Effect of aloe-emodin on the proliferation and apoptosis of human synovial MH7A cells; a comparison with methotrexate

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Abstract. Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by synovial hyperplasia. Methotrexate (MTX), an antifolate derivative, is used for the treatment of RA, as it exerts antiproliferative efftects on lymphocytes and synovial cells. Aloe-emodin (AE) is a primary component of anthraquinones in *Aloe vera* and exerts antiproliferative and apoptotic effects on various tumor cells. In the present study, the effect of AE on the proliferation and apoptosis of MH7A human RA synovial cells was examined. In addition, the effect of AE was compared with that of the established RA therapeutic MTX. MH7A cells were incubated with 5, 10, 20 or 40 μM AE, or 0.01, 0.05, 0.1 or 1 μM MTX, for 24, 48 or 72 h. Subsequently, total cell numbers were assessed using trypan blue staining and Cell Counting kit-8. Furthermore, MH7A cells incubated with AE or MTX for 48 h were evaluated for apoptosis following Annexin V/propidium iodide (PI) staining, and for cell cycle distribution following PI staining. The results indicated that ≥10 μM AE and ≥0.05 μM MTX effectively decreased the numbers of viable MH7A cells. In addition, 40 μM AE and 1 μM MTX induced apoptosis in MH7A cells. Cell cycle analysis revealed that ≥20 μM AE induced G2/M phase arrest, whereas ≥0.1 μM MTX induced S phase arrest. These observations suggested that AE treatment inhibited the growth of MH7A cells by arresting the cell cycle at a different checkpoint compared with MTX treatment. Thus, AE may be a potential therapeutic agent for the treatment of RA, and may be complimentary to MTX, based on its antiproliferative effect on synovial cells.

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Abbreviations: RA, rheumatoid arthritis; AE, aloe-emodin; MTX, methotrexate; PI, propidium iodide; DMSO, dimethyl sulfoxide; ROS, reactive oxygen species

Key words: aloe-emodin, rheumatoid arthritis, synovial cells, methotrexate, proliferation, apoptosis, cell cycle

Introduction

Rheumatoid arthritis (RA) is a chronic autoimmune and inflammatory disease characterized by persistent synovitis; symptoms include joint pain, stiffness and swelling. In RA, chronic inflammation leads to synovial hyperplasia termed 'pannus', which erodes bone and cartilage, thereby destroying articular cartilage (1).

Synovial hyperplasia is caused by alterations in cell proliferation and cell death, involving increased proliferation and insufficient apoptosis of synovial cells (2-4). The proliferation of synovial cells is regulated by growth factors and cytokines produced in the local milieu (5,6). The insufficient apoptosis of synovial cells is caused by the overexpression of transformation-associated proteins, such as tumor protein p53 (7,8). Therefore, inhibiting proliferation and inducing apoptosis of synovial cells may constitute a promising strategy for the treatment of RA (3).

Methotrexate (MTX), an antifolate derivative, which inhibits DNA synthesis and induces apoptosis of tumor cells, is widely used as a therapeutic agent for malignant tumors. MTX is additionally utilized as a first-line RA treatment, as it exerts anti-inflammatory and antiproliferative effects on lymphocytes and synovial cells (9).

Aloe-emodin (AE; the chemical structure of which is presented in Fig. 1) is a primary bioactive component of *Aloe vera*, *Aloe arborescens* and certain Chinese herbs, including *Rheum officinale*. AE has been demonstrated to modulate various functions of host cells; AE exerts anti-inflammatory effects by inhibiting the activation of macrophages (10,11) and antiproliferative effects on human tumor cells, including neuroblastoma, hepatoma, leukemia, tongue squamous cancer and colon cancer cells (12-17). In HL-60 human leukemia cells, AE exerts antitumor effects by inhibiting cell proliferation, and inducing apoptosis and cell cycle arrest (14). Based on these findings, the present study aimed to investigate whether AE may inhibit proliferation and induce apoptosis in synovial cells, which are important in the pathogenesis of RA. The effect of AE on cell growth,

apoptosis and cell cycle distribution of synovial cells was evaluated using MH7A human RA synovial cells. In addition, the effect of AE was compared with the effect of MTX, an established first-line RA treatment.

Materials and methods

Reagents. AE and MTX were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany). An 100 mM AE stock solution was prepared by dissolving AE in dimethyl sulfoxide (DMSO), and was stored at -20˚C. The AE stock solution was further diluted to the indicated concentrations in culture media immediately prior to each experiment. The final concentrations of AE in the culture media were 5, 10, 20 and 40 μM. These concentrations were selected since AE at a concentration range of 6.25-50 μM has been reported to induce G2/M cell cycle arrest and apoptosis through the activation of caspase-6 in human colon cancer cells (16). MTX was dissolved in DMSO at 100 mM, and was further diluted in culture media. The final concentrations of MTX in the media were 0.01, 0.05, 0.1 and 1 μM.

Cell culture. MH7A human RA synovial fibroblast-like cells (18) were provided by the RIKEN BioResource Center (Tsukuba, Japan) through the National Bio-Resource Project of the Ministry of Education, Culture, Sports Science and Technology Japan, and maintained in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Nichirei Biosciences, Inc., Tokyo, Japan), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C in an atmosphere of 5% CO_2. Cells were passaged every 3-4 days.

Measurement of viable cells. MH7A cells ($5x10^4$ cells/well) were seeded into 12-well plates and cultured for 24 h. Cells were subsequently incubated with 5, 10, 20 or 40 μM AE, 0.01, 0.05, 0.1 or 1 μM MTX, or DMSO as a vehicle control (0.04% for AE and 0.001% for MTX), for 3 days. Following incubation, cells were harvested with trypsin/EDTA, stained with 0.25% trypan blue and counted using an automated cell counter (Thermo Fisher Scientific, Inc.).

Alternatively, MH7A cells ($1x10^4$ cells/well) were seeded into 48-well plates and cultured for 24 h. Cells were subsequently incubated with AE, MTX or DMSO, as aforementioned. Following incubation, live cells were evaluated using Cell Counting kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. The absorbance of each well was measured at a wavelength of 450 nm using a microplate reader (xMark™; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Analysis of cell death. MH7A cells ($2x10^5$ cells/well) were seeded into 6-well plates and cultured for 24 h. Cells were subsequently incubated with AE, MTX or DMSO for 2 days. Following incubation, cells were harvested with trypsin/EDTA, and apoptosis was measured using an Annexin V-fluorescein isothiocyanate/propidium iodide (PI) assay kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan). Cells were analyzed for early and late apoptosis, and necrosis, using a flow cytometer (FACSCalibur; BD Biosciences, San Jose, CA, USA) and the software BD CellQuest™ Pro version 6.0 (BD Biosciences).

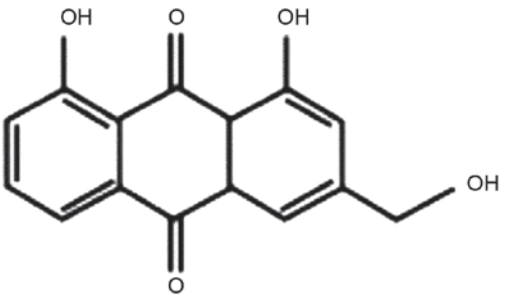

Figure 1. Chemical structure of aloe-emodin.

Analysis of cell cycle phase distribution. MH7A cells ($2x10^5$ cells/well) were seeded into 6-well plates and cultured for 24 h. Cells were subsequently incubated with AE, MTX or DMSO for 2 days. Following incubation, cells were harvested with trypsin/EDTA, fixed and permeabilized with 100% ethanol, stained with PI (Cell Cycle Phase Determination kit; Cayman Chemical Company, Ann Arbor, MI, USA) and analyzed by flow cytometry.

Statistical analysis. Data are presented as the mean ± standard deviation. The statistical significance of the differences between groups was assessed using one-way analysis of variance followed by a post hoc Bonferroni test for multiple comparisons. Statistical analysis was performed using GraphPad Prism software version 5 (GraphPad Software, Inc., La Jolla, CA, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of AE and MTX treatment on the proliferation of MH7A cells. To evaluate the effect of AE and MTX on the proliferation of synovial cells, MH7A cells were cultured for 3 days in the absence or presence of 5-40 μM AE or 0.01-1 μM MTX, and the number of viable cells was measured by trypan blue exclusion staining. As presented in Fig. 2, the total number of live MH7A cells increased ~8-fold in 3 days of culture in the absence of AE and MTX. AE treatment inhibited the increase in cell number in a dose-dependent manner (Fig. 2A); however, this effect was not significant. MTX treatment significantly inhibited the increase in MH7A cell numbers at concentrations ≥0.05 μM ($P<0.05$; Fig. 2B).

In addition, the effect of AE and MTX on MH7A cells was analyzed by CCK-8 assay. CCK-8 assay is based on reduction of tetrazolium salt by live and metabolically active cells, and is therefore utilized for the quantification of living cells in culture. As presented in Fig. 3, the number of viable MH7A cells increased ~6-fold in 3 days in the absence of AE and MTX. When measured using the CCK-8 assay, 10-40 μM AE (Fig. 3A) and 0.05-1 μM MTX (Fig. 3B) significantly inhibited the increase in the number of live cells in a dose-dependent manner ($P<0.05$). These observations indicate that AE and MTX inhibit the proliferation of MH7A cells.

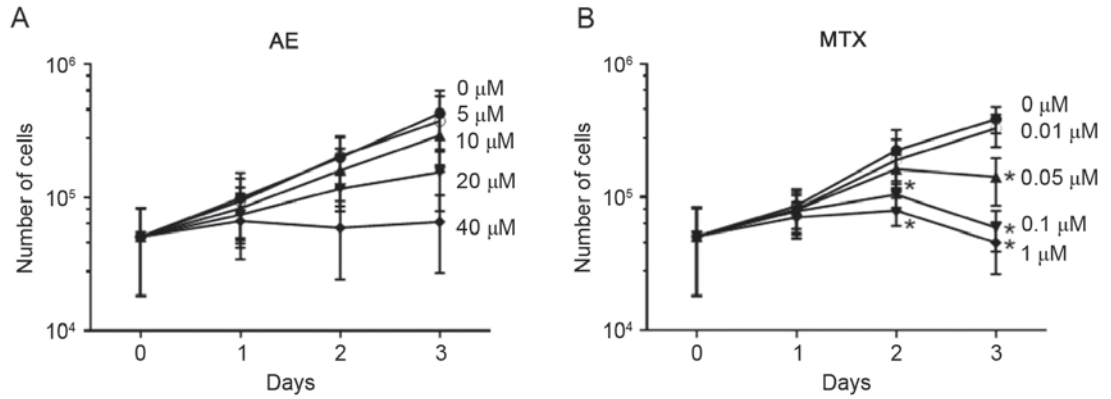

Figure 2. Evaluation of the effect of AE and MTX on MH7A cell proliferation by trypan blue staining. MH7A cells ($5x10^4$ cells/well) were seeded into 12-well plates for 24 h, and treated with (A) AE (5, 10, 20 or 40 μM), or (B) MTX (0.01, 0.05, 0.1 or 1 μM) for 1, 2 and 3 days. Cells treated with the vehicle dimethyl sulfoxide served as a control (0 μM). The number of live cells was measured by trypan blue exclusion staining using an automated cell counter. Data are presented as the mean ± standard deviation of 3 (AE) or 4 (MTX) independent experiments. *P<0.05 vs. 0 μM. AE, aloe-emodin; MTX, methotrexate.

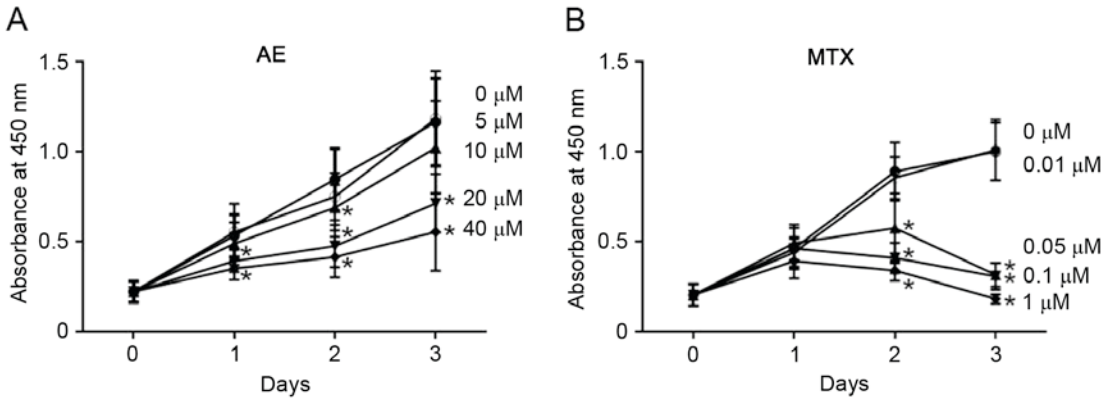

Figure 3. Evaluation of the effect of AE and MTX on MH7A cell proliferation by CCK-8 assay. MH7A cells ($1x10^4$ cells/well) were seeded into 48-well plates for 24 h, and treated with (A) AE (5, 10, 20 or 40 μM), or (B) MTX (0.01, 0.05, 0.1 or 1 μM) for 1, 2 and 3 days. Cells treated with the vehicle dimethyl sulfoxide served as a control (0 μM). Live cell numbers were determined by CCK-8 assay. Data are presented as the mean ± standard deviation of 3 (AE) or 4 (MTX) independent experiments. *P<0.05 vs. 0 μM. AE, aloe-emodin; MTX, methotrexate; CCK-8, Cell Counting kit-8.

Effect of AE and MTX treatment on MH7A cell death. The effect of AE and MTX treatment on MH7A cell death was examined. MH7A cells were incubated in the absence or presence of 5-40 μM AE or 0.01-1 μM MTX for 2 days, and apoptosis and necrosis were analyzed by Annexin V/PI staining (Fig. 4A). Flow cytometric analysis demonstrated that treatment with 40 μM AE significantly increased the percentage of Annexin V^+/PI^- (early apoptotic; 5.6±2.4 vs. 1.2±0.4%) and Annexin V^+/PI^+ (late apoptotic; 6.2±1.0 vs. 2.1±0.4%) cells, compared with untreated MH7A cells (P<0.05; Fig. 4B). This indicated that AE treatment induces early and late apoptosis in MH7A cells. Similarly, treatment with 1 μM MTX increased the percentage of Annexin V^+/PI^- (11.2±2.4 vs. 1.8±0.4%) and Annexin V^+/PI^+ (8.6±2.6 vs. 1.0±0.3%) cells, compared with untreated cells (P<0.05; Fig. 4B). Concentrations ≤20 μM AE and ≤0.1 μM MTX exhibited no effect on apoptosis (Fig. 4B). These observations indicated that only 40 μM AE and 1 μM MTX induced apoptotic cell death in MH7A cells. AE and MTX did not induce necrotic cell death (Annexin V^-/PI^+ cells) in MH7A cells even at high concentrations evaluated in the present study (data not shown).

Effect of AE and MTX treatment on cell cycle phase distribution of MH7A cells. Cell proliferation is regulated by cell cycle progression. Thus, to clarify the underlying mechanism involved in the inhibitory action of AE or MTX on the proliferation of MH7A cells, the effect of AE and MTX on cell cycle phase distribution was examined. MH7A cells were cultured for 2 days in the absence or presence of 5-40 μM AE or 0.01-1 μM MTX, and stained with PI to determine the DNA content of the cells (Fig. 5A). The results demonstrated that treatment with 40 μM AE increased the percentage of cells in G2/M phase and decreased the percentage of cells in G1 phase compared with untreated cells (P<0.05; Fig. 5B), suggesting that AE induced G2/M phase arrest in MH7A cells. Conversely, treatment with 1 μM MTX significantly increased the percentage of cells in S phase and decreased the percentage of cells in G2/M phases compared with untreated cells (P<0.05; Fig. 5B). In addition, 1 μM MTX slightly decreased the percentage of cells in G1 phase, although this effect was not significant. These observations suggested that MTX induced S phase arrest in MH7A cells. The effects of AE and MTX on the cell cycle were dose-dependent, with AE demonstrating significant effects at concentrations ≥20 μM and MTX demonstrating significant effects at concentrations ≥0.05 μM (P<0.05; Fig. 5B), compared with untreated cells. Notably, 20 μM AE and 0.05 and 0.1 μM MTX significantly induced G2/M and S

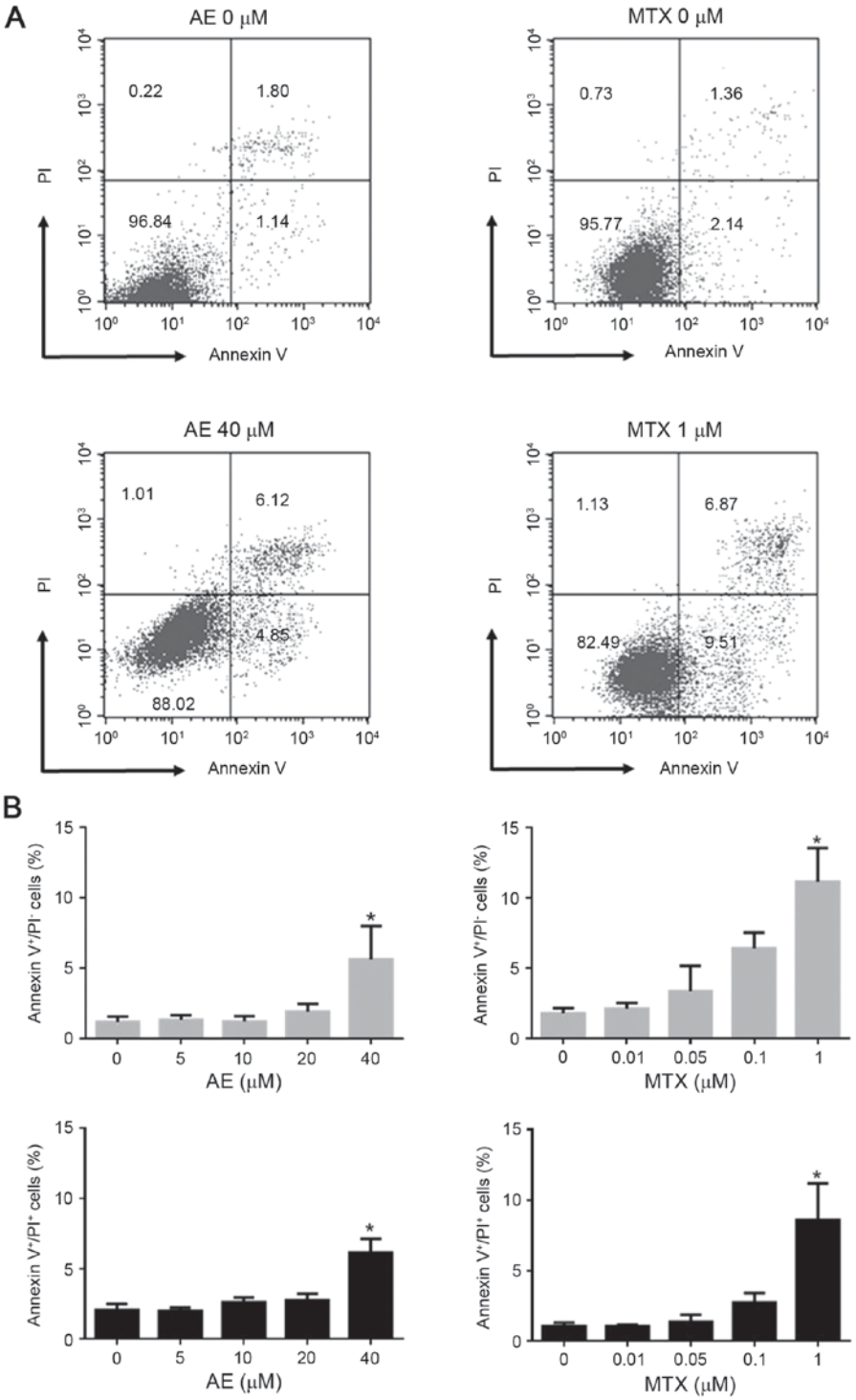

Figure 4. Evaluation of the effect of AE and MTX on cell death. MH7A cells ($2x10^5$ cells/well) were seeded into 6-well plates for 24 h, and treated with AE (5, 10, 20 or 40 μM), or MTX (0.01, 0.05, 0.1 or 1 μM) for 48 h. Cells treated with the vehicle dimethyl sulfoxide served as a control (0 μM). Apoptosis was evaluated by Annexin V/PI staining followed by flow cytometric analysis. (A) Representative plots of control cells and cells treated with 40 μM AE or 1 μM MTX. (B) Quantification of the percentage of early ($AnnexinV^+/PI^-$) and late ($AnnexinV^+/PI^+$) apoptotic cells. Data are presented as the mean ± standard deviation of 4 independent experiments. *P<0.05 vs. 0 μM. AE, aloe-emodin; MTX, methotrexate; PI, propidium iodide.

phase arrest, respectively, whereas the same concentrations did not induce apoptotic cell death (Fig. 4).

Discussion

RA is a chronic inflammatory disease characterized by synovial hyperplasia, in which the proliferation of synovial cells and infiltrating macrophages and lymphocytes is increased, and apoptosis of these cells is decreased (3). MTX, an antifolate derivative, is presently a first-line RA treatment, as it exerts anti-inflammatory and antiproliferative effects on lymphocytes and synovial cells (19). However, the administration of MTX induces a variety of adverse effects, including potentially life-threatening hepatotoxicity, nephrotoxicity,

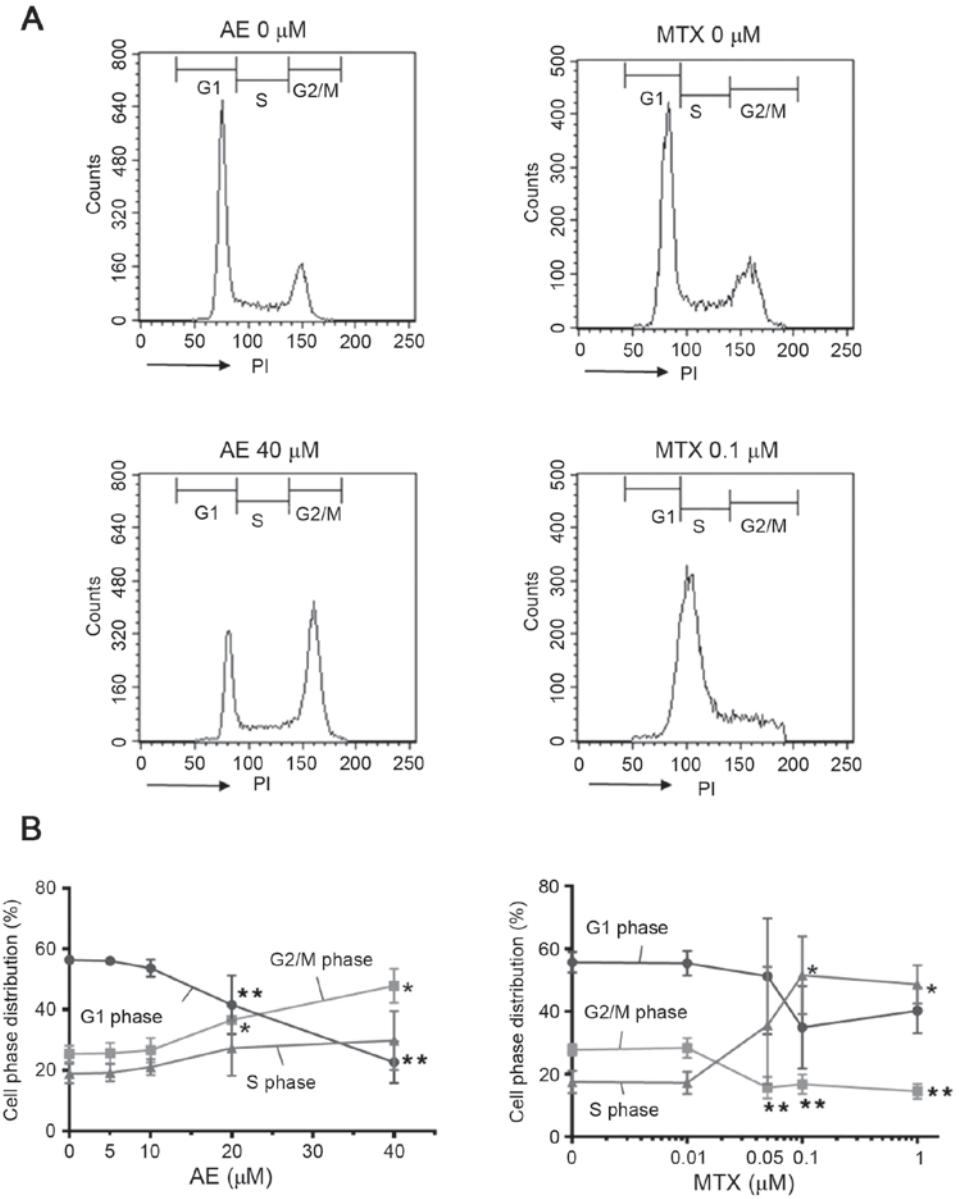

Figure 5. Evaluation of the effect of AE and MTX on cell cycle distribution. MH7A cells ($2x10^5$ cells/well) were seeded into 6-well plates for 24 h, and treated with AE (5, 10, 20 or 40 μM), or MTX (0.01, 0.05, 0.1 or 1 μM) for 48 h. Cells treated with the vehicle dimethyl sulfoxide served as a control (0 μM). Cell cycle distribution was evaluated by PI staining followed by flow cytometric analysis. (A) Representative plots of control cells and cells treated with 40 μM AE or 0.1 μM MTX. (B) Quantification of the percentage of cells in the G1, G2/M and S phases. Data are presented as the mean ± standard deviation of 4 independent experiments. *P<0.05 and **P<0.01 vs. 0 μM. AE, aloe-emodin; MTX, methotrexate; PI, propidium iodide.

pulmonary damage and myelosuppression (20). Thus, RA patients are utilizing complementary and alternative medicine to treat RA symptoms (21,22). AE, a bioactive component of Aloe, is hypothesized to act as an antitumor reagent (17), based on its suppressive action on the cell growth of various human tumor cells (12). In the present study, the effect of AE on the growth of synovial cells was examined, and it was revealed that ≥10 μM AE significantly decreased the numbers of live MH7A cells, based on the CCK-8 assay. A Trypan blue assay also revealed that AE decreased the viability of MH7A cells; however, the deviations of the data were high, and significance was not detected with regards to the Trypan blue assay.

Previous studies have demonstrated that AE induces apoptosis in various human tumor cell lines, including SJ-N-KP neuroblastoma, Hep G2 hepatoma and HL-60 leukemia cells (12-14). Thus, to elucidate whether AE induces the apoptosis of MH7A cells, apoptosis analysis was performed in AE-treated MH7A cells using AnnexinV/PI double staining followed by flow cytometry. The results indicated that following treatment for 2 days with 40 μM AE, 5.6 and 6.2% of cells were early and late apoptotic, respectively, whereas the same concentration and duration of treatment inhibited MH7A cell proliferation by 69%. In addition, AE did not induce apoptosis at concentrations <40 μM, although AE significantly inhibited the proliferation of MH7A cells by 25% at 10 μM and 59% at 20 μM, compared with untreated cells. Therefore, the inhibitory effects of AE on cell proliferation may not be fully explained by the induction of apoptosis.

Notably, AE has been reported to modulate the cell cycle, thereby suppressing the proliferation of cancer cells (14-16).

Thus, to further clarify the underlying mechanism involved in the inhibitory effect of AE on the proliferation of MH7A cells, the effect of AE treatment on cell cycle progression was examined. The majority of cells analyzed in the untreated group were in G1 phase (in preparation for mitosis). AE treatment decreased the percentage of cells in G1 phase and increased the percentage of cells in G2/M phase (prior to/in mitosis) in a dose-dependent manner compared with the untreated group, suggesting that AE induced G2/M arrest in MH7A cells. These observations indicated that AE-treated MH7A cells may not complete mitosis. AE at a dose of 20 μM induced G2/M arrest, whereas the same dose was not sufficient to induce apoptotic cell death. Furthermore, 40 μM AE induced cell cycle arrest in the G2/M phase by 23% among MH7A cells compared with untreated cells, whereas the same dose induced apoptosis in only 12% (5.6 and 6.2% of early and late apoptotic cells) of total cell population. These observations suggested that the G2/M phase arrest may be more important than the induction of apoptosis for the inhibition of cell proliferation by AE.

Consistent with the results of the present study, AE has been reported to induce G2/M phase arrest in other cells, including HL-60 and WiDr colon adenocarcinoma cells (14,16). In WiDr cells, the promoter activity and protein expression levels of cyclin B1, an essential factor for cell cycle progression from G2 to M phase, were strongly suppressed by AE treatment (16). Therefore, AE-mediated suppression of cyclin B1 may be involved in the inhibition of MH7A cell proliferation. By contrast, AE has been demonstrated to induce G1 phase arrest in Hep G2 human hepatoma cells (13), and S phase arrest in FaDu human pharyngeal squamous carcinoma, H1299 human lung cancer and MG-63 human osteosarcoma cells (23). These observations suggested that the cell cycle progression-associated target molecules of AE may differ in various cell types (17).

AE is a 1,8-dihydroxyanthraquinone compound, having an anthraquinone ring and two phenolic hydroxyl groups. Quinones are highly redox-active molecules that lead to the generation of reactive oxygen species (ROS). AE has been demonstrated to induce the loss of mitochondrial membrane potential and caspase-dependent apoptosis in SCC-4 human tongue squamous cancer cells, through increased ROS production (15). It is possible that ROS production may be involved in the AE-induced apoptosis of MH7A cells observed in the present study. However, MH7A cells are more resistant to AE compared with the cancer cells previously examined; 40 μM AE induces 50% apoptosis in SCC-4 cells (15), and 40% apoptosis in CH27 human lung squamous carcinoma cells (24), whereas 40 μM AE induced only 12% apoptosis in MH7A cells.

The effects of AE treatment were compared throughout the present study with the effects of 0.01-1 μM MTX. The concentrations of MTX were selected based on the concentrations detected in synovial fluids of RA patients treated with low-dose MTX (~1.3 μM) (25). The results indicated that ≥0.05 μM MTX inhibited proliferation of MH7A cells, and 1 μM MTX almost completely inhibited proliferation (~90% in 3 days), compared with untreated cells. These observations are in agreement with previous reports that 2 μM MTX inhibited cell growth by ~80% in human synovial fibroblasts (6) and 1 μM MTX inhibited cell growth by ~80% in adherent cells of rheumatic synovial tissue (26).

To understand the mechanism of MTX-induced inhibition of cell proliferation, the effect of MTX on MH7A cell death was examined in the present study. The results indicated that 1 μM MTX treatment for 2 days induced early and late apoptosis in 11.2 and 8.6%, respectively, of MH7A cells. By contrast, 1 μM MTX inhibited the proliferation of MH7A cells by ~80% at the same time point, suggesting that the induction of apoptosis only partially contributed to the inhibition of cell proliferation by MTX, similar to AE.

The effect of MTX on the cell cycle progression of MH7A cells was examined. In contrast to the action of AE, MTX decreased the percentage of cells in G2/M phase and increased the percentage of cells in S phase in a dose-dependent manner, suggesting that MTX induces S phase arrest in MH7A cells. Notably, MTX induced cell cycle arrest in the S phase by 34% in MH7A cells at 0.1 μM, a dose at which apoptotic cell death was not observed. A similar increase in S phase cells was observed following treatment with 1 μM, at which dose apoptotic cell death was induced in 20% (11.2 and 8.6% of early and late apoptotic cells) of cells. These observations suggested that MTX-induced S phase arrest is more important than apoptosis in the MTX-mediated inhibition of proliferation.

As MTX is an antimetabolite that interferes with folate metabolism and DNA synthesis (6), explaining the induction of cell cycle arrest at the S phase. Apoptosis is additionally regulated by molecules involved in cell division and cell cycle progression (27). Thus, MTX-mediated S phase arrest may induce apoptosis in MH7A cells. In addition, AE-mediated G2/M phase arrest may induce apoptosis in MH7A cells.

In conclusion, the results of the present study revealed that AE, and MTX, treatment inhibited the proliferation of MH7A synovial cells. Furthermore, AE and MTX treatment induced apoptosis and cell cycle arrest (at G2/M and S phases, respectively) compared with untreated MH7A cells. For efficient therapeutic intervention in RA, it is essential to suppress proliferation and induce apoptosis of synovial and inflammatory cells in the RA joints (2,3). Thus, the present results suggested that AE, a natural component of Aloe, may be a safe and effective agent for the treatment of RA. Future studies are required to examine more fully the therapeutic potential of AE, including *in vivo* studies in animal models of RA.

Acknowledgements

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