Antitumor effects of a water-soluble extract from Maitake (*Grifola frondosa*) on human gastric cancer cell lines

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Abstract. We investigated the effects of a water-soluble extract of Maitake (*Grifola frondosa*), a Japanese edible mushroom, on the proliferation and cell death of four human gastric cancer cell lines (TMK-1, MKN28, MKN45 and MKN74). The Maitake extract (ME) inhibited the proliferation of all four cell lines in a time-dependent manner. The inhibition was most pronounced in TMK-1 cells, which exhibited up to 90% inhibition after treatment with 10% ME for 3 days. Staining of ME-treated TMK-1 cells with Hoechst 33258 revealed increased numbers of nuclear condensations and apoptotic bodies. Induction of apoptosis was confirmed by fluorescence-activated cell sorting analyses. Western blot analyses of TMK-1 cells after ME treatment revealed increases in intracytoplasmic cytochrome c and cleavage of caspase-3 and poly(ADP-ribose) polymerase, but no expression of p21 or Bax. The caspase-3 protease activities in lysates of TMK-1 cells treated with 1% or 10% ME were about three times higher than those in control cells. The proliferation of TMK-1 cells was hardly affected by the caspase-3 inhibitor z-DEVD-fmk. Taken together, these results suggest that ME induces apoptosis of TMK-1 cells by caspase-3-dependent and -independent pathways, resulting in potential antitumor effects on gastric cancer.

Introduction

Maitake (*Grifola frondosa*) is an edible mushroom that is also used as a Chinese medicine called 'Keisho'. A water-soluble extract of Maitake mushrooms has been confirmed to contain substances with anti-diabetic activity (1). The standardized product, namely the MD-fraction containing a ß-glucan extracted from Maitake, has been analyzed for various medicinal properties such as its antitumor (2-7),

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antihyperlipoid (8) and antihepatitis (9) effects. There is a growing consensus that the MD-fraction strongly enhances immunocompetent cell activities (2-6,8,9). These reports suggest that some components of the Maitake extract may represent potential cancer chemopreventive substances. However, there are few reports on the effects of the Maitake extract on human cancer cell lines (7).

In the present study, we investigated the effects of a water-soluble Maitake extract on four human gastric cancer cell lines, in terms of their cell proliferation, cell cycle and apoptosis.

Materials and methods

Preparation of the Maitake extract. A water-soluble extract of Maitake (5.25% w/v) was obtained from the Industrial Research Institute of Tottori Prefecture (Sakaiminato, Japan). The extract and 4 volumes of 95% ethanol were mixed overnight and the deposit after centrifugation was dried to a powder at room temperature. The powder was dissolved in ion-exchanged water and freeze-dried to remove the remaining ethanol. The freeze-dried powder was dissolved in ion-exchanged water to a concentration of 0.56% (w/v), and the resulting solution was termed the Maitake extract (ME).

Cell lines and culture conditions. Four cell lines derived from human gastric cancer (TMK-1, MKN28, MKN45 and MKN74) were used in this study. All the cell lines were cultured in RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 292 μg/ml L(+)-glutamine in a humidified 5% CO_2 incubator at 37˚C.

Cell viability and detection of apoptotic cells. Approximately 1×10^5 cells were seeded into 9.6-cm dishes, and incubated with 10% ME for 0, 24, 48 or 72 h. After the required incubation, the cells were washed twice with phosphate-buffered saline (PBS), trypsinized and harvested. The numbers of viable cells were counted under a phase-contrast microscope (Olympus, Tokyo, Japan). Non-viable cells exhibiting vesicles or wrinkled appearances were distinguished morphologically. To detect apoptotic cells, both adherent and floating cells were collected, fixed with 100 μl of Clarke's fixative (ethanol:acetic acid = 3:1) for 5 min at 4˚C, allowed to dry

in air and then stained with 1 mg/ml Hoechst 33258 for 10 min in the dark at room temperature. After staining, the cells were washed three times with distilled water for 5 min each. The apoptotic index (AI) was defined as follows: AI (%) = (apoptotic cells/200 cells) x 100. Data represent the means of three independent experiments.

Cell cycle analysis by flow cytometry. For cell cycle analysis, TMK-1 cells were cultured for 2 days. Subsequently, the medium was exchanged for fresh medium with or without 10% ME and the cells were cultured for a further 24 or 48 h. The cell cycle was analyzed as described previously (9). Briefly, the cells were lysed in 0.5 ml of 0.1% sodium citrate solution containing 50 μg/ml propidium iodide (PI), 0.05% Nonidet-P 40 and 10 μg/ml RNase A in PBS(-), and then incubated on ice for 20 min. Nuclei were collected and resuspended in 0.5 ml of PBS(-) containing 1% FBS. The nuclear DNA content was analyzed by flow cytometry using an EPICS XL flow cytometer (Coulter Electronics Inc., Hialeah, FL). The proportion of cells in the pre-G1 fraction of the cell cycle was determined using the Multitime software (Phoenix Flow Systems, San Diego, CA).

Detection of cytochrome c release by Western blot analysis. Approximately 2.5x10^6 cells were seeded in 7.9-cm dishes. After ME treatment, the cells were harvested and solubilized in 100 μl of buffer A (20 mM HEPES-KOH pH 7.5, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM DTT, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 250 mM sucrose) (8). After lysis, the supernatants (cytosolic fractions) were collected by centrifugation at 14,900 rpm for 10 min at 4˚C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analyses were performed as described below. The protein concentration was determined by the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA) using bovine serum albumin (Wako, Osaka, Japan) as the standard. Subsequently, appropriate protein amounts were separated by SDS-PAGE under reducing conditions using a 12% gel and electrotransferred onto a polyvinylidene difluoride membrane (Millipore Co., Bedford, MA). After blocking with 5% skim milk in PBS(-) containing 0.01% Tween-20 (T-PBS), the membrane was blotted with a mouse anti-human cytochrome c monoclonal antibody (clone 7H8.2C12; 1:500; BD Biosciences, San Jose, CA) overnight at 4˚C. The membrane was washed with T-PBS once for 15 min and three times for 5 min each, and then incubated with peroxidase-labeled anti-mouse IgG (1:1000; MBL Co., Ltd., Nagoya, Japan) for 30 min at room temperature. After washing in T-PBS as described above, signals were detected with an enhanced chemiluminescence system (ECL Western Blot Analysis System; Amersham Pharmacia Biotech, Buckinghamshire, UK).

Detection of apoptosis-related proteins by Western blotting. For total protein extracts, 1x10^6 cells were lysed in 150 μl of lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.1% SDS, 1% sodium deoxycholate, 1% Triton X-100, 1 mM PMSF, 5 mg/ml aprotinin, 1 μg/ml leupeptin). Next, aliquots containing 50 μg of protein were separated by SDS-PAGE. Western blot analyses were performed using the following

antibodies: mouse anti-CPP32/caspase-3 monoclonal antibody (1:1000; Transduction Laboratories, Lexington, KY); rabbit anti-human caspase-7 and anti-cleaved caspase-7 antibodies (1:1000, respectively; Cell Signaling Technology, Beverly, MA); rabbit anti-human caspase-8 antibody (1:200; Santa Cruz Biotechnology Inc., Santa Cruz, CA); rabbit anti-human caspase-9 and cleaved-caspase-9 polyclonal antibody and rabbit anti-human poly(ADP-ribose) polymerase (PARP) and anti-cleaved-PARP polyclonal antibody (1:1000, respectively; Cell Signaling Technology); mouse anti-bcl-2 monoclonal antibody (clone 124; 1:250; Dako A/S, Glostrup, Denmark); mouse anti-human Bax monoclonal antibody (clone 4F11; 1:500; MBL Co., Ltd.); mouse anti-p21 monoclonal antibody (F-5; 1:200; Santa Cruz Biotechnology). The membranes were developed with peroxidase-labeled anti-rabbit IgG (1:1000; MBL Co., Ltd.) or anti-mouse IgG (1:1000; MBL Co., Ltd.).

Caspase-3 activation assay. A CPP32/Caspase-3 Colorimetric Protease Assay Kit (MBL Co., Ltd.) was used to investigate caspase-3 activation in ME-treated TMK-1 cells. The assay was performed according to the manufacturer's instructions after 24 h of incubation with 0, 0.1, 1 or 10% ME. Caspase-3 activation led to cleavage of the provided colorimetric substrates (substrate peptides conjugated to pNA; DEVD-pNA for caspase-3), which could be measured photometrically at 405 nm.

Z-DEVD-fmk, a caspase-3 inhibitor, was obtained from Peptide Institute (Osaka, Japan). For caspase-3 inhibition experiments, TMK-1 cells were treated with 10% ME for 0, 24, 48 or 72 h. For these experiments, the cells were incubated with 50 μM z-DEVD-fmk for 2 h before the addition of ME. After incubation with ME for the required times, the cells were harvested and the numbers of viable cells were counted.

Results

Induction of apoptosis by G. frondosa. To determine their susceptibilities to ME, the four gastric cancer cell lines were exposed to 10% ME for 24, 46 and 72 h. As shown in Fig. 1, the number of viable cells decreased in a time-dependent manner in all four cell lines. The affected cells all floated owing to loss of adherence to the flasks and exhibited 'blebbing' (vesicle formation) (Fig. 2A) compared with control cells (Fig. 2B). Therefore, ME exerted cytotoxic effects on all four gastric cancer cell lines. TMK-1 cells exhibited the most striking effects, and their cell viability was approximately 10% after 72 h of treatment. Based on these findings, we used TMK-1 cells for further analyses.

To determine whether the suppression of TMK-1 cell viability induced by ME was associated with cell cycle arrest or apoptosis, we examined the cell cycle distribution by flow cytometry analysis. Fig. 3 shows the DNA content frequency histogram for PI-stained TMK-1 cells following incubation with or without ME for 24 and 48 h. Treatment with 10% ME induced an increase in the pre-G1 fraction, corresponding to apoptotic cells, and the percentage reached 13.3% after 48 h.

Next, we examined the cellular morphological changes after ME treatment for 0, 24 and 48 h using Hoechst 33258
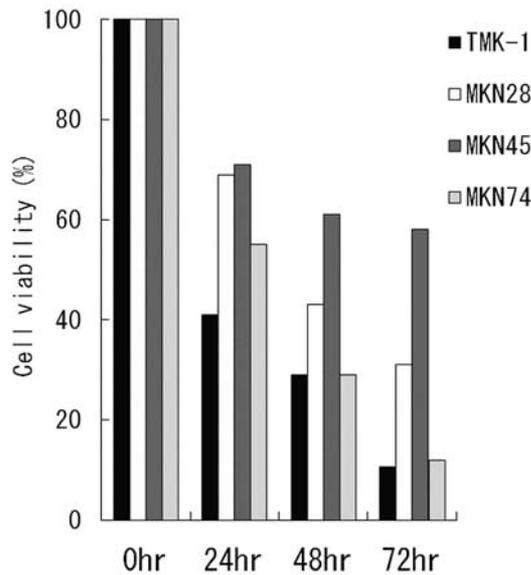
Figure 1. Effects of ME on the viability of four gastric cancer cell lines. ME induces cell death in a time-dependent manner in all four cell lines. The data shown are the means of three independent experiments. Closed bars, TMK-1; open bars, MKN28; dark gray bars, MKN45; thin gray bars, MKN74.
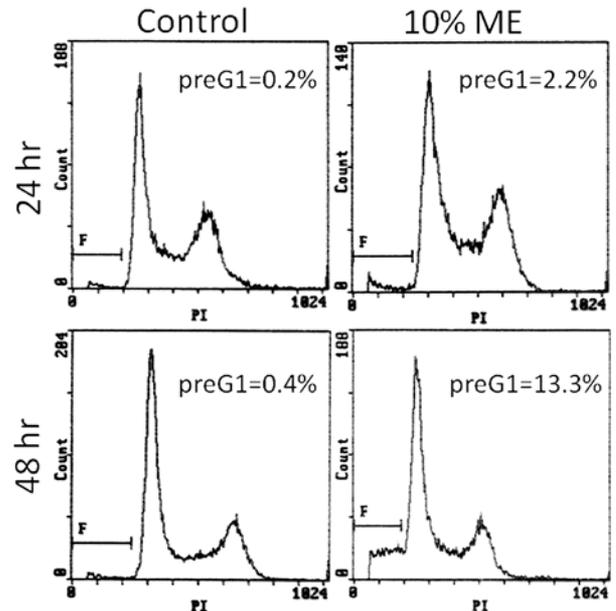

Figure 3. Analysis of the cell cycle using flow cytometry. TMK-1 cells were incubated with (10% ME) or without (control) 10% ME for 24 and 48 h. The percentages of cells in the pre-G1 phase are indicated in each cell cycle profile.
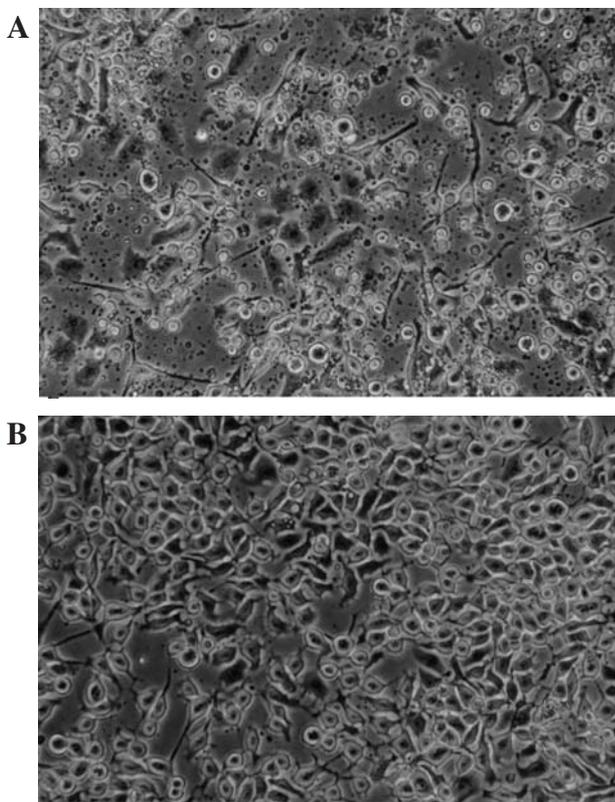
Figure 2. Photomicrographs of 10% ME-treated (A) and untreated (B) TMK-1 cells in culture.
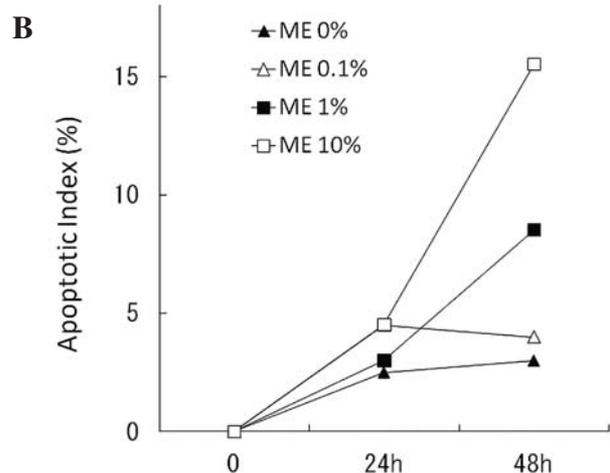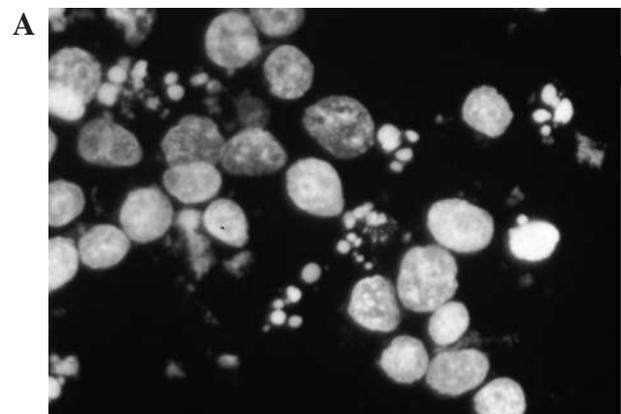

Figure 4. (A) Morphological analysis of TMK-1 cells treated with 10% ME for 48 h. Cells were stained with Hoechst 33258 (x800). Some cells show apoptosis with fragmented and condensed nuclei. (B) AI values induced by ME. TMK-1 cells were incubated with 0% (closed triangles), 0.1% (open triangles), 1% (closed squares) or 10% (open squares) ME for 24 and 48 h. The data shown are the means of three independent experiments. The AI increases in a time- and dose-dependent manner.

staining. Condensation of heterochromatin and nuclei was variably detected (Fig. 4A). The AI increased in a time- and dose-dependent manner (Fig. 4B). The AI values were 15.5% in 10% ME-treated cells, 8.5% in 1% ME-treated cells, 4% in 0.1% ME-treated cells and 3% in control cells after 48 h.
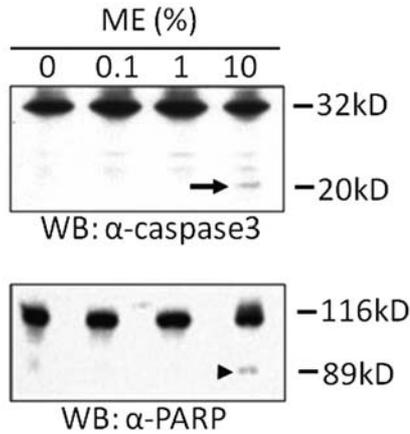
Figure 5. Detection of cleavage of caspase-3 and PARP during apoptosis by Western blotting. TMK-1 cells were treated with 0, 0.1, 1 or 10% ME for 24 h. Cleavage of caspase-3 (arrow) and PARP (arrowheads) is detected after treatment with 10% ME.
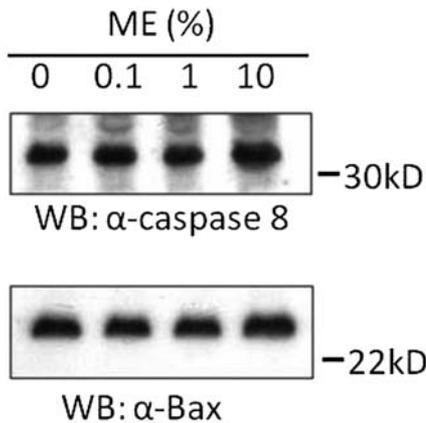

Figure 6. Detection of caspase-8 (above) and bax (bottom) during apoptosis by Western blotting. TMK-1 cells were treated with 0, 0.1, 1 or 10% ME for 24 h. The expression levels of caspase-8 and bax proteins remain unchanged by treatment with ME.

Expression of apoptosis-related proteins in ME-treated TMK-1 cells. Activation of caspase-3 in whole cell lysates was analyzed by Western blotting using an antibody that recognized the 20-kDa cleaved fragment of activated caspase-3 (Fig. 5, arrow). Treatment with 10% ME for 24 h induced caspase-3 cleavage and activation in conjunction with cleavage of PARP (Fig. 5, arrowhead). On the other hand, the expression levels of caspase-8 and bax protein remained unchanged by treatment with ME (Fig. 6). Detectable levels of bcl-2, p21, caspase-7 and caspase-9 were not observed in TMK-1 cells by Western blotting (data not shown).

Cytochrome c release into the cytoplasm of TMK-1 cells during ME treatment. To investigate whether ME induces the release of cytochrome c from mitochondria into the cytosol, we determined the amounts of cytochrome c in the cytosolic fractions of TMK-1 cells exposed to 0, 0.1 or 10% ME by Western blot analysis. The amount of cytochrome c in the cytosol increased in a dose-dependent manner after 24 h (cytosol), although the expression level remained unchanged in the total cell lysates (cellular) (Fig. 7).
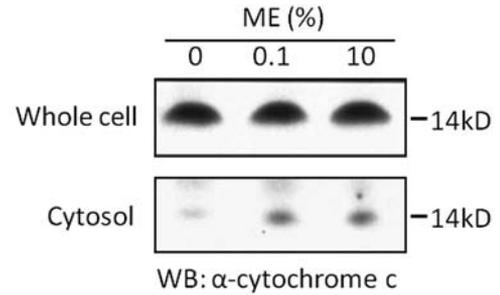

Figure 7. Detection of cytochrome c release by Western blotting. Cytochrome c is released after treatment of TMK-1 cells with 0.1 or 10% ME for 24 h (Cytosol), but the total amount of cytochrome c at the cellular protein level remains unchanged (Cellular).
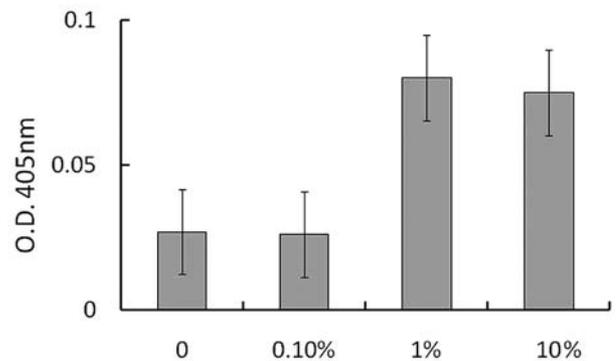

Figure 8. Colorimetric assay of caspase-3 activation. The activity of caspase-3 is increased in TMK-1 cells after treatment with 1 and 10% ME but remains unchanged after treatment with 0.1% ME.
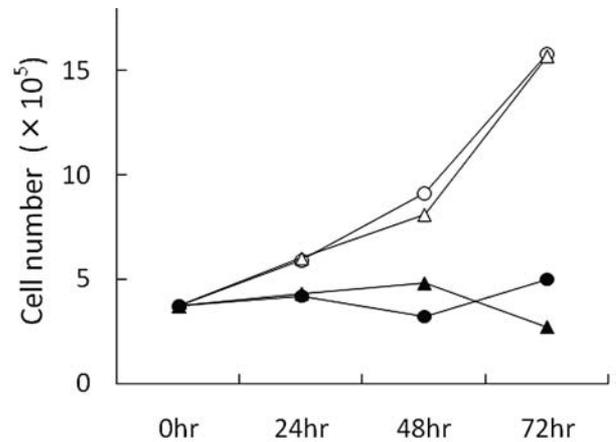

Figure 9. ME-mediated cell growth inhibition is not blocked by the caspase-3 inhibitor z-DEVD-fmk in TMK-1 cells. Cells were incubated with (open and closed triangles) or without (open and closed circles) 50 μM z-DEVD-fmk for 2 h, followed by the addition of 0% (open triangles and circles) or 10% (closed triangles and circles) ME. The data shown are the means of three independent experiments.

Caspase-3 activation assay. The activity of caspase-3 in TMK-1 cells treated with 1 or 10% ME was increased by about 3-fold compared with control cells (Fig. 8). Therefore, the activation of caspase-3 preceded the induction of apoptosis. The caspase-3 activity remained unchanged in cells treated with 0.1% ME.

Caspase-3 inhibition assay. The above findings indicate that ME treatment of TMK-1 cells results in apoptosis via activation of caspase-3. Since z-DEVD-fmk inhibits caspase-3 activation, we examined whether z-DEVD-fmk could block ME-mediated cell death. Compared with ME treatment (10%), preincubation of TMK-1 cells with z-DEVD-fmk (50 μM) for 2 h before ME treatment resulted in no significant increase in cell growth (Fig. 9).

Discussion

It is well known that the ß (D)-glucan derived from *G. frondosa* increases the activity of the immune system (14,15). We examined the direct antitumor effects of our ME on gastric cancer cell lines, and clearly detected decreases in their cell viabilities. These results suggest that ME inhibits cell proliferation, regardless of the histological type or p53 gene status of the cell lines.

When we cultured TMK-1 cells with ME, the cellular morphology became round and cells floated in the culture medium in a time-dependent manner, suggesting that apoptosis occurred in these experiments. Fluorescence-activated cell sorting (FACS) analysis revealed that about 13% of the total cells were in the pre-G1 phase when TMK-1 cells were cultured with 10% ME for 48 h. Hoechst staining confirmed apoptosis with many oil drops in the nuclei of cells treated with 1% or 10% ME, in contrast to the apparent absence of apoptosis among the cells treated with 0.1% or 0% ME. The AI values confirmed by Hoechst staining were similar to those based on the results of the FACS analysis. On the other hand, the viability of TMK-1 cells cultured with 10% ME for 48 h was decreased by about 70% compared with control cells. These results cannot be explained by the FACS and AI analyses. This discrepancy may partly arise through necrosis and/or cell cycle arrest, similar to the effects of other anticancer agents (15).

It is well known that there are many signal transduction pathways in apoptosis (16,17). Activation of caspase-3, a component of the downstream apoptosis pathway, is essential for the induction of apoptosis (16). Cleavage of PARP, an endogenous substrate of caspase-3, provides definite evidence of apoptosis (17). In the present study, cleavage of caspase-3 and PARP was identified in 10% ME-treated TMK-1 cells by Western blot analysis, confirming activation of the apoptosis signal pathway at the molecular level. In addition, we investigated various apoptosis-related proteins, namely cytochrome c, caspase-9 and Apaf-1, to confirm the presence of apoptosis signaling in the upstream of caspase-3. Li *et al* (18) reported that caspase-9 is the most critical upstream molecule of the apoptotic protease cascade, and becomes activated by cytochrome c and dATP. Although the total expression level of cytochrome c remained constant regardless of ME treatment, the cytoplasmic level of cytochrome c was increased by ME treatment compared with control cells. These results suggest that cytochrome c is released from mitochondria into the cytoplasm.

Caspase-7 acts as an apoptosis-related protein in parallel with caspase-3 and caspase-9 in the downstream of cytochrome c (18). Bcl-2 is mainly located on the outer membrane of mitochondria and prevents the release of cytochrome c,

thereby preventing caspase activation (19,20). Caspase-8 activates downstream caspases by direct or indirect cleavage of Bid and induces cytochrome c release from mitochondria (21). The present study revealed almost the same expression levels of these molecules, suggesting that they do not participate in the apoptosis induced by ME. Recently, many studies on chemotherapeutic agents have demonstrated that there are two major cell-intrinsic pathways for apoptosis induction, namely ligation of cell surface death receptors and involvement of release of mitochondrial cytochrome c (22-25). ME may trigger apoptosis directly or indirectly by inducing the release of cytochrome c from mitochondria without caspase-8 activation.

To investigate the molecular mechanism underlying the changes observed in the cell kinetics, we evaluated the activation of caspase-3. Since DEVE-dependent cleavage activity exhibits similar kinetics, colorimetric methods were applied. Our findings revealed that ME induced significant caspase-3-dependent apoptosis in TMK-1 cells. However, coincubation with a caspase-3 inhibitor did not totally abolish the apoptosis, suggesting the presence of both caspase-3-dependent and -independent pathways.

In conclusion, we have clearly demonstrated *in vitro* antitumor effects of a water-soluble ME of Maitake on gastric cancer cell lines, in which apoptosis was induced in a dose- and time-dependent manner. Further analyses using *in vitro* experimental models and prospective cohort studies are required to confirm these antitumor effects.

Acknowledgments

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