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ORIGINAL ARTICLE

Protective effect of aged garlic extract (AGE) on the apoptosis of intestinal epithelial cells caused by methotrexate

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Abstract
Purpose Methotrexate (MTX) causes intestinal damage, resulting in diarrhea. The side effects often disturb the cancer chemotherapy. We previously reported that AGE protected the small intestine of rats from the MTX-induced damage. In the present paper, the mechanism of the protection of AGE against the MTX-induced damage of small intestine was investigated, using IEC-6 cells originating from rat jejunum crypt.
Methods The viability and apoptosis of IEC-6 cells were examined in the presence of MTX and/or AGE.
Results The viability of IEC-6 cells exposed to MTX was decreased by the increase of MTX concentration. The MTX-induced loss of viable IEC-6 cells was almost completely prevented by the presence of more than 0.1% AGE. In IEC-6 cells exposed to MTX, the cromatin condensation, DNA fragmentation, caspase-3 activation and cytochrome *c* release were observed. These were preserved to the control levels by the presence of AGE. MTX markedly decreased intracellular GSH in IEC-6 cells, but the presence of AGE in IEC-6 cells with MTX preserved intracellular GSH to the control level. IEC-6 cells in G2/M stage markedly decreased 72 h after the MTX treatment, which was preserved to the control level by the presence of AGE. These results indicated that AGE protected IEC-6 cells from the MTX-induced damage.
Conclusions The MTX-induced apoptosis of IEC-6 cells was shown to be depressed by AGE. AGE may be useful for the cancer chemotherapy with MTX, since AGE reduces the MTX-induced intestinal damage.

Keywords Methotrexate · IEC-6 cells · Apoptosis · Aged garlic extract · Intestinal damage

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Introduction

Methotrexate (MTX) is widely used as a chemotherapeutic agent for treatment of many cancers, demonstrating consistent activity against a number of malignant tumors [1]. MTX has also been found to play a major therapeutic role in nonneoplastic diseases as an anti-inflammatory and immunosuppressive agent [2]. However, MTX treatment is often accompanied by side effects such as nausea, vomiting, diarrhea, stomatitis, gastrointestinal ulceration and mucositis [3, 4]. The therapeutic use of MTX has been limited by its toxicity for the proliferating cells, especially the rapidly dividing cells of intestinal crypts. MTX is a dihydrofolate reductase (DHFR) inhibitor that blocks DNA synthesis by depleting the intracellular-reduced folate pools required for the biosynthesis of purines and thymidine, and leads to cell cycle arrest and apoptosis in many cell types [5, 6]. MTX inhibited intestinal epithelial proliferation and induced apoptosis in small intestinal crypts [7, 8]. However, there is no effective treatment to reduce the MTX-induced gastrointestinal toxicity.

The intestinal epithelial cell, IEC-6, is an immortalized epithelial cell line derived from neonatal rat ileum [9]. IEC-6

cells have the characteristics of crypt-type intestinal cells but do not exhibit differentiated morphology or gene expression [9] by the lack of transcription factor *Drosophila* caudal (Cdx2) expression [10]. IEC-6 cells have been extensively used as an in vitro intestinal model such as for gastrointestinal regeneration [11, 12] and for the study of folate and its derivatives transport including MTX [13–15]. In the present study, we investigated the mechanism of MTX-induced gastrointestinal toxicity and tried to combat the cytotoxic action of MTX using IEC-6 cells.

Garlic (*Allium sativum*) extracts have come under intensive study because of their possible beneficial effects on several disease processes. Garlic derivatives have various biological properties such as antimicrobial and antithrombotic activities, immune system enhancement and antitumor potential [16]. Aged garlic extract (AGE) and its constituents have been shown to prevent oxidative injury in endothelial cells [17] and suppress cancer growth [18, 19]. We have previously shown that AGE protects the small intestine of rats from MTX-induced damage [20, 21]. Therefore, it has been suggested that there is different effect of AGE for tumor cells and normal intestine cells. In addition, we recently suggested the antiapoptosis action of AGE in IEC-6 cells [22].

In the present study, we investigated the effect of AGE on the MTX-induced cytotoxicity using IEC-6 cells and showed that MTX-induced apoptosis in IEC-6 cells, which was inhibited by AGE. Our results indicate that AGE prevents the MTX-induced toxicity, suggesting that AGE may be useful for the cancer chemotherapy by reducing the antitumor drugs-induced intestinal damage.

Materials and methods

Materials

MTX was kindly supplied from Wyeth Lederie Ltd (Tokyo, Japan). N-Ac-Asp-Glu-Val-Asp-AFC (Ac-DEVD-AFC) colorimetric caspase-3 substrate was purchased from Biomol Research *Laboratories* Inc. (Plymouth Meeting, Pennsylvania). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and other chemicals were obtained from Sigma Chemical Co. (St Louis, MO). AGE was prepared by Wakunaga Pharmaceutical Co. Ltd (Osaka Japan) as described elsewhere [23].

Cell culture

IEC-6 cells were obtained from American Type Culture Collection (Rockville, MD). IEC-6 cells were grown in DMEM (Sigma) containing 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 5% FBS, 100 units/l bovine insulin (Wako Pure Chemical Industries Ltd, Tokyo, Japan) and 20 mg/l gentamicin sulfate (Nacalai Tesque, Inc. Kyoto, Japan). Cells were incubated at 37°C in 5% CO_2 and 95% air. The culture medium was changed every 2 days.

MTT assay

The viability of IEC-6 cells was determined by the MTT assay. Briefly, IEC-6 cells were plated on a 96-well multiplate and treated with MTX in the presence and absence of AGE for 24, 48 and 72 h. MTT solution (5 mg/ml, 1:10) was added to each well. Following 4-h incubation at 37°C, the produced formazan was dissolved with acid–isopropanol solution (0.04 N HCl/isopropanol). The absorbance at 570 nm (reference at 630 nm) was determined by a microplate reader MULTISKAN JX (Themo LabSystems Inc, Massachusetts).

Hoechst staining

Cells were harvested and centrifuged at 500×*g* for 5 min. Cell pellets were washed once with cold phosphate buffered saline solution (PBS) and fixed for 2 h in 4% (vol/vol) formaldehyde in PBS. Cells were washed with PBS and stained with Hoechst 33342. Cells were examined by fluorescence microscopy at an excitation wavelength, 346 nm and an emission wavelength, 460 nm.

Caspase activity assay

Cells were harvested and centrifuged at 500×*g* for 5 min. Cell pellets were washed once with cold PBS, resuspended in lysis buffer containing 50 mM Tris–HCl (pH 7.5), 10% sucrose, 10 mM EDTA, 0.1% Triton X-100, 10 mM dithiothreitol (DTT), and incubated on ice for 40 min. Lysates were centrifuged at 15,000×*g* for 15 min at 4°C. Caspase-3 activity was determined for each sample by using 100 µl of cell lysate incubated with caspase-3 specific fluorogenic substrate Ac-DEVD-AFC at a final concentration of 50 µM for 1 h at room temperature. The reaction was stopped by diluting the sample with 900 µl PBS. Sample fluorescence was measured at an excitation wavelength, 400 nm and an emission wavelength, 505 nm with the F-2000 fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan).

Preparation of mitochondria and cytosol

Cells were scraped off the flasks and centrifuged at 1,000×*g* for 10 min at 4°C. Cell pellets were resuspended in cold PBS and centrifuged at 700×*g* for 10 min at 4°C. The cell pellets were resuspended in 1 ml of lysis buffer containing 20 mM Hepes-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride,

20 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml pepstatin. Cells were lysed by 15 passage through a 26-gauge needle. The homogenates were centrifuged at 1,000×*g* for 5 min at 4°C. The supernatants were then centrifuged at 10,000×*g* for 15 min at 4°C. The resulting mitochondrial pellets were resuspended in 50 μl of cold cell lysis buffer. The supernatant from the 10,000×*g* spin fraction was further centrifuged at 100,000×*g* for 1 h at 4°C to produce a supernatant corresponding to the cytosolic fraction.

Western blot analysis of cytochrome *c* release

The mitochodria and cytosolic fractions were used for the detection of cytochrome *c*. These fractions were separated on 15% polyacrylamide slab gel containing 0.1% SDS. The fractionated proteins were transferred to Immobilon Transfer Membrane (Millipore) by electroblotting. The blotted membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 and 3% bovine serum albumin for 1 h at room temperature and probed for 1 h at room temperature with cytochrome *c* mouse antibody (1:500) diluted with Tris-buffered saline containing 0.05% Tween 20 and 0.1% bovine serum albumin. The membranes were then allowed to bind donkey anti-mouse IgG (Santa Cruz Biotech. Inc., Santa Cruz, CA) conjugated with horseradish peroxidase (Amersham Biosciences Corp., Piscataway, NJ) (1:1000) at room temperature for 1 h and analyzed using an enhanced chemiluminescence (ECL) kit (Amersham Biosciences Corp.).

Intracellular GSH

Cells were washed once with PBS and incubated for 30 min in the buffer containing 5.6% metaphosphoric acid and 0.08% EDTA. Cells were scraped and centrifuged at 15,000×*g* for 5 min. The supernatant was filtrated through a membrane filter (pore size 0.45 μm) and GSH was determined by HPLC as described previously [24]. Briefly, the HPLC conditions were as follows: pump and detector, two LC-10AD liquid chromatographs with a RF-535 fluorescence HPLC monitor (Shimadzu Ltd, Kyoto, Japan). column, Inertsil ODS column (4.6 mm inner diameter × 250 mm, GL Sciences Ltd, Tokyo, Japan); mobile phase, 0.1% trifluoroacetic acid-methanol (18:1); flow rate, 1.0 ml/min; reaction solution, 2.3 mM *o*-phthalaldehyde and 14.4 mM 2-mercaptoethanol in 10% methanol/100 mM carbonate buffer (pH 10.5) which was delivered at 0.2 ml/min. The mixture was then passed through a stainless-steel coil at 70°C to facilitate derivatization. Fluorescence detection was at an excitation wavelength, 355 nm and an emission wavelength, 425 nm. Protein contents of cell digests were determined according to Lowry et al. [25] using bovine serum albumin as the standard.

Cell cycle distribution

Cell cycle profiles were assessed by measuring the DNA contents of individual cells using flow cytometry. Cells were collected and washed with PBS and fixed with 70% cold ethanol (−20°C). The samples were then treated with RNase A, stained with propidium iodide and analyzed using a cell sorter (MoFlo, DakoCytomation).

Statistical analysis

Data are presented as mean ± SD. Statistical analysis of data for multiple comparisons was performed by ANOVA. For single comparison, the significance of differences between means was determined by unpaired Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Effect of AGE on the MTX-induced loss of IEC-6 cell viability

The effect of AGE and/or MTX on the viability of IEC-6 cells was determined according to the MTT assay. IEC-6 cells treated with MTX for 24 h decreased their viability slightly. The exposure of IEC-6 cells to MTX for 48 and 72 h significantly decreased the cell viability, depending on the exposure time and concentration of MTX (Fig. 1a). AGE (0.1, 0.5, 1%) prevented the decrease of cell viability caused by MTX (Fig. 1b).

The MTX-induced apoptosis of IEC-6 cells and its prevention by AGE

The MTX-induced apoptosis of IEC-6 cells was examined by fluorescence technique. The fluorescence microscopy of IEC-6 cells with the Hoechst 33342 nuclear stain was measured to clarify whether the nucleus undergoes the condensation due to apoptosis. IEC-6 cells exposed to 1 μM MTX for 72 h showed the nucleus condensation by Hoechst staining (Fig. 2a, c). The addition of 0.5% AGE to the medium containing 1 μM MTX inhibited the MTX-induced nucleus condensation (Fig. 2d), while AGE alone caused no change of nucleus (Fig. 2b).

Caspase-3 and cytochrome *c* of IEC-6 cells exposed to MTX and/or AGE

Caspase-3 activity was examined using the specific fluorogenic substrate Ac-DEVD-AFC. As shown in Fig. 3a, the exposure of IEC-6 cells to MTX caused an increase in

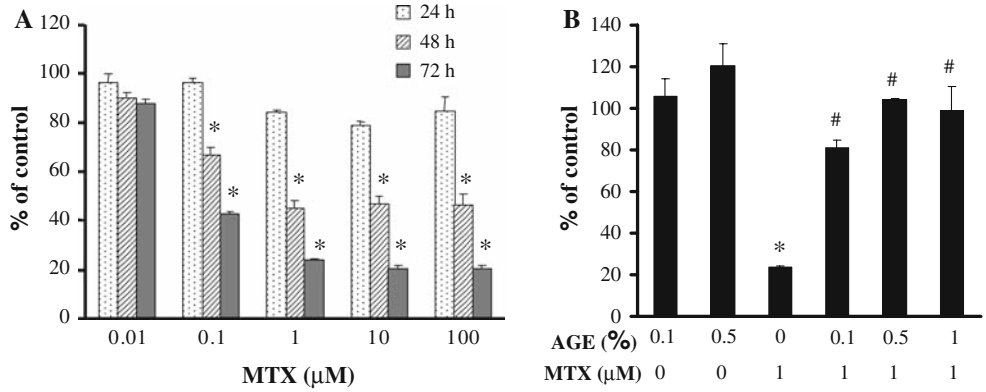

Fig. 1 Effect of AGE on MTX-induced loss of IEC-6 cell viability. IEC-6 cells treated with MTX at different concentrations for 24, 48, 72 h (**a**), and with 1 μM MTX in the presence of different concentrations of AGE for 72 h (**b**). Viable cells were determined by the MTT assay. The data are expressed as the percentage of control cells treated with medium only and as the mean ± SD of at least four independent experiments. *$p < 0.01$, compared to the control (without MTX). #$p < 0.01$, compared to the cells treated with MTX

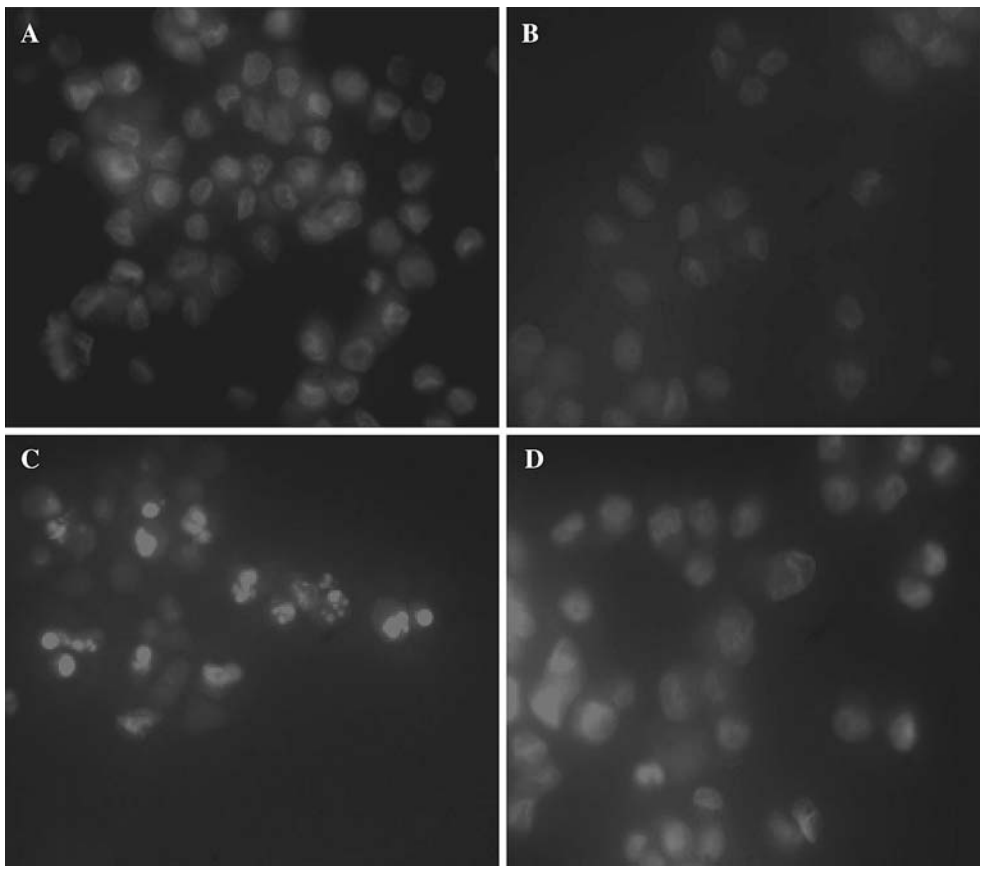

Fig. 2 Effect of AGE on nuclei in MTX treated IEC-6 cells. IEC-6 cells were treated with AGE and/or MTX for 72 h. Cells were fixed and stained with Hoechst 33342, and visualized under a fluorescence microscope. **a** Control, **b** 0.5% AGE, **c** 1 μM MTX, **d** 1 μM MTX + 0.5% AGE. Experiments were repeated thrice with similar results

caspase-3 activity in a dose-dependent manner. Caspase-3 activity in IEC-6 cells exposed to MTX for more than 48 h was markedly up-regulated (Fig. 3b). AGE (0.5%) significantly reduced the activation of caspase-3 induced by MTX, but AGE itself had no effect on caspase-3 activity (Fig. 3b). The release of cytochrome *c* from mitochondrial intermembraneous space into cytosol is a prominent downstream manifestation of the evolution of apoptotic cell death. As shown in Fig. 4, MTX induced the release of cytochrome *c* from mitochondria into cytosol, and AGE prevented the MTX-induced release of cytochrome *c*.

Effect of AGE on the MTX-induced decrease of intracellular GSH

The exposure of IEC-6 cells to MTX decreased the contents of intracellular GSH in an MTX concentration-dependent manner (Fig. 5a). The effects of MTX (1 mM) and/or AGE

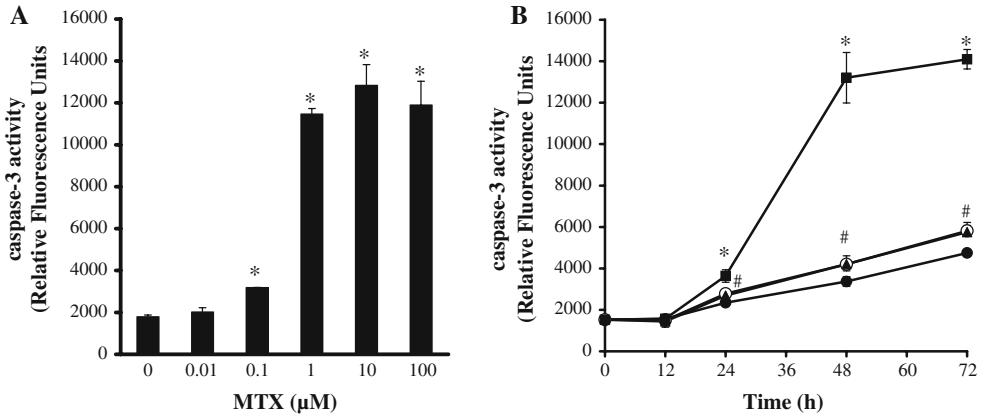

Fig. 3 Effect of AGE and/or MTX on caspase-3 activation. IEC-6 cells were treated with MTX at different concentrations for 72 h (**a**), and treated with 1 μM MTX in the absence and presence of 0.5% AGE for 0–72 h (**b**). Caspase-3 activation was measured as described in "Materials and methods". *Closed circle* control, *open circle* 0.5% AGE, *filled square* 1 μM MTX, *filled triangle* 1 μM MTX + 0.5% AGE. The data are expressed as the mean ± SD of three independent experiments. *$p < 0.05$, compared to the control (without MTX). #$p < 0.05$, compared to the cells treated with MTX

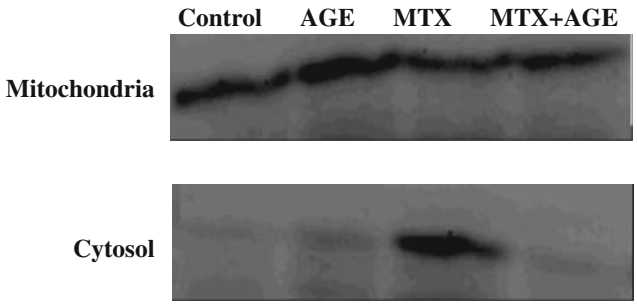

Fig. 4 Effect of AGE and/or MTX on cytochrome *c* release from mitochondria to cytosol. IEC-6 cells were treated with 0.5% AGE and/or 1 μM MTX for 72 h. The amounts of cytochrome *c* in mitochondria and cytosol fractions were detected by Western blotting as described in "Materials and methods". Experiments were repeated thrice with similar results

(0.5%) on intracellular GSH were examined by changing the exposure periods of IEC-6 cells with MTX and/or AGE (Fig. 5b). IEC-6 cells treated with AGE (0.5%) itself significantly increased the amounts of intracellular GSH. The treatment of IEC-6 cells with MTX (1 μM) and AGE (0.5%) prevented the MTX-induced decrease of intracellular GSH, which rather increased the amounts of intracellular GSH to the level of intracellular GSH which was induced by AGE alone. The effect of AGE-induced increase of intracellular GSH against the MTX-induced decrease of its GSH was dependent on the AGE concentration (Fig. 5c).

Effect of AGE and/or MTX on the cell cycle

The cell cycle distribution of IEC-6 cells treated with MTX (1 μM) and/or AGE (0.5%) was examined by separating them using the cell sorter (Fig. 6). The number of IEC-6 cells in G_2/M stage decreased markedly 72 h after the MTX treatment as shown by an arrow (Fig. 6c). This was preserved to the control level by the presence of AGE with MTX (Fig. 6d). AGE itself did not affect the cell cycle of IEC-6 cells (Fig. 6b). The similar results were obtained from the cell cycle distribution of IEC-6 cells treated with MTX and/or AGE for 48 h, while no clear change in the cell cycle distribution was observed in the IEC-6 cells treated for 24 h (data not shown).

Discussion

MTX plays an important role in the treatment of a variety of malignancies and is well understood with respect to its molecular mechanism of action [26]. However, the clinical application of this drug is limited by its toxic dose-related side effects. In the present study, we have shown that MTX induced IEC-6 cell death through apoptosis, as demonstrated by nuclear condensation, in addition to an increase in DNA fragmentation [22]. DNA of IEC-6 cells treated with MTX (0, 0.01, 0.1, 1 and 10 μM) and/or AGE (0, 0.5 and 1%) was isolated and analyzed by agarose gel electrophoresis. MTX-induced DNA fragmentation in a concentration-dependent manner, while AGE inhibited the MTX-induced DNA fragmentation in a concentration-dependent manner [22]. MTX also decreases the contents of intracellular GSH and populations in the G_2/M phase of the cell cycle. AGE protects the IEC-6 cells from the MTX-induced toxicity. These results suggest that AGE may be useful for the cancer chemotherapy with MTX.

Our results indicate that the MTX treatment of IEC-6 cells increases the cell death and decreases the cell number in a time- and concentration-dependent manner. MTX induces the cell nuclear condensation and an increase in

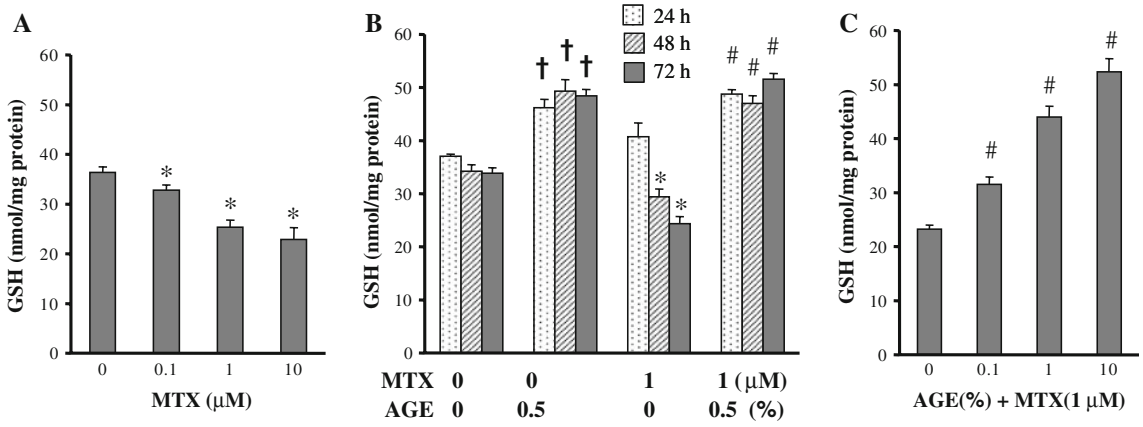

Fig. 5 Effect of AGE on MTX-induced decrease of intracellular GSH. IEC-6 cells treated with MTX at different concentrations for 72 h (**a**), with AGE and/or MTX for 24, 48, 72 h (**b**), and with MTX in the presence of different concentrations of 0.5% AGE for 72 h (**c**). GSH was determined as described in "Materials and methods". The results are expressed as the mean ± SD of at least four independent experiments. *,†$p < 0.05$, compared to the control (without MTX and AGE). #$p < 0.05$, compared to the cells treated with MTX and without AGE

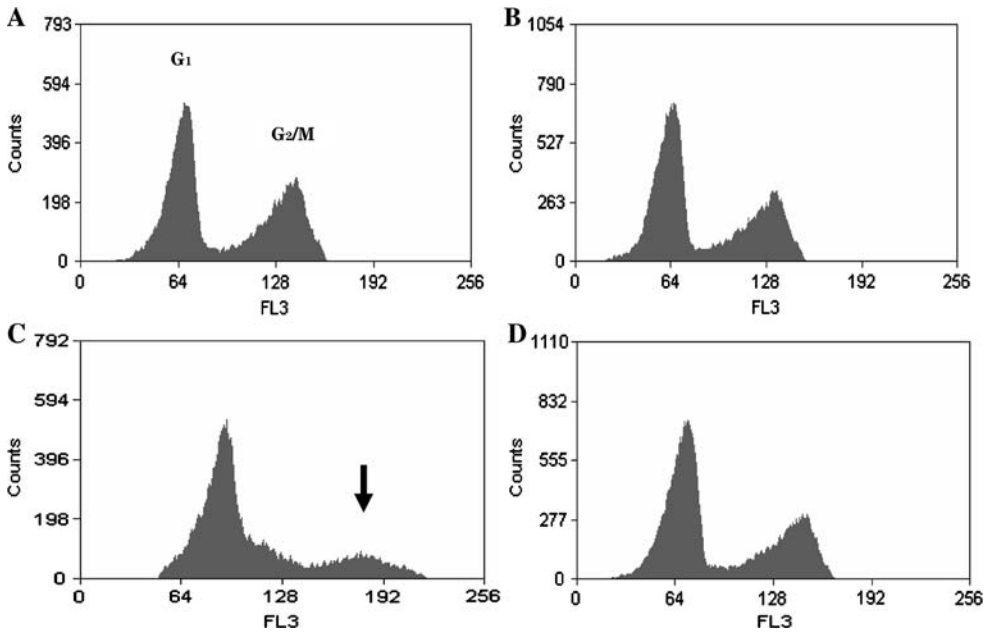

Fig. 6 Effect of AGE on MTX-induced cell cycle arrest in IEC-6 cells. IEC-6 cells were treated for 72 h without MTX and AGE (control) (**a**), with 0.5% AGE (**b**), with 1 μM MTX (**c**) and with 0.5% AGE and 1 μM MTX (**d**). Cell cycles were determined as described in "Materials and methods"

DNA fragmentation in a dose-dependent manner, indicating that MTX induced the IEC-6 cell death through apoptosis. These results are consistent with previous observation that MTX induces apoptosis in the small intestine [8]. Furthermore, our results showed that MTX increased the activity of caspase-3 of IEC-6 cells in a time- and concentration-dependent manner, and induced cytochrome *c* release from mitochondria into cytosol. We evidence that caspase-3, a key enzyme for the execution of apoptosis in many instances, also plays a central role for MTX-induced apoptosis in IEC-6 cells. Mitochondria play an essential role in many forms of apoptosis by releasing apoptogenic factors, such as cytochrome *c* [27, 28] and apoptosis-inducing factor [29] from the intermembrane space into the cytoplasm. Cytochrome *c* binds to the cytoplasmic protein Apaf-1 via the COOH terminus in the presence of ATP, resulting in an oligomer complex [29]. This complex recruits procaspase-9 and induces the self-activation of caspase-9 [29, 30]. Active caspase-9 cleaves and activates downstream caspases-3, -6, and -8, leading to apoptosis [31]. In the present study, the MTX treatment of IEC-6 cells was shown to increase the release of cytochrome *c* from mitochondria into cytosol, and to activate caspase-3, leading to the apoptosis.

The present study showed that the MTX treatment also resulted in a significant decrease in GSH level in a dose-dependent manner and in the decrease of the G_2/M phase and accumulation of cells in the S phase of the cell cycle. Reduced GSH participates directly in several intracellular functions, including detoxification of xenobiotics, protection from free radicals-induced damages, transport of amino acids, synthesis of DNA, microtubule assembly, and cell motility [32, 33]. Since GSH is also important for functional maintenance of mitochondria, the MTX-induced decrease of intracellular GSH may lead to the damage of mitochondria as shown by the release of cytochrome *c* from mitochondria. The present result that MTX decreases the contents of intracellular GSH of IEC-6 cells in a concentration-dependent manner is consistent with our previous observation that MTX induces intracellular GSH depletion in the small intestine [34]. GSH is connected with the oxidative stress of cells and the oxidative stress is a factor of cell damage. We have evidence that MTX caused the oxidative stress in small intestinal membranes [34, 35].

AGE is produced by storing sliced raw garlic in 15–20% ethanol for 20 months. This whole process causes a considerable loss of allicin and increases the activity of certain newer compounds, such as S-allylcysteine, S-allylmercaptocysteine, allixin and selenium which are stable, bioavailable and antioxidative [36]. AGE is effective against growth of cancer [18, 19]. We previously showed that AGE protected the small intestine of rats and IEC-6 cells from the MTX-induced damage [21, 37]. The present results showed that the MTX induced loss of viable IEC-6 cells was almost completely prevented by the presence of more than 0.1% AGE. The extents of cromatin condensation, DNA fragmentation, caspase-3 activation and cytochrome *c* release caused by MTX were preserved to their control levels by the presence of AGE. These results suggest that AGE inhibits the MTX-induced apoptosis in the IEC-6 cells. Moreover, AGE prevented the decrease of intracellular GSH caused by MTX and preserved the intracellular GSH to the normal level. MTX produces reactive oxygen species in small intestinal membranes [34, 35]. The MTX-induced apoptosis in the IEC-6 cells suggests the apoptosis was mediated by the reactive oxygen species, because it is inhibited by AGE which has the antioxidative effect as we reported previously [23].AGE was also found to keep the cell cycle of IEC-6 cells normal even in the presence of MTX. Thus, the protective effect of AGE may possibly be associated with the depression of the oxidative stress.

These cell culture studies indicated that AGE protected IEC-6 cells from the MTX-induced damage. These protective effect of AGE found in IEC-6 cells originating from crypt cells of rat small intestine can explain the reason why AGE administered to rats protects the small intestine of rats from the damage of small intestine induced by MTX administration [20, 21]. AGE may possibly be useful for the cancer chemotherapy with MTX because of reducing the MTX-induced intestinal damage.

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