1080 cells treated with magnolol at 100 μM for 18 h and 24 h showed typical morphological characteristics of apoptosis, such as swelling-shape (Fig. 3). These changes were not observed at 30 μM. Furthermore, magnolol-induced apoptosis was confirmed by observation of DNA fragmentation after 24 h treatment with magnolol at 100 μM (Fig. 4). DNA fragmentation was observed, and only magnolol at 100 μM exhibited the apoptotic changes in HT-1080, B16-BL6 and THP-1 cells. However, BAE cells treated with 100 μM of magnolol did not show apoptotic morphological changes (Fig. 3) and the formation of typical DNA ladder (Fig. 4).

Activation of Caspases in Magnolol-Induced Apoptosis To investigate the involvement of caspases in magnolol-induced apoptosis, we examined caspase activity in magnolol-treated cells by measurement of the fluorescence released by the cleavage of the caspase substrate: DEVD-MCA or IETD-MCA. When the caspase-3 and caspase-8 activities in human fibrosarcoma HT-1080 treated with magnolol were measured at time course, the activation of caspase-3 and caspase-8 was observed later than 18 h after magnolol treatment (Fig. 5). In

Fig. 3. Microscopical Observations in Cultured B16-BL6 (A, B, C), HT-1080 (D, E, F) and BAE (G, H, I) Cells were treated with magnolol at 30 μ M (B, E, H) and 100 μ M (C, F, I) or without magnolol (A, D, G) for 24 h.

Fig. 4. DNA Fragmentation Induced by Magnolol

Cells were treated with various doses of magnolol for 24 h and then collected. DNA fragmentation of treated cells was detected on 2% agarose gel electrophoresis. DNA ladder of the untreated controls (lanes 1, 4, 7, 10), DNA ladder of the cells treated with 30 μ M of magnolol (lanes 2, 5, 8, 11) and with 100 μ M of magnolol (lanes 3, 6, 9, 12). 100 bp DNA ladder (lane 0).

Fig. 5. Effect of Magnolol on Caspase-3 and Caspase-8 Activation

HT-1080 (1×10^6) were treated with magnolol for 15 min, 3, 6, 12, 18 and 24 h, then collected and washed with PBS. Cell lysate was incubated at 37°C with DEVD-MCA or IETD-MCA for 1 h. After incubation, the fluorescence intensity was measured. Excitation and emission wavelengths are 380 nm and 460 nm, respectively

addition, the apoptotic DNA fragmentation by magnolol at 100 μ M was inhibited by the pre-treatment of caspases inhibitor, z-VAD-fmk (Fig. 6).

Fig. 6. Effect of Caspase Inhibitor, z-VAD-fmk on Magnolol-Induced Apoptosis

HT-1080 cells were treated z-VAD-fmk (25 μ M) for 6 h, and then added to magnolol at 100 μ M. After incubation for 24 h, treated cells were collected and DNA fragmentation was detected on 2% agarose gel electrophoresis. Lane 1 = z-VAD-fmk only, 2 = control, 3 = z-VAD-fmk + magnolol, 4 = magnolol only.

DISCUSSION

We previously reported that magnolol showed the regression of tumor growth caused by subcutaneous injections of B16-BL6 melanoma cells into the right hind footpad.⁸⁾ The anti-tumor ability of magnolol was so strong that we suspected its contribution to the apoptosis induction. To clarify the mechanism of tumor growth inhibition by the administration of magnolol, we investigated the apoptosis induction ability of magnolol in the tumor cells. We demonstrated that magnolol induced apoptotic cell death, and inhibited cells growth *in vitro*. We previously reported that the co-injection of magnolol at 30 μ M and the tumor cell significantly suppressed angiogenesis and tumor growth.⁹⁾ The magnolol concentration at which co-injection of magnolol suppressed tumor growth was lower than the concentration that inhibited the tumor cell proliferation and induced the apoptosis. These

results suggest that the anti-tumor effect of magnolol is also associated with both of apoptotic induction and anti-angiogenic activity.

We also demonstrated that the HT-1080 cells treated with magnolol at 100 μ M showed the typical morphological characteristics of apoptosis and led to DNA fragmentation. Furthermore, the activation of caspase-3 and caspase-8 was observed later than 18 h after magnolol treatment. Because the times of activation of caspase and formation of DNA fragmentation are almost coincide, it is suggested that the magnolol induces caspase-3 and caspase-8 dependent apoptosis. In addition, magnolol-induced apoptosis was blocked by the caspases inhibitor. However, we could not confirm the route of apoptosis from these data, and further investigation is needed. Magnolol has many biological activities such as hydroxyl radical scavenging,⁴⁾ Ca^{2+} -channel blocking⁵⁾ and cytosolic free Ca^{2+} -elevation inducing.¹¹⁾ Calcium entry into cells appears to be a critical early event in apoptosis.¹²⁾ The endonuclease that causes characteristic apoptotic cleavage of chromatin is calcium dependent.¹³⁾ McConkey *et al.* reported that increase of calcium triggered to stimulate glucocorticoid and T cell receptor in thymocytes and cell death was prevented by blocking of the calcium increase.^{14,15)} Moreover, Vignaux reported an importance of extracellular Ca^{2+} and intracellular Ca^{2+} for Fas-mediated apoptotic cell death.^{16,17)} Activated Fas receptor recruits Fas-associated death domain protein (FADD), and this leads to proteolytic activation of the caspase-8 and caspase-3. These reports may support our conclusion that magnolol may induce apoptosis through Fas-mediated death pathway. Indeed, we observed the activation of caspase-3 and caspase-8 after magnolol treatment.

Magnolol is contained in the bark of *Magnolia obovata*, which has been used in Chinese traditional medicine for the relief of fever, headache, anxiety and stroke. Though data are not shown, i.p. injection of magnolol at 100 mg/kg to mice did not show any toxicity to mice in the blood test, such as

serum aspartate transaminase, serum alanine transaminase, serum total bilirubin, and total protein. Magnolol is expected to be harmless enough to have no serious side effect.

Our study found that magnolol induced apoptotic cell death with the activation of caspase, and suppressed tumor cells growth *in vitro*. The inhibitory mechanisms of magnolol on tumor growth were due to not only anti-angiogenic action but also apoptotic induction. Therefore, magnolol has both of anti-tumor activity and anti-metastatic activities, and may be used as a powerful tool for clinical cancer therapy.

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