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Mycelia from *Antrodia camphorata* in Submerged Culture Induce Apoptosis of Human Hepatoma HepG2 Cells Possibly through Regulation of Fas Pathway

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The objective of this study was to investigate the antiproliferative effect and the mechanism of the methanol extracts of mycelia (MEM) form *Antrodia camphorata* in submerged culture toward HepG2 cells. The results showed that MEM-induced cell apoptosis involved up-regulation of Fas and down-regulation of Bcl-2, DR3, DR4, TNFRI, and TNFRII in HepG2 cells, while no changes on the levels of Bax, Bid, Bad, and Bak protein were observed. On the basis of these results, the involvement of the Fas/Fas ligand (FasL) death-receptor pathway, in MEM-induced apoptosis in HepG2 cells, was investigated. The apoptosis inducing activity was significantly enhanced by a Fas activator and inhibited by a Fas antagonist. To know about the effect of MEM on the activation of the apoptotic pathway, the adenovirus transfected with Bcl-2 was infected on HepG2 cells. The data showed that the percentage of apoptotic cells induced by MEM in Bcl-2-infected HepG2 (Bcl-2 overexpression) was not significantly different from that of uninfected HepG2. These results demonstrate that MEM induces HepG2 apoptosis through inhibition of cell growth and up-regulation of Fas/FasL to activate the pathway of caspase-3 and -8 cascades.

KEYWORDS: ***Antrodia camphorata*; apoptosis; Fas/FasL; Bcl-2**

INTRODUCTION

There has been an increasing interest in phytochemicals that may exhibit anticancer activity. *Antrodia camphorata* is known as niu-chang-chih or niu-chang-ku. Niu-chang is the Chinese common name for *Cinnamomum kanehirai*, which is one of the endangered species in Taiwan. Ku in Chinese means mushroom, and chih refers to a *Ganoderma*-like fungus. *A. camphorata* is a new species of the genus *Antrodia* (family Polyporaceae, Aphyllophorales) that is parasitic on the inner cavity of the endemic species Cinnamomum kanehirai Hay. (*1*). *A. camphorata* was early identified as a new *Ganoderma* species, *Ganoderma camphoratum* (*2*). Traditionally, it is used as a remedy for food, alcohol, and drug intoxication, diarrhea, abdominal pain, hypertension, skin itches, and liver cancer among Chinese (*3*).

Recent studies indicate that widely used chemotherapeutic agents induce apoptosis in susceptible cells. It was suggested that chemotherapeutic agents can regulate the Fas/Fas ligand (FasL) system to activate the dowmstream apoptotic pathways (*4*). Fas/FasL is one of the most relevant pathways in the liver (*5*). Fas belongs to the tumor necrosis factor receptor superfamily, and the Fas ligand induces apoptosis through Fas (CD95), a 45-kDa cell surface protein, inducing caspase cascade activation, first caspase 8 followed by caspase 3 (*6*).

The Bcl-2 family is composed of anti-apoptotic (Bcl-2 and Bcl-X_L) and pro-apoptotic (Bax, Bad, and Bak) factors (*7*). Bcl-2 and its homologous proteins, such as Bax and Bcl-X_L, modulate apoptotic process. These molecules interact with each other to form homo- and heterodimers (*8*). Once anti-apoptotic molecules, Bcl-2 or Bcl-X_L, interact with Bax (and other pro-apoptotic molecules such as Bad), Bcl-2/Bax, or Bcl-X_L/Bax, the heterodimer inhibits the apoptotic effect of Bax (*9*, *10*). Previous reports demonstrated that some Bcl-2 family members are located on mitochondrial membranes and can alter the permeability of the mitochondrial membrane, which triggers the realease of cytochrome *c* (*7*) or caspases (*11*) and activates the post-mitochondrial caspase cascade leading to apoptotic cell death.

Chen and Yang (*12*) revealed that Zhankuic acid (a type of steroid acid) in the fruiting bodies of *A. camphorata* is cytotoxic to P 388 murine leukemia. Recently, Hseu et al. (*13*) reported that *A. camphorata* exerts antiproliferative action and growth inhibition on HL-60 cells through apoptosis induction. In our previous studies, *A. camphorata* of a submerged culture (ACSC) has potent antioxidant activity in vitro and in vivo (*14*, *15*). The aqueous extract of ACSC significantly decreases the viability of HL-60 cells but not the human umbilical vein

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endothelial cells (*16*). In addition, the methanol extract of mycelia (MEM) from ACSC has no obvious cytotoxic effect on normal hepatocytes below the concentration of 100 μg/mL (*17*). The results also indicated that MEM induced HepG2 apoptosis through activation of caspase-3 and -8 cascades and regulation the cell cycle progression to inhibit hepatoma cell proliferation (*17*). We hypothesized that a death receptor (DR)-regulated pathway may be the possible mechanism for MEM-mediated apoptosis in HepG2 cells; however, the exact mechanism for MEM to induce apoptosis of hepatoma cell lines is not well-understood. Thus, the objective of this study was to elucidate the possible regulation pathway of apoptosis induced by MEM. The inhibitory effect of Fas expression and promotion of Bcl-2 expression on hepatoma cell lines was also examined.

MATERIALS AND METHODS

Materials and Chemicals. *A. camphorata* An5 in submerged culture (BCRC 930032) and human hepatoma cell line (HepG2, CCRC 60025) was purchased from Food Industry Research and Development Institute, Hsin Chu, Taiwan). Anti-Bid, Anti-Bad, Anti-Bcl-2, anti-Bcl-X_L, anti-Bax and anti-Bak, anti-Fas, anti-Fas ligand/CD95L, anti-DR3, anti-DR4, anti-TNFRI, anti-TNFRII, anti-β-actin antibodies, and secondary antibodies (horseradish peroxidase-conjugated anti-goat, anti-mouse, and anti-rabbit IgG) were purchased form BD Pharmingen (San Diego, CA). Antagonistic anti-Fas (clone ZB4) antibody and angonistic (activator) anti-Fas (clone CH 11) antibody were purchased from Upstate Biotechnology (Lake Placid, NY). Adenovirus/Bcl-2 was kindly provided by Dr. Song-Kun Hsu of the Institute of Biomedical Science of Academia Sinica (Taipei, Taiwan).

Culture Conditions of *A. camphorata*. *A. camphorata* hyphae was separated from the fruiting bodies and was inoculated into a culture medium that was composed of 2.5% corn starch, 2% sucrose, 0.5% yeast extract, 0.1% KH_2PO_4, 0.3% $MgSO_4$, 0.3% $(NH_4)_2SO_4$, and 0.05% citric acid in distilled water and adjusted to an initial pH range of 5.3−5.5. Each shaking flask culture was carried out in a 2 L Erlenmeyer flask containing 1 L of medium and incubated at 27−30 °C for 7 days. Thereafter, 3.5 L of the shaking flask cultures was inoculated into a 500 L fermented tank containing 350 L of culture medium and then cultured at 27−30 °C for 7 days with a 0.5 vvm aeration rate (aeration volume/medium volume (L)/min) by shaking at 50 rpm with a rotary shaker to obtain a mucilaginous medium containing mycelia. The residual sugar concentration detected by using the phenol−H_2SO_4 method was ~0.1 g/L after cultivation for 7 days. The mycelia were collected by centrifugation (4 °C, 8000 rpm for 15 min) and then washed with distilled water. Finally, the mycelia were freeze-dried to a powder form. The yield of mycelia in submerged culture was 1.1 g of dry weight of mycelia/100 g of ACSC.

Preparation of Mycelial Extracts from ACSC. The methanol extracts of mycelia (MEM) from ACSC were obtained by extracting freeze-dried mycelia (10 g) with methanol three times at 30 °C. The extracts were filtered through Whatman No. 2 filter paper and then concentrated to dryness. The yield of MEM was 3.0 g.

Cell Culture. Human hepatoma HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Flow Laboratories), supplemented with 10% fetal bovine serum, 2 mM glutamine, and antibiotics (100 U/mL pencillin and 100 μg/mL streptomycin) at 37 °C in a humidified atmosphere of 5% CO_2. The medium was changed every 2 days. Cells were seeded at a density of 1×10^5 cells/well onto a 12-well-plate (FALCON, Becton Dickinson, NJ) 24 h prior to MEM treatment. MEM was added to the medium at various times and concentrations. An agonist anti-Fas (clone CH 11) antibody, which induces apoptosis, and an antagonist anti-Fas (clone ZB4) antibody, which inhibits activation of Fas, were purchased from Upstate Biotechnology (Lake Placid, NY). The antagonist anti-Fas antibody was added to the culture medium for 1 h before the exposure of the cells to MEM or the agonist anti-Fas antibody. After incubation, cells were washed with phosphate buffered saline (PBS). Morphological changes were observed after the addition of MEM using a phase-contrast inverse microscope (IMT-2, Olympus Co., Ltd., Tokyo, Japan).

Flow Cytometric Analysis. Flow cytometric analysis of hepatoma cells (HepG2) was carried out using FACScan (Becton-Dickinson Immunocytometery Systems, San Jose, CA) with propidium iodide staining, as previously described by Chiu et al. (*18*). The sample was incubated with 0.5 mL (v/v) of staining solution A (50 μg/mL propidium iodide, 3% poly(ethylene glycol) 8000, 0.1% Triton X-100, 1 mg/mL RNase, 4 mM citrate buffer, pH 7.2) and incubated at 37 °C for 20 min, followed by the addition 0.5 mL (v/v) of solution B (50 μg/mL propidium iodide, 3% poly(ethylene glycol) 8000, 0.1% Triton X-100, 0.4 M NaCl, pH 7.2). The sample was then stored at 4 °C for at least 1 h and filtered before being analyzed using a flow cytometer. The percentage of sub-G1 cells was calculated by using FACScan software.

Protein Preparation and Western Blot Analysis. The protein preparation and Western blot analysis were modified from the method of Agarwal et al. (*19*) and Laemmli (*20*). To prepare proteins for immunoblotting, untreated or MEM-treated cells were lysed in protein lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM ethylenediamine-tetraacetic acid (EDTA), 1 mM ethylene glycol-bis-(aminoethyl ether)-tetraacetic acid (EGTA), 0.5 mM dithiothreitol, 1% NP-40, 0.3% deoxycholate, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL soybean trypsin inhinbitor, 0.5 mM phenylmethylsulfonylfluoride), and the protein concentration was determined using the Bradford method. Total protein (10 μg) was loaded onto an SDS polyacrylamide gel (7.5−12%), electrophoretically separated, and transferred onto a PVDF membrane (Millpore). The membrane was blocked with 5% nonfat dry milk in TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) and incubated overnight at 4 °C with specific primary antibodies, including anti-human Bcl-2, Bcl-X_L, Bax, Bak, Bid, Fas ligand/CD95L, DR3, DR4, TNFRI, and TNFRII antibodies. Subsequently, the membrane was washed with TBST buffer and incubated with appropriate secondary antibody (horseradish peroxidase-conjugated anti-goat, anti-mouse, or anti-rabbit IgG). Determinations were performed using enhanced chemiluminescence kits (Amersham; ECL kits).

Statistical Analysis. Values are expressed as means ± standard deviation and analyzed statistically using SAS. All data are obtained from three separate experiments. P values of <0.05 are considered statistically significant.

RESULTS

Effect of MEM on Fas/FasL Protein Expression. When HepG2 was treated with MEM for 24 h, the Fas protein expression increased with increasing MEM concentration (0−200 μg/mL) **(Figure 1A)**. The FasL protein expression was not significantly increased until the concentration of MEM was higher than 100 μg/mL. When HepG2 was treated with 50 μg/mL MEM for 24, 48, and 72 h, Fas protein expression was significantly increased with the incubation time **(Figure 1B)**. However, the FasL expression was significantly increased only when HepG2 was treated with MEM for 72 h.

Effect of MEM on Other Death Receptors (DRs) Protein Expression. Figure 2A shows that the expression of DRs (DR3, DR4, TNFRI, and TNFRII) was significantly decreased by MEM in a concentration-depentent manner (0−200 μg/mL). When HepG2 was treated with 50 μg/mL MEM for 24, 48, and 72 h, other DRs were decreased with incubation time (**Figure 2B)**. In contrast to Fas/FasL, the expression of DR3, DR4, TNFRI, and TNFRII was decreased in a dose- and time-dependent manner (**Figure 2**).

Effect of MEM on Bcl-2 Protein Expression. Figure 3A shows that when HepG2 was treated with MEM for 24 h, the protein expression of Bcl-2 was decreased with increasing MEM concentration (0−200 μg/mL). When HepG2 was treated with 50 μg/mL MEM for 24, 48, and 72 h, Bcl-2 protein expression was significantly decreased, as compared to that of untreated HepG2 cells, but the effect was not time-dependent (**Figure 3B)**.

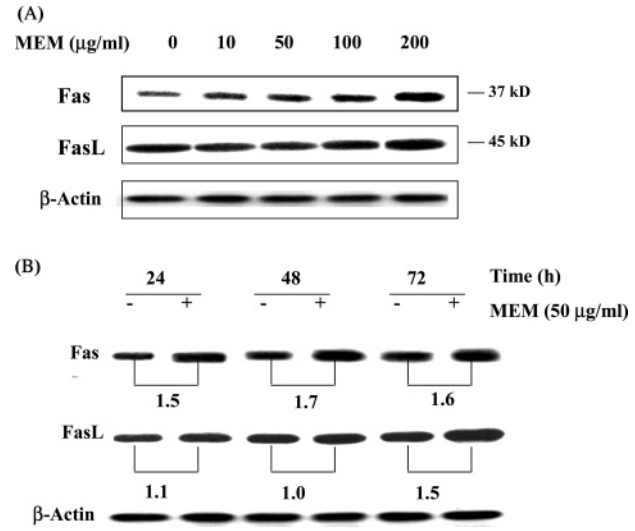

Figure 1. MEM induces an increased expression of Fas/FasL proteins in HepG2 cells. HepG2 cells were treated with different concentrations of MEM (0, 10, 50, 100, and 200 μg/mL) for 24 h (**A**) and 50 μg/mL MEM for 24, 48, and 72 h (**B**). β-Actin was used as an internal loading control to confirm equal loading. Similar results were obtained in three separate experiments.

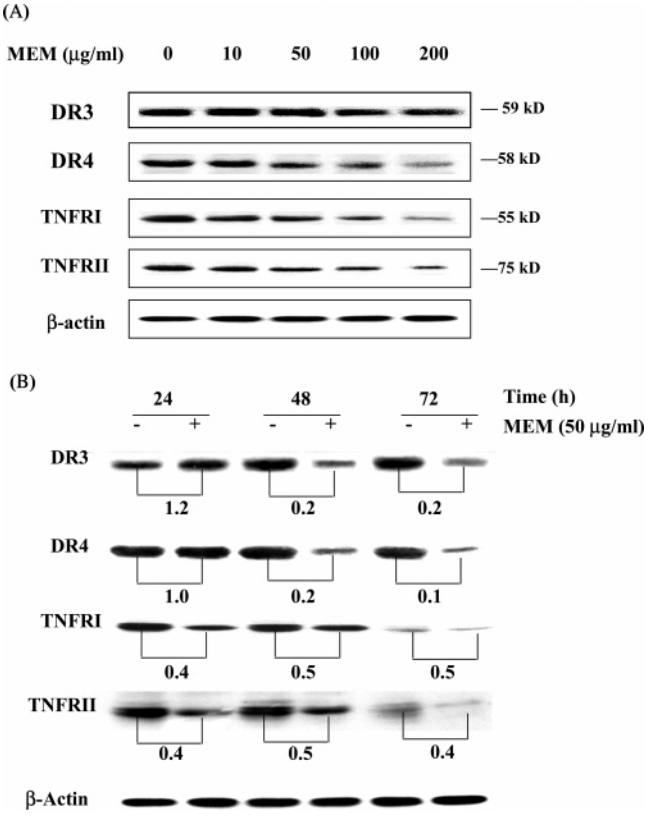

Figure 2. MEM induces a decreased expression of death receptor proteins (DR3, DR4, TNFRI, and TNFRII) in HepG2 cells. HepG2 cells were treated with different concentrations of MEM (0, 10, 50, 100, and 200 μg/mL) for 24 h (**A**) and 50 μg/mL MEM for 24, 48, and 72 h (**B**). Similar results were obtained in three separate experiments.

Effect of MEM on Antiapopotic Factors Protein Expression. When HepG2 was treated with different concentrations (0−200 μg/mL) of MEM for 24 h, the pro-apopotic factors protein expression, such as Bad, Bax, Bak, and Bid, was not significantly different (**Figure 4A**). However, Bcl-X_L was slightly increased when the MEM concentration was higher than

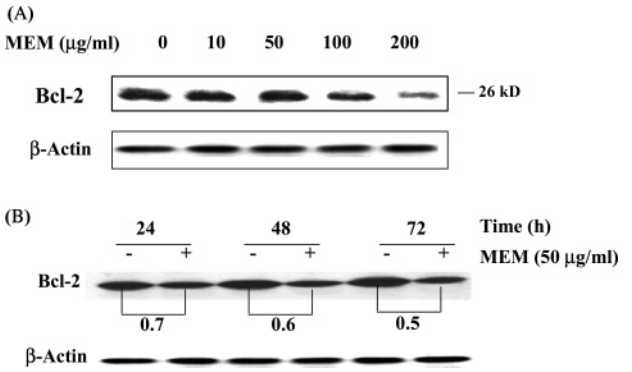

Figure 3. MEM induces a decreased expression of Bcl-2 proteins in HepG2 cells. HepG2 cells were treated with different concentrations of MEM (0, 10, 50, 100, and 200 μg/mL) for 24 h (**A**) and 50 μg/mL MEM for 24, 48, and 72 h (**B**). β-Actin was used as an internal loading control to confirm equal loading. Similar results were obtained in three separate experiments.

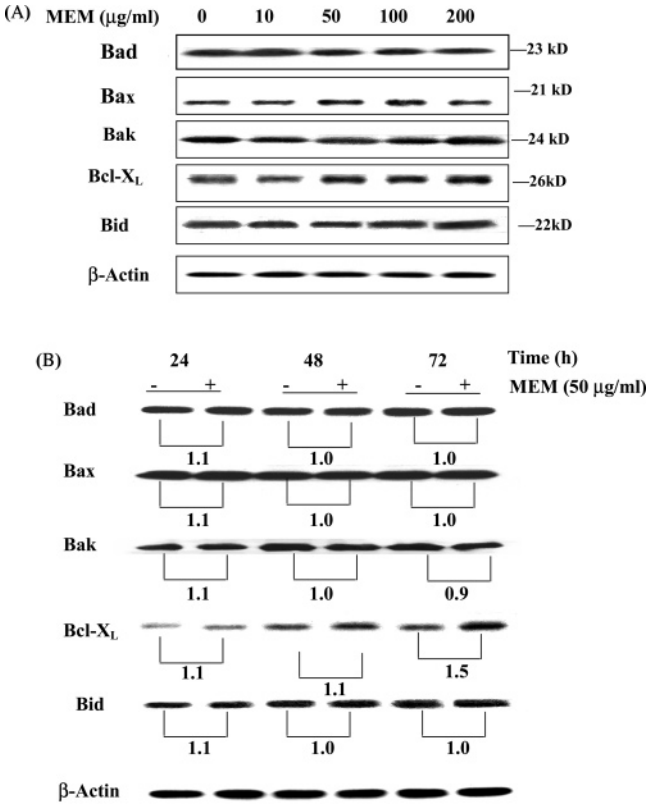

Figure 4. Expression of Bad, Bax, Bak, Bcl-X_L, and Bid in $HepG_2$ cells treated with different concentrations of MEM (0, 10, 50, 100, and 200 μg/mL) for 24 h (**A**) and with 50 μg/mL MEM for 24, 48, and 72 h (**B**). β-Actin was used as an internal loading control to confirm equal loading. Similar results were obtained in three separate experiments.

50 μg/mL. As shown in **Figure 4B**, except Bcl-X_L, there were no observable differences in Bad, Bax, Bak, and Bid expression when HepG2 cells were treated with MEM for 24, 48, and 72 h. However, Bcl-X_L was markedly increased by MEM, and the effect was time-dependent.

Effect of MEM on Regulation of Fas/FasL and Bcl-2 Protein Expresion. Flow cytometry was used to quantitate the induction of apoptosis at different concentrations of MEM (0−200 μg/mL) and to evaluate the relationship of apoptosis with Fas/FasL or with Bcl-2 protein expression in HepG2 cells. Cells treated with different concentrations of MEM for 24 h were labeled with propidium iodide and analyzed by flow cytometry.

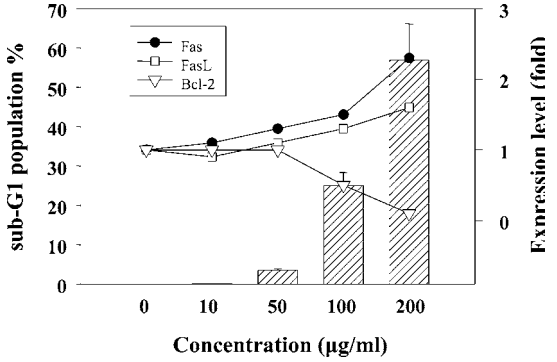

Figure 5. MEM-mediated sub-G1 population % (Bar plots) and Fas, FasL, and Bcl-2 (straight lines) protein expression levels of HepG2 cells. HepG2 cells were incubated with different concentrations of MEM (0, 10, 50, 100, and 200 μg/mL) for 24 h. After incubation, the cell sub-G1 population % was calculated by using FACScan software. Similar results were obtained in three separate experiments.

MEM treatment increased the number of sub-G1 hypodiploid cell populations in a dose-dependent manner (**Figure 5**). When HepG2 cells were treated with 10, 50, 100, and 200 μg/mL MEM for 24 h, the sub-G1 hypodiploid cell populations were 0.1, 0.2, 3.6, 25.1, and 56.9%, respectively. **Figure 5** also shows that at concentrations of 50−200 μg/mL, MEM exhibited a dose-dependent increase in Fas/FasL expression and a decrease in Bcl-2 expression. These results suggest that either an increase in Fas/FasL or a decrease in Bcl-2 expression can induce HepG2 cells apoptosis.

Effect of MEM on Fas Activator and Antagonist. As shown in **Figure 6A**, when the Fas activator (10 ng/mL) and MEM (50 μg/mL) were incubated alone with HepG2 cells for 48 h, the sub-G1 hypodiploid cell populations were 5.1 and 7.4%, respectively. However, the sub-G1 hypodiploid cell populations increased to 18.8% when the Fas activator and MEM were co-incubated with HepG2 cells for 48 h. As stated previously, it was revealed that the Fas activator might enhance the cell cytotoxic effect of MEM-mediated apoptosis. Furthermore, when the Fas antagonist antibody (2 ng/mL) and MEM were co-incubated with HepG2 cells, the sub-G1 hypodiploid cell populations induced by MEM were significantly decreased to 3.5%. Apoptotic cells were reduced by half as compared to those of MEM-treated cells (**Figure 6B**), indicating that the Fas antagonist antibody can diminish the cytotoxic effect mediated by MEM.

Effect of MEM on Bcl-2 Overexpression. When the adenovirus transfected with Bcl-2 was infected on HepG2 cells for 2 h followed by incubation with MEM (50 μg/mL), the percentage of sub-G1 hypodiploid cell populations was not significantly different between adeno-Bcl-2 vector and adenoviral vector ($p > 0.05$). The sub-G1 hypodiploid cell populations were all around 33−37% when cells were incubated with MEM for 72 h (**Table 1**).

DISCUSSION

Many mechanisms for developing resistance to apoptosis have been suggested in malignant cells, including the following: lack of cell-surface Fas protein expression, synthesis of anti-apoptotic proteins such as the Bcl-family of proteins, alterations in intracellular Fas-signaling pathways, and production of a soluble form of Fas (*21*). In the present study, we examined the regulation pathway of apoptosis induced by MEM and the effect of inhibition of Fas expression and promotion of Bcl-2 expression on hepatoma cell lines. Our preliminary study demonstrated that MEM induced HepG2 by inducing apoptosis through activation of caspase-3 and -8 cascades (*17*), and we hypothesized that the DR-regulated pathway may be the major mechanism of MEM-mediated apoptosis in HepG2 cells. One study indicated that apoptosis induced by anticancer agents through caspase-8 activation is related the Fas/FasL stimulation (*22*). Here, we showed that the expression of Fas in HepG2 cells treated by MEM was significantly increased in a dose-dependent manner, suggesting that Fas stimulation is the DR-regulated pathway of apoptosis induced by MEM. By contrast, other DRs (DR3, DR4, TNFRI, and TNFRII) might not be the major regulating effectors in MEM-induced cell apoptosis because they were decreased rather than increased by MEM treatment. However, it is unclear why they are decreased by MEM treatment.

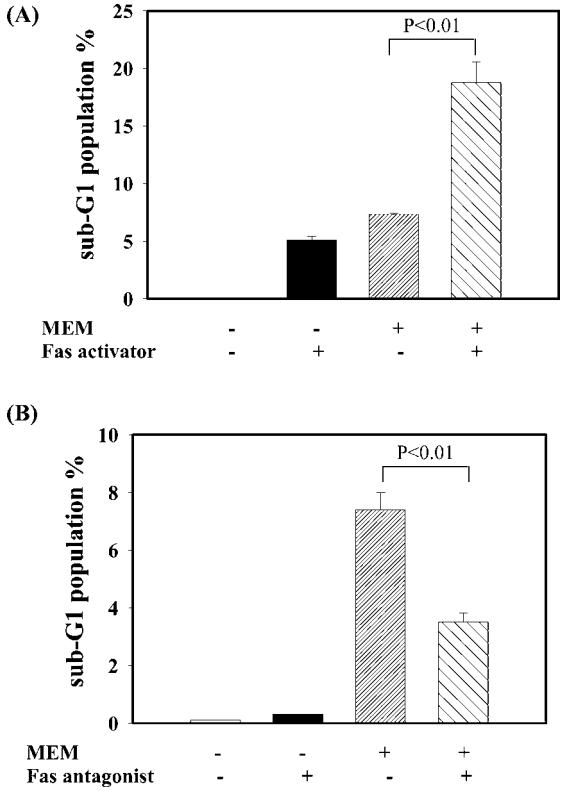

Figure 6. MEM-mediated sub-G1 population % of anti-Fas activator (**A**) and antagonist (**B**) on HepG2 cells. Cells were coincubated with MEM (50 μg/mL) and anti-Fas activating (10 ng/mL) or antagonist antibody (2 ng/mL) for 48 h. After incubation, the cell sub-G1 population % was calculated by using FACScan software. All values represent the mean ± SD from triplicate experiments and analyzed using one-way ANOVA. $P <$ 0.01 as compared to the MEM-treated group.

Table 1. MEM-Mediated Apoptosis in HepG2 Cells with Bcl-2 Overexpression (HepG2/Bcl-2 Cells)

treatment[a]	sub-G1 population %[b]
MEM	30.2 ± 3.0[c]
adenoviral vector (10 MOI)[d]	2.1 ± 0.9
MEM + adeno-Bcl-2 vector (10 MOI)	33.4 ± 2.8
adenoviral vector (25 MOI)	3.4 ± 0.7
MEM + adeno-Bcl-2 vector (25 MOI)	36.8 ± 3.9

[a] HepG2 cells were infected with adenovirus/Bcl-2 for 2 h prior to MEM treatment (50 μg/mL for 72 h). [b] Sub-G1 population % was calculated by using FACScan software. [c] Each value represents the mean ± SE of three independent experiments. [d] MOI, multiplicity of infection.

It has been suggested that hepatocarcinoma cells may escape from apoptosis due to decreased Fas gene expression (*23*, *24*). However, Lamboley et al. (*25*) have reported that overexpression of Fas gene in human hepatoma cells overcomes their resistance to Fas-mediated apoptosis. To determine whether MEM induces apoptosis through the activation of Fas receptor signaling, both a Fas-activation (agonist) antibody (clone CH11) and an antagonist antibody (clone ZB4) were used. We found that the percentage of apoptosis cells in HepG2 cells induced by MEM was significantly correlated with Fas expression. Furthermore, co-incubation of HepG2 cells with MEM and the Fas activation antibody increased apoptotic cells more than MEM treatment alone. By contrast, co-incubation of HepG2 cells with MEM and the Fas antagonist antibody decreased apoptotic cells more than MEM treatment alone. Thus, our results demonstrate that the expression of Fas is an important pathway of MEM-mediated apoptosis in HepG2 cells.

Many reports have pointed out that the ability of inducing apoptosis on tumor cells by anticancer agents (such as taxol, curcumin, and retinoid *N*-(4-hydroxyphenyl) retinamide) correlates well with the ability of decreasing expression of Bcl-2 (*26*, *27*). In the present study, we showed that the expression of Bcl-2 was decreased in a concentration-dependent manner correlated with the percentage of apoptotic cells treated by MEM in HepG2 cells, suggesting that Bcl-2 may play a role in MEM-mediated apoptosis of HepG2 cells. Overexpression or stable tranfection of Bcl-2 suppresses drug-induced apoptosis in various cells and prevents activation of the caspase cascade (*28*, *29*). Overexpression of Bcl-2 would cause mitochondrial damage, followed by the activation of caspases. The activated caspases cleave Bcl-2 into a Bax-like cytotoxic effector, which in turn accelerates cytotoxicity, possibly by further damaging the mitochondrial membrane (*30*). To examine whether the decrease of Bcl-2 expression regulates the apoptosis pathway of MEM-mediated apoptosis in HepG2 cells, Bcl-2 protein expression by infection to HepG2 cells was determined. Our results demonstrated that the percentage apoptotic cells induced by MEM in Bcl-2-infected HepG2 (Bcl-2 overexpression) was not significantly different from that in uninfected HepG2. The result indicates that the decrease in Bcl-2 expression may not play an important role in the apoptotic pathway of HeG2 cells induced by MEM.

Mitochondrial-dependent apoptosis is often through the activation of a pro-apoptotic factor of the Bcl-2 family. Bax or Bad (pro-apoptotic factors) have been shown to homodimerize as well as heterodimerize with Bcl-2 and Bcl-X_L (anti-apoptotic factors). When in excess, Bax (or Bad) counters the ability of Bcl-2 (or Bcl-X_L) to repress cell death (*10*). Bid is activated from truncated-Bid (t-Bid) by caspase-8, and the mitochondrial-dependent pathway is activated to proceed with cell apoptosis (*31*). Previous reports have documented that the ratio of pro- and anti-apoptotic proteins determines in part the susceptibility of cells to a death signal (*32*). However, we found no dose- and time-response in the expression of the other pro-apoptotic factors (such as Bad, Bax, Bak, and Bid). Furthermore, the expression of Bcl-X_L was slightly increased. Although the release of cytochrome *c* was not measured in the present study, these results suggest that the mitochondria-dependent pathway may not be a major pathway of MEM-induced apoptosis.

In conclusion, our study in HepG2 cells demonstrates that MEM induces cell apoptosis possibly by involving up-regulating Fas expression, which promotes the ligation of Fas and FasL and then passes the death message to cytosolic messengers. As a result, procaspase-8 is activated to caspase-8, which triggers the caspase activation cascade. In addition, our results demonstrate that the MEM-induced apoptotic pathway may not be related to the mitochondria pathway.

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