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Induction of apoptosis and cell-cycle arrest in human colon cancer cells by meclizine

Jiunn-Chang Lin [a,c], Yuan-Soon Ho [b], Jie-Jen Lee [a], Chien-Liang Liu [a,d], Tsen-Long Yang [a], Chih-Hsiung Wu [c,e,*]

[a] Department of Surgery, Mackay Memorial Hospital, Taipei, Taiwan
[b] Institute of Biomedical Technology, Taipei Medical University, Taipei, Taiwan
[c] Graduate Institute of Medical Sciences, Taipei Medical University, Taipei, Taiwan
[d] Mackay Medicine, Nursing and Management College, Taipei, Taiwan
[e] Department of Surgery, Taipei Medical University Hospital, Taipei, Taiwan

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Abstract

Meclizine (MEC), a histamine H1 antagonist, is used for the treatment of motion sickness and vertigo. In this study, we demonstrate that MEC dose-dependently induced apoptosis in human colon cancer cell lines (COLO 205 and HT 29 cells). Results of a DNA ladder assay revealed that DNA ladders appeared with MEC treatment in COLO 205 cells at dosage of >50 μM. In addition, the total cell number decreased dose-dependently after treatment with MEC in COLO 205 and HT 29 cells. Using flow cytometry, the percentage of COLO 205 cells arrested at G0/G1 phase increased dose-dependently. Analysis of changes in cell-cycle arrest-associated proteins with Western blotting showed that p53 and p21 were upregulated after treatment with MEC. The kinase activities of cyclin-dependent kinase 2 (CDK2) and CDK4 were suppressed in MEC-treated cells. As for apoptosis, MEC may induce upregulation of p53 and downregulation of Bcl-2, thus causing the release of cytochrome C from mitochondria and the translocation of apoptosis-inducing factor (AIF) to the nucleus. This resulted in the activation of caspase 3, 8, and 9. Our results provide the molecular basis of MEC-induced apoptosis and cell-cycle arrest in human colon cancer cells.

Keywords: Meclizine; Apoptosis; Cell-cycle arrest

1. Introduction

Meclizine (MEC) is an H-1 piperazine antihistamine derivative with anticholinergic, antiemetic, antispasmodic, central nervous system depressant, and local anesthetic effects. The chemical name of MEC is 1-(4-chlorobenzhydryl)-4-(3-methylbenzyl) piperazine dihydrochloride, and its structure is shown in Fig. 1. Its antiemetic effects come from suppression of the emetic center in the brain stem, with little or no effect on the chemoreceptor trigger zone (Wood et al., 1981). Because MEC also reduces the excitability of neurons in the vestibular nucleus, it is often prescribed for motion sickness, vertigo, radiation dizziness, Meniere's disease, and nausea and vomiting during pregnancy; however, it is ineffective at preventing chemotherapy-induced nausea and vomiting.

Malignant neoplasms still remain the leading cause of death in many countries. Because carcinogenesis is a complex process, finding effective therapies often relies on new discoveries about the underlying cellular mechanisms. Other studies are examining pharmaceutical agents that might interfere with the cellular mechanisms in cancerous cells so as to block the growth of tumors and which can

* Corresponding author. Address: Department of Surgery, Taipei Medical University Hospital, Taipei, Taiwan. Tel.: +886 2 27372181; fax: +886 2 27389524.
E-mail address: chwu@tmu.edu.tw (C.-H. Wu).

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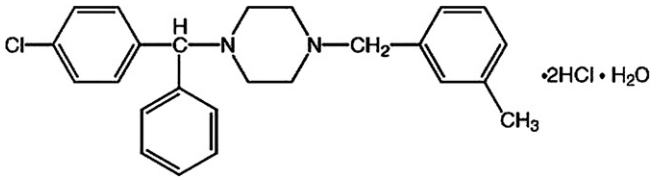

Fig. 1. Structure of meclizine (MEC).

thereby be used as therapeutic agents. One such pharmaceutical agent is MEC. In the present study, we found that when MEC is added to cultures of a human colon cancer cell line (COLO 205 and HT 29 cells), cell proliferation was inhibited. It causes cell-cycle arrest at the G0/G1 phase. MEC further induced elevations of cell cycle-related protein levels of p53 and p21, and inhibited the activities of cyclin-dependent kinase 2 (CDK2) and CDK4. In addition, MEC treatment also downregulated the Bcl-2 antiapoptotic gene and caused the translocation of cytochrome C from mitochondria to the cytoplasm and apoptosis inducing factor (AIF) from mitochondria to the nucleus. Subsequently, apoptosis occurred due to the increased caspase activity. For comparison, untransformed human cells (FCH; a normal human colon epithelial cell line) were cultured and treated with MEC; in these noncancerous cells, proliferation was not suppressed. These experiments shed new light on the molecular mechanisms involved in MEC-induced apoptosis.

2. Materials and methods

2.1. Cell culture

The HT 29 and COLO 205 cell lines were isolated from human colon adenocarcinoma (Semple et al., 1978) (HTB-38 and CCL-222; American Type Culture Collection (ATCC)). The cell line FCH, a homozygous familial hypercholesterolemia cell (CRL-1831; ATCC), was derived from primary cultures of normal human colonic mucosa (Siddiqui and Chopra, 1984). The p53 gene in both COLO 205 and FCH cells was the wild type. Cell lines were grown at 37 °C in a 5% carbon dioxide atmosphere in RPMI 1640 medium for COLO 205 and HT 29 cells, and in DMEM/Ham's Nutrient Mixture F-12, 1:1 with 2.5 mM L-glutamine, 1.2 g/L sodium bicarbonate, 15 mM HEPES and 0.5 mM sodium pyruvate for FCH cells, and both cultures were supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 25 μg/ml amphotericin B. The p53 gene in COLO 205 and FCH cells was of the wild-type (Ho et al., 1996). In HT-29 cells, p53 is mutated in codon 273 (Niewolik et al., 1995).

2.2. Determination of cell growth curve

COLO 205 and HT 29 cells (1×10^5 cells/well) and FCH cells (CRL-1831) (1×10^5 cells/well) were plated in 35-mm Petri dishes. The next day, the medium was changed and MEC (10–100 μM) was added. Control cells were treated with the vehicle, dimethyl sulfoxide (DMSO), in a final concentration of 0.05% (v/v). The incubation medium was renewed every day during the experiment. At the end of incubation, cells were harvested for cell counting with a hemocytometer.

2.3. Formazan-based viable cell mass assay (MTT Assay)

COLO 205, HT 29 cells, and FCH cells were treated with MEC (20–75 μM) alone or combination of MEC and 100 ng/ml 12-O-tetradecanoylphorbol-13-acetate (TPA). Cell viability was determined at 24 h based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-^{2}H-tetrazollium bromide (MTT) assay. Briefly, cells were seeded in a 96-well plate at a density of 1×10^4 cells/well and allowed to adhere overnight. After removing the medium, 200 μl of fresh medium per well, containing 10 mmol/L HEPES (pH 7.4) was then added. Then 50 μl of MTT was added to the wells and the plate was incubated for 2–4 h at 37 °C in the dark. The medium was removed and 200 μl DMSO and 25 μl Sorensens' glycine buffer were added to the wells. Absorbance was measured using an ELISA plate reader at 570 nm.

2.4. Cell synchronization, drug treatment, and flow cytometry analysis

At 24 h after plating of cells, the culture medium was removed. Cells were washed three times with phosphate-buffered saline (PBS) and then incubated with medium containing 0.04% FCS for 24 h. Under these conditions, cells were arrested in the G0/G1 phase as determined by flow cytometry. After serum starvation, the low-serum (0.04% FCS) medium was removed, and cells were then stimulated by adding medium which contained 10% FCS. MEC solutions were prepared by dissolving this compound in a final concentration of 0.05% DMSO. Treated cells were harvested and fixed with 1 ml ice-cold 70% ethanol at 4 °C for 1 h. The cell-cycle stages in MEC- and mock-treated groups were measured by flow cytometry. Cells were incubated with 20 μg/ml propidium iodide (Sigma, St. Louis, MO) for 30 min, and DNA content was measured using a FACScan laser flow cytometer analysis system (Becton-Dickinson, San Jose, CA); 15,000 events were analyzed for each sample.

2.5. Preparation of whole cell protein lysate

COLO 205 and HT 29 cells were plated on 10-cm Petri dishes for 24 h and then were treated with MEC and 0.05% DMSO for 24 h. Cells were rinsed three times with ice-cold PBS, pelleted at 800 g for 5 min and lysed in 500 μl of freshly prepared extraction buffer (20 mM Tris–HCl (pH 7.9), 137 mM NaCl, 10 mM NaF, 5 mM EDTA, 5 mM DTT, 2 mM PMSF, 1 mM EGTA, 1 mM Na_3VO_4, 10% Glycerol, 1 mM sodium pyrophosphate, 0.1 mM β-glycerophosphate, 10 μg/ml aprotinine, 10 μg/ml leupeptin, and 1% Triton X-100) for 30 min on ice. The extracts were cleared by centrifugation for 30 min at 12,000*g*.

2.6. Extraction of cytosol and mitochondria lysate

To determine cytochrome C translocation, cytosolic and mitochondrial extracts were prepared from cells that had undergone drug treatment. Cells were treated and harvested as described above. Then cells were suspended in buffer A (20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose) for 30 min on ice. Cells were sonicated five times during this period. After centrifugation 10 min at 3500*g*, the supernatants were collected and were further centrifugated for 15 min at 1400*g*. The supernatant was the cytosol lysate, and the pellet was suspended in protein extraction buffer and centrifugated to obtain the mitochondrial lysate.

2.7. Extraction of cytosol and nucleus lysate

To determine AIF translocation, cytosolic and nuclear extracts were prepared from cells that had undergone drug treatment. Cells were treated and harvested as described for the previous method. Cells were suspended with buffer A for 30 min on ice, homogenized with a glass Dounce homogenizer (40 strokes), and centrifugated for 10 min at 12,000*g*. The supernatant was the cytosol lysate. The nuclei were pelleted, and the pellet was washed with hypotonic buffer (10 mM HEPES (pH 7.2), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 200 μM Na_3VO_4, 1 mM PMSF, and 10 mM NaF) one time and centrifuged for 10 min at 12,000*g*. The pellet was vortex-mixed with Triton X lysis buffer (10 mM Tris–HCl, 150 mM NaCl, 10 mM NaF, 100 mM Na_3VO_4, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 5 mM EDTA, 1 mM PMSF, and 1% Triton X-100) and was placed in a water bath sonicator for

1 min and in an ice bath for 30 min, then centrifuged for 10 min at 12,000*g* to obtain the nuclear lysate.

2.8. Western blot analysis

Proteins were normalized to 50 μg/lane, resolved on 12.5% (w/v) SDS-PAGE, and blotted onto Immobilon P membranes with a semidry electroblotting apparatus (TE70; Hoefer Scientific Instruments, San Francisco, CA). The membrane was blocked overnight at room temperature with blocking reagent (20 mM Tris (pH 7.4), 125 mM NaCl, 0.2% (v/v) Tween 20, 4% (w/v) nonfat dry milk, and 0.1% (w/v) sodium azide). Antibodies were used for immunoblot assays, including polyclonal rabbit antisera specific for the human bad, poly (ADP-ribose) polymerase (PARP), Bcl-2, cyclin-A, and cyclin E proteins. Mouse monoclonal antibodies included antisera for the other human proteins.

Filters were incubated for 1 h with the primary antibody, washed three times, and then incubated with an alkaline phosphatase-conjugated secondary antibody (immunoglobulin G) in PBS and 0.5% (v/v) Tween 20 for another 45 min with gentle shaking. After three final washes, the proteins were visualized by incubating with the colorigenic substrates nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate (Sigma). Video densitometry quantification was performed with an OPTIMAS-6 image analysis system.

2.9. Analysis of DNA fragmentation

Treated cells and cells receiving mock treatment were grown in 10-cm Petri dishes. Both attached and unattached cells were harvested and washed three times with ice-cold PBS. Cells were dissolved in 100 μl of DNA lysis buffer (50 mM Tris–HCl (pH 8.0), 10 mM EDTA, 0.5% N-Lauroylsarcosine, and 2 mg/ml proteinase K) and were incubated for 3 h at 55 °C, and RNAse A (Amresco, Solon, Ohio) was added for another 3 h. The DNA was extracted twice with equal volumes of phenol and once with chloroform-isoamylalcohol (24:1, v/v). The DNA was then precipitated with 0.1 volume of sodium acetate (pH 4.8) and 2.5 volumes of ethanol at −20 °C overnight and pelleted at 12,000*g* for 1 h. Samples were electrophoresed in a 1.5% (w/v) agarose gel, and DNA was visualized by ethidium bromide staining.

2.10. Kinase activity assay

As described previously (Wu et al., 1996), MEC-treated cells were lysed in protein extraction buffer, and immunoprecipitated with an anti-CDK2 or anti-CDK4 antibody (2 μg/ml). The protein complexes in the beads were washed twice with buffer and then once with Rb kinase assay buffer (50 mM HEPES-KOH (pH 7.5), containing 2.5 mM EGTA, 10 mM β-glycerophosphate, 1 mM sodium fluoride, 0.1 mM sodium

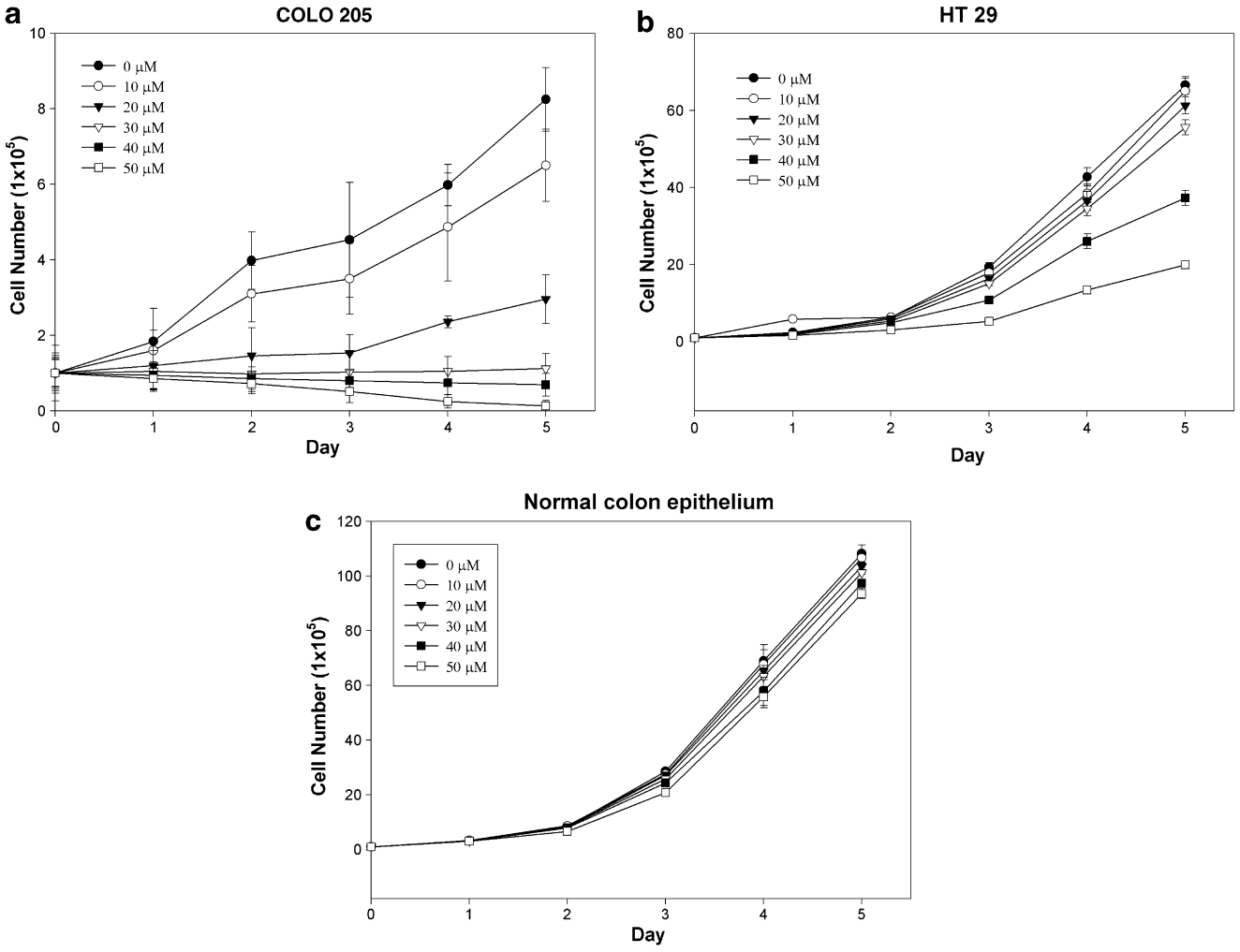

Fig. 2. Effects of MEC on the cell number in human colon cancer cells and normal human colon epithelial cells. Cell proliferation was dose-dependently inhibited in COLO 205 cells (a) and HT 29 cells (b) after treatment with MEC (10–50 μM). However, MEC had a minor influence on cell proliferation in normal colon epithelial cells FCH (CRL-1831). Media with and without MEC were changed daily until cells were counted. Each data point represents the mean ± SE of three independent experiments.

orthovanadate, 10 mM $MgCl_2$, and 1 mM DTT). The levels of phosphorylated of Rb (for pRb), histone H1 (for CDK2) and glutathione S-transferase-Rb fusion protein (for CDK4) were measured by incubating the beads with 40 μl of hot Rb kinase solution (0.25 μl (2 μg) of Rb-GST fusion protein (Santa Cruz Biotechnology, Santa Cruz, CA), 0.5 μl of (γ-32 P) ATP (Amersham), 0.5 μl of 0.1 mM ATP, and 38.75 μl of Rb kinase buffer) at 37 °C for 30 min, and then reaction was stopped by boiling the samples in SDS sample buffer for 5 min. Samples were analyzed by 12% SDS-PAGE, and the gels were then dried and subjected to autoradiography.

3. Results

3.1. MEC inhibited cell proliferation in COLO 205 and HT 29 cells, but not FCH cells

In this study, we examined the antiproliferative effect of MEC on colon cancer cells. Treatment of COLO-205 and HT 29 cells with MEC (0–50 μM) for 24 h induced a decrease in cell number in a dose-dependent manner (Fig. 2a and b). In contrast, proliferation of FCH cells (CRL-1831) was minimally affected by MEC treatment (Fig. 2c). Such results suggest that MEC has a growth inhibitory effect on human colon cancer cells, but normal human colon epithelial cells are more resistant to MEC treatment.

3.2. MEC had no synergistic effect with TPA

TPA has been studied widely as a tumor promoter (Armuth and Berenblum, 1972). For investigating the effect of MEC on the level of TPA toxicity (in terms of cancer promotion), MTT assay was done to compare cell viability after treatment with MEC alone or combination of MEC and TPA. Fig. 3a–c shows that there is no synergistic effect between MEC and TPA on FCH, COLO 205, and HT 29 cells.

3.3. MEC induced cell-cycle arrest in the G0/G1 phase

To further examine the actions of MEC on the cell mitotic cycle, COLO 205 cells were incubated in culture media with 0.04% FCS for 24 h so as to synchronize their mitotic

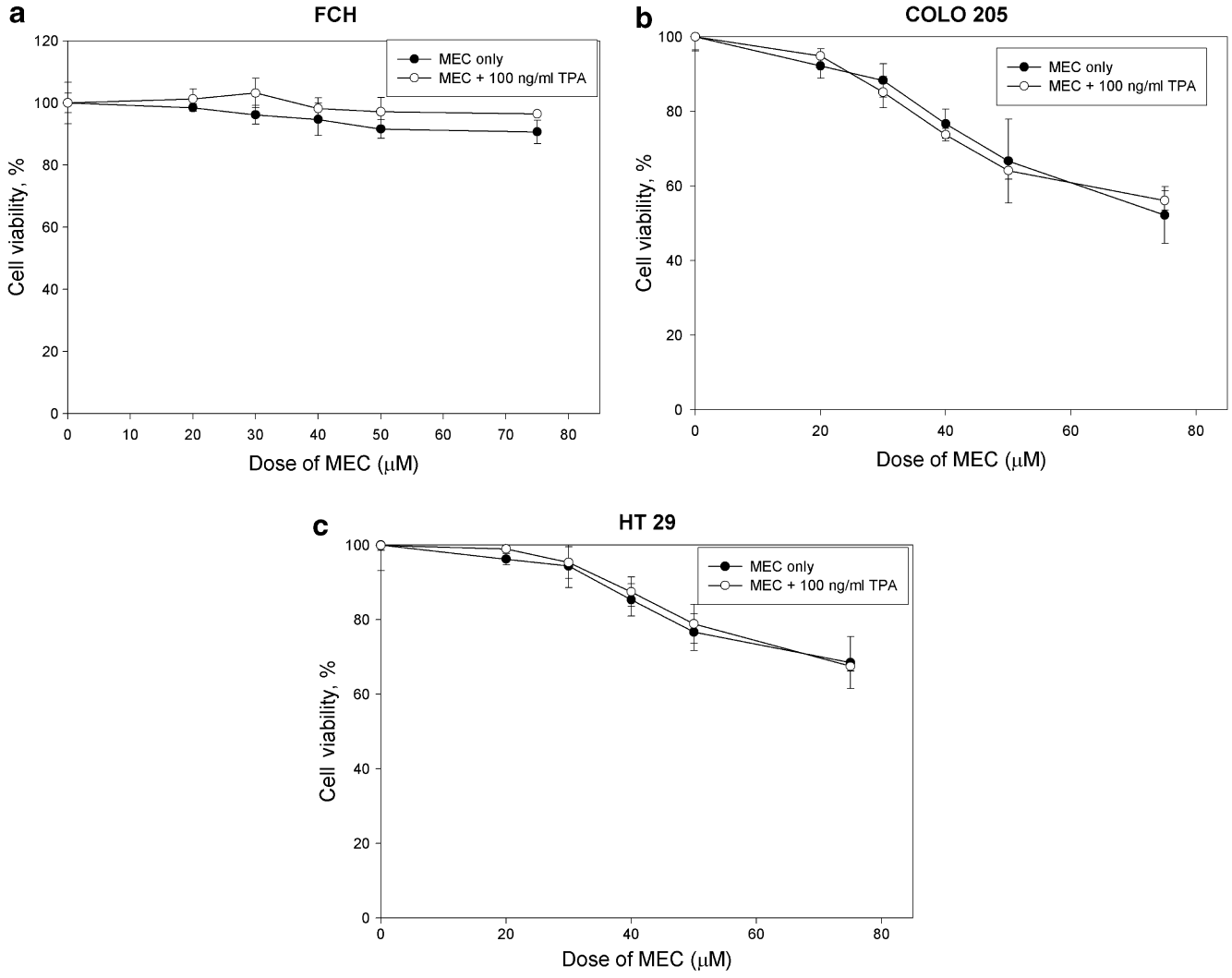

Fig. 3. Effects of MEC alone or combination of MEC and TPA on the cell viability in human colon cancer cells and normal human colon epithelial cells. No synergistic effects were found between MEC and TPA. Each data point represents the mean ± SE of three independent experiments.

activities at the G0/G1 phase. They were returned to media supplemented with 10% FCS and were treated with 0.1% DMSO and 50 μM MEC for various time periods. Fig. 4a shows a representative cell-cycle change in COLO 205 cells after treatment with 0.1% DMSO for various time periods. At 15 and 18 h, the percentage of cells in the G0/G1 phase was lowered and percentage of cells in the S phase was increased. After MEC treatment for various time periods, the percentage of cells at the G0/G1 phase remained constant, indicating that the cell cycle was arrested at the G0/G1 phase (Fig. 4b). Then COLO 205 cells were treated with various concentrations of MEC (10–100 μM). Fig. 5 shows that the G0/G1 arrest effect increased dose-dependently.

3.4. MEC upregulated p53 and p21/CIP1 but not p27/KIP1 proteins in COLO 205 and HT 29 cells

To clarify the molecular mechanism of the MEC-induced inhibition of COLO-205 cell proliferation, we further examined the levels of cell-cycle regulatory proteins in MEC-treated COLO-205 and HT 29 cells. Progression of cell-cycle activity is regulated by coordinated successive activation of certain CDKs, which is in turn modulated by association with a number of regulatory subunits called cyclins and with a group of CDK-inhibitory proteins. Accordingly, we examined changes in cyclin, CDK, and CDK-inhibitory protein levels in MEC-treated COLO-205 cells. Cells were synchronized with culture medium containing 0.04% FCS for 24 h and then were treated with various concentrations of MEC (10–100 μM) for 24 h. Fig. 6a shows the dose-dependent upregulation of p53 and p21/CIP1, but no changes in the levels of p27, CDK1, CDK2, CDK4, Wee1, 14-3-3σ, PCNA, or cyclins A, B, D1, D3, and E were seen. In MEC-treated HT 29 cells, upregulation of p53 and p21/CIP1 were also found to be dose-dependent, but protein level of p27 was unchanged (Fig. 6b). These findings suggest that upregulation of p53 and p21/CIP1 is involved in MEC-induced G0/G1 cell-cycle arrest.

3.5. MEC inhibits CDK2 and CDK4 kinase activities in COLO 205 cells

Although protein levels of CDK2 and CDK4 were not significantly influenced by MEC treatment, we further

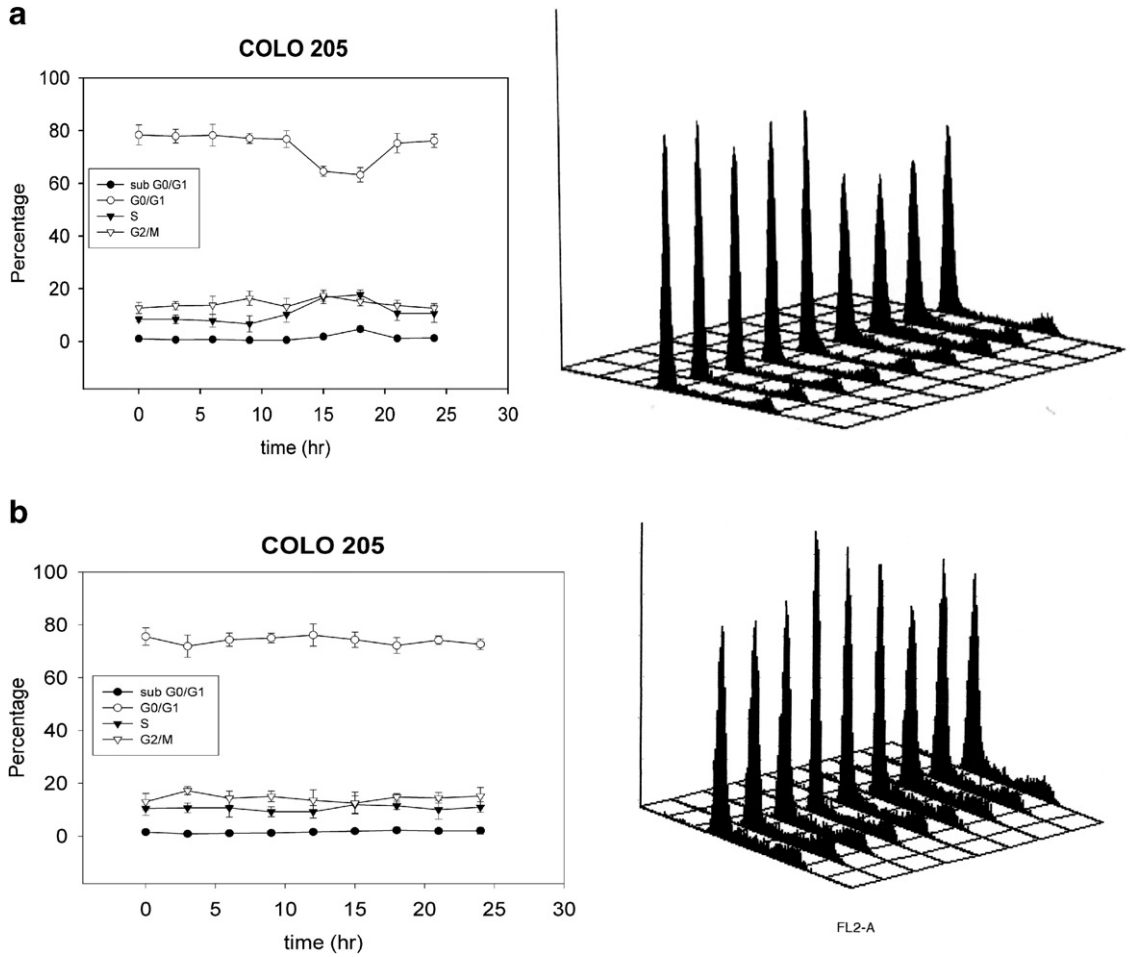

Fig. 4. Time-dependent response of MEC-induced arrest of COLO 205 cells at the G0/G1 phase. FACS analysis of DNA content was performed after COLO 205 cells were release from quiescence by incubation in culture media supplemented with 10% FCS and (a) 0.1% DMSO or (b) 50 μM MEC in 0.1% DMSO for various durations. Percentage of cells in the G0/G1, S, and G2/M phase were determined using established CellFIT DNA analysis software.

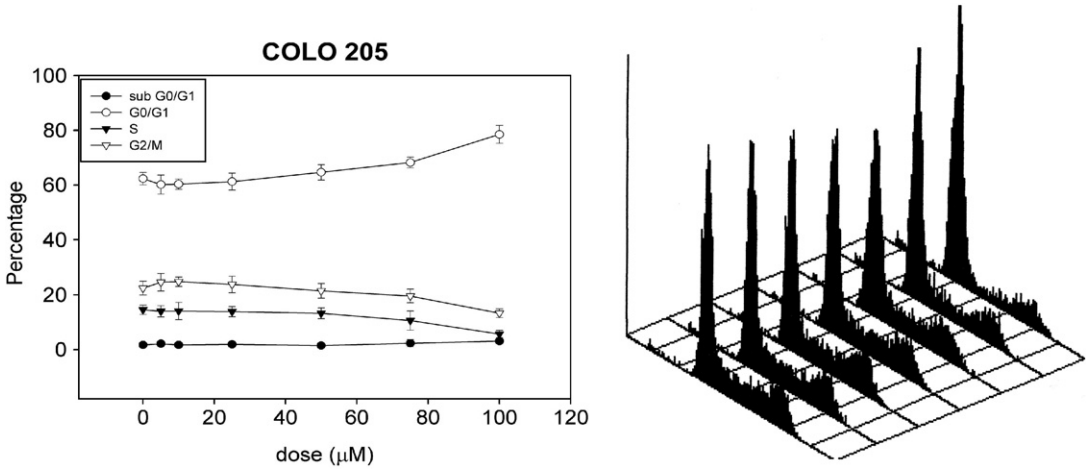

Fig. 5. Dose-dependent response of MEC-induced G0/G1 phase arrest in COLO 205 cells. FACS analysis of DNA content was performed after COLO 205 cells were incubated with various concentrations of MEC (10–100 μM). Percentages of cells in the G0/G1, S, and G2/M phases were determined using established CellFIT DNA analysis software.

explored whether CDK kinase activity was changed by MEC. CDK2 kinase activity was examined using histone H1 and CDK4 activity using the glutathione S-transferase-Rb fusion protein as a substrate. Fig. 7 shows that the assayable CDK4 kinase activity was significantly decreased in COLO-205 cells treated with MEC for 24 h. However, CDK2 kinase activity was only slightly inhibited by MEC treatment. The P21/CIP1 protein is a potent inhibitor of CDK2 and CDK4. Combining these results with those from Western blotting indicates that MEC upregulated p53 and p21/CIP1, but not p27, and CDK2 and CDK4 kinase activities were suppressed so that the cell-cycle was arrested in the G0/G1 phase.

3.6. MEC induced apoptosis in COLO 205 cells

We examined COLO 205 cells treated with MEC to determine whether apoptosis had occurred. COLO 205 cells treated with various concentrations of MEC (10–100 μM) exhibited morphological changes which were accompanied by progressive internucleosomal degradation of DNA to yield DNA ladder fragments (Fig. 8) in 50 and 100 μM MEC treated cells. This result confirms that MEC induces apoptosis in COLO 205 cells.

3.7. MEC activated Caspase 3, 8, and 9 in COLO 205 and HT 29 cells

Apoptosis requires the activation of caspases, so the involvement of caspase activation was investigated in MEC-induced apoptosis in colon cancer cells by Western blot analysis. COLO 205 and HT 29 cells were treated with various concentrations of MEC (10–100 μM), and whole-cell lysates were collected for analysis. Fig. 9a shows that the protein levels of the active subunits of caspase 3, 8, and 9 were elevated. PARP degradation supports the observation of caspase activation. These results indicate that caspase 3, 8, and 9 were activated in MEC-treated COLO 205 cells. Similar changes were seen in MEC-treated HT 29 cells (Fig. 9b).

3.8. MEC upregulates p53 and downregulates Bcl-2 in COLO 205 cells

To clarify the molecular mechanism of MEC-induced apoptosis, the levels of apoptosis regulatory proteins were examined in the MEC-treated COLO-205 cells. Accordingly, we examined the changes in p53, Bcl-2 family members, and cytochrome C. Cells were harvested after treatment with various concentrations of MEC (10–100 μM) for 24 h. Fig. 10 illustrates the dose-dependent upregulation of p53. Bcl-2 downregulation was seen when a high concentration (100 μM) of MEC was administered. No changes in the levels of Bad, AIF, cytochrome C, and Apaf-1 were noted. These findings suggest that p53 upregulation and Bcl-2 downregulation are involved in MEC-induced apoptosis.

3.9. MEC induced cytochrome C release from mitochondria

It has been shown that activation of caspase 9 occurs during the release of cytochrome C from mitochondria to the cytosol (Shi, 2002b). Although the whole-cell cytochrome C protein level did not change after MEC treatment, subcellular extraction (cytosolic and mitochondrial) lysates were examined to clarify whether cytochrome C translocation occurred with MEC-induced apoptosis in COLO 205 cells. Fig. 11 shows that MEC treatment resulted in a significant shift of cytochrome C from the mitochondrial fraction to the cytosolic fraction of cell extracts. This MEC-induced elevation of cytosolic cytochrome C was noted to occur in a dose-dependent manner. Evidently, translocation of cytochrome C from mitochon-

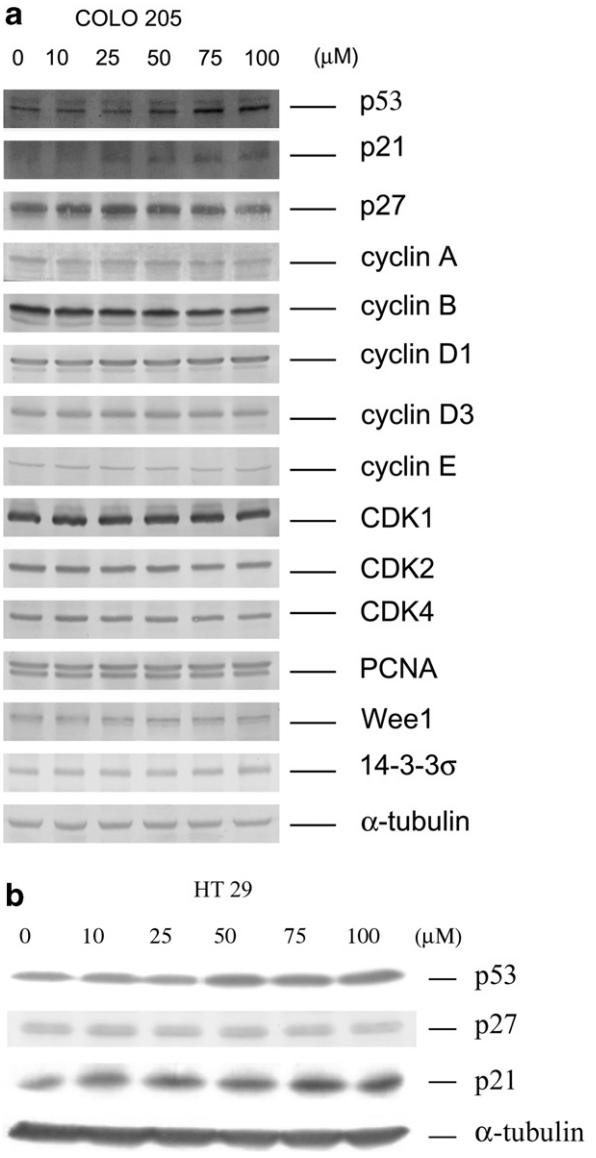

Fig. 6. Dose-dependent response of MEC-induced cell-cycle regulatory protein levels in COLO 205 cells (a) and HT 29 cells (b). Whole-cell lysates were prepared from COLO 205 and HT 29 cells treated with various concentrations of MEC (10–100 μM) for 24 h. Protein extracts (50 μg/lane) were separated by SDS-PAGE, immunoblotted with antibodies, and detected using the BCIP/NBT system.

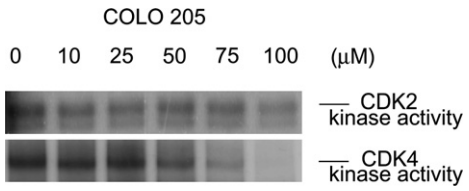

Fig. 7. CDK2 and CDK4 kinase activity of COLO 205 cells after treatment with various concentrations of MEC (10–100 μM) for 24 h. Protein lysates (50 μg/ml) were mixed with the CDK2 (or CDK4) antibody, agarose beads, and immunoprecipitation buffer, then reacted with a hot Rb kinase solution, separated by SDS-PAGE, and demonstrated with autoradiography. MEC strongly decreased assayable CDK4 kinase activity in a dose-dependent manner and only slightly inhibited CDK2 activity.

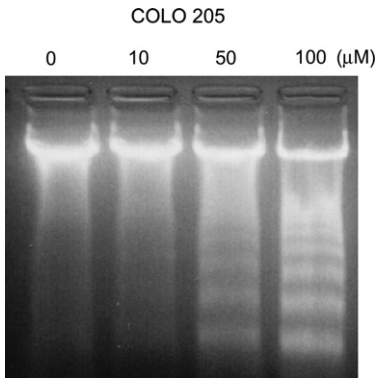

Fig. 8. Induction of apoptosis in COLO 205 cells after MEC treatment was shown by DNA fragmentation, using electrophoresis of genomic DNA. DNA fragmentation was examined at 24 h after MEC treatment.

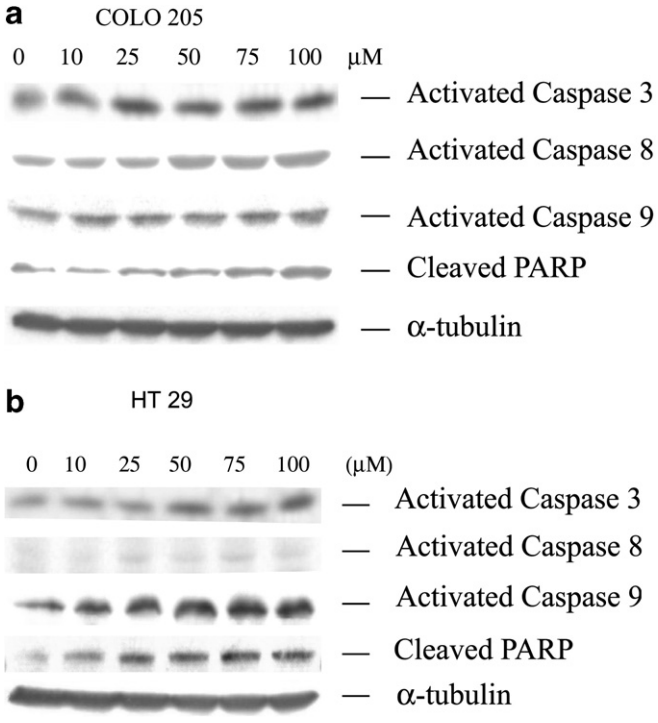

Fig. 9. Dose-dependent response of MEC-induced caspase activation in COLO 205 (a) and HT 29 (b) cells. Whole-cell lysates were prepared from COLO 205 and HT 29 cells treated with various concentrations of MEC (10–100 μM) for 24 h. Protein extracts (50 μg/lane) were separated by SDS-PAGE, immunoblotted with antibodies, and detected using the BCIP/NBT system. Protein levels of activated Caspase 3, 8, and 9 and cleaved PARP were increased after MEC treatment.

dria to the cytosol occurred in MEC-treated COLO 205 cells first, and activation of caspase 8 and 9 and DNA fragmentation followed thereafter.

3.10. MEC induced AIF translocation from the cytosol to the nucleus

It has been shown that AIF translocation from mitochondria to the nucleus occurs in apoptosis (Daugas

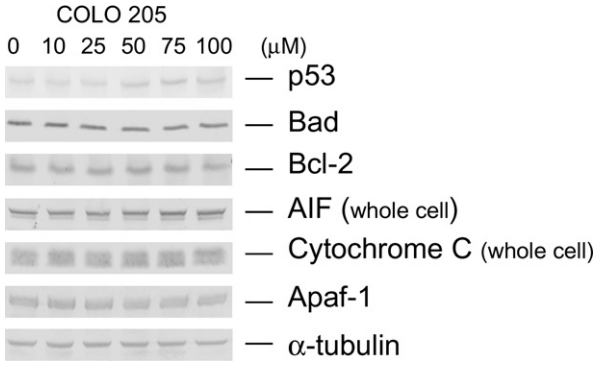

Fig. 10. Dose-dependent response of MEC-induced apoptosis regulatory protein levels in COLO 205 cells. Whole-cell lysates were prepared from COLO 205 cells treated with various concentrations of MEC (10–100 μM) for 24 h. Protein extracts (50 μg/lane) were separated by SDS-PAGE, immunoblotted with antibodies, and detected using the BCIP/NBT system.

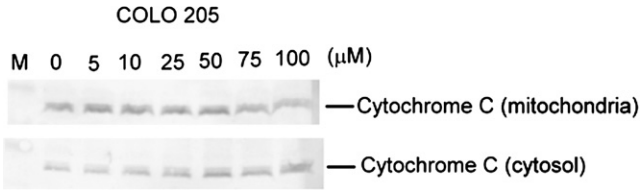

Fig. 11. Dose-dependent response of cytochrome C levels in COLO 205 cells. Cytosol and mitochondria lysates were prepared from COLO 205 cells treated with various concentrations of MEC (10–100 μM) for 24 h. Protein extracts (50 μg/lane) were separated by SDS-PAGE, immunoblotted with antibodies, and detected using the BCIP/NBT system.

et al., 2000). Although the whole-cell AIF protein level was not significantly influenced by MEC treatment, subcellular extraction (nuclear and mitochondrial) lysates were examined to clarify whether AIF translocation occurred or not with MEC-induced apoptosis in COLO 205 cells. Fig. 12 shows that the protein level of AIF from the mitochondrial fraction of cell extracts decreased and that of the nuclear fraction increased after MEC treatment. This MEC-induced translocation of AIF from mitochondria to the nucleus may also play a role in apoptosis of COLO 205 cells.

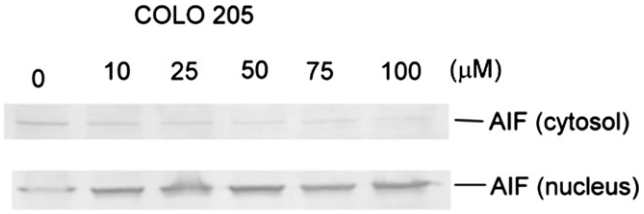

Fig. 12. Dose-dependent response of AIF levels in COLO 205 cells. Cytosol and nucleus lysates were prepared from COLO 205 cells treated with various concentrations of MEC (10–100 μM) for 24 h. Protein extracts (50 μg/lane) were separated by SDS-PAGE, immunoblotted with antibodies, and detected using the BCIP/NBT system.

4. Discussion

MEC is a commercially available antihistamine commonly used for motion sickness and vertigo. The most common adverse effects are sedation, drowsiness, dry mouth, and blurred vision (Dahl et al., 1984; Schmitt and Shaw, 1986), but incidences of these side effects are low, and it is considered a relatively safe drug. Wargovich et al. (2000) reported that rat colon aberrant crypt foci (ACF) induced by the carcinogen, azoxymethane, can be prevented by several potential agents. Among 76 investigated drugs, 20 of them were classified as strong inhibitors of ACF, including MEC. This was the first report that mentioned the antitumor activity of MEC. Other antihistamines such as loratadine (Chen et al., 2006) and terfenadine (Liu et al., 2003) have also been demonstrated to have antitumor effects through various mechanisms. The present study used an in vitro experiment to study the molecular mechanism of the anticancer effect of MEC for the first time.

TPA has different effects in various cell lines. Our previous research showed that TPA upregulates p21 and induces cell-cycle arrest at the G0/G1 phase in COLO 205 cells (Lin et al., 2002). Lewis et al. demonstrated TPA-induced apoptosis through a caspase-independent pathway in COLO 205 cells (Lewis et al., 2003). Choi et al. showed TPA did not influence cell proliferation in "original" HT 29 cells, but TPA increased doubling time and inhibited cell proliferation in PKC-overexpressing variant HT 29 cells (Choi et al., 1990). TPA did not stimulate proliferation of normal colonic epithelial cells (Friedman et al., 1984). So concerning about these 3 cell lines used in our study, TPA seems have no cancer promotion effect. MTT assay was performed to evaluate the interaction of MEC and TPA in these 3 cell lines and results are shown in the Fig. 3. It seems that MEC and TPA have no synergistic effects.

Cyclin dependent kinases (CDKs) control most if not all of the major cell-cycle transitions of eukaryotes (Tannoch et al., 2000). The CDKs associated with the G1/S phase transition in mammalian cells are CDK2, CDK4, and CDK6 (Reed, 1997). Activities of these CDKs are influenced by multiple layers of regulation, including the availability of cyclins (Johnson and Walker, 1999; McGill and Brooks, 1995), phosphorylation and dephosphorylation of CDKs, and CDK inhibitors (Vidal and Koff, 2000). In our data, the protein levels of cyclins did not change after MEC administration. p53 and p21/CIP1 were upregulated by MEC treatment, but p27/KIP1 was not influenced (Fig. 6a and b). p21/CIP1 is usually transcriptionally activated by p53 and is a universal cyclin-CDK inhibitor (el-Deiry, 1998; Gartel and Tyner, 1999). Our data also demonstrated that CDK2 and CDK4 kinase activities were inhibited by MEC (Fig. 7). So it is evident that MEC results in cell-cycle arrest at the G0/G1 phase through suppression of CDK2 and CDK4 kinase activities by the upregulation of p53 and p21/CIP1.

Apoptosis is a normal cell mechanism in development and homeostasis. It plays a role in sculpting the developing

organism, in the precise regulation of cell numbers, and in the defense mechanism to remove unwanted and potentially dangerous cells (Hale et al., 1996; Kerr et al., 1972). Dysregulation of apoptosis can result in autoimmune diseases, neurodegenerative disorders, and cancer (Fadeel et al., 1999; Reed and Tomaselli, 2000). Important features of apoptotic cell death are cell shrinkage, chromatin condensation, DNA fragmentation, and apoptotic bodies (Saraste and Pulkki, 2000). Internucleosomal DNA fragmentation is a significant feature of apoptotic cell death, and this phenomenon is caused by caspase-activated DNAse and endonuclease G which cleave DNA between nucleosome (Nagata, 2000). We first demonstrated that MEC induces DNA fragmentation in COLO 205 cells (Fig. 8). Caspases play a central role in apoptosis (Cohen, 1997; Earnshaw et al., 1999). When caspases are activated, they cleave a wide variety of proteins, including certain key substrates in the cell resulting in cell death through apoptosis (Earnshaw et al., 1999). Our data showed that caspase 3, 8, and 9 were all activated after MEC treatment in COLO 205 and HT 29 cells (Fig. 9a and b). These three pathways have been shown to participate in the process of apoptosis: whether through a death receptor-mediated (Greenstein et al., 2002), mitochondrion-mediated (Wang, 2001), or endoplasmic reticulum-mediated pathway (Nakagawa et al., 2000). Caspase 8 is thought to be involved in the death receptor pathway. Ding et al. (2000) revealed that caspase 8 is activated following p53 activation and is independent of FADD in an S100 cell-free system. This implies that caspase 8 might also be activated by p53 through an unknown mechanism. In our study, apoptosis regulatory proteins associated with the mitochondrial pathway were examined. Bcl-2 was downregulated but the proapoptotic member, Bax, remained unchanged. Protein levels of cytochrome C and AIF did not change when whole-cell extracts were analyzed. However, subcellular translocation of these two molecules plays an important role in MEC-induced apoptosis. Cytochrome C released from the intermembrane space of mitochondria into the cytosol allows the formation of apoptosome, a high-molecular-weight complex that consists of the adapter protein, Apaf-1, and caspase 9. Caspase 9 is activated following recruitment into apoptosome (Shi, 2002a). Active caspase 9 then cleaves and activates the effector caspases, such as caspases 3, which execute the cell-death response program.

There exists a discrepancy in our data. In COLO 205 cells, upregulation of p53 (Fig. 10), translocation of cytochrome C (Fig. 11), and activation of caspases (Fig. 9a) are shown when MEC was added at a concentration of 50 μM. However, translocation of AIF (Fig. 12) is noted at a concentration as low as 10 μM. This implies that different concentrations of MEC might have different effects and mechanisms. Previous literature showed that AIF released from the intermembrane space of mitochondria into the nucleus induces nuclear chromatin condensation and large-scale (~50 kb) DNA fragmentation in a caspase-independent fashion (Daugas et al., 2000; Evan and Vousden, 2001). It is possible that low dose MEC mobilizes AIF from the mitochondria to the nucleus in a caspase-independent manner, and high dose MEC induces release of cytochrome C, followed by activation of caspase 9 and caspase 3.

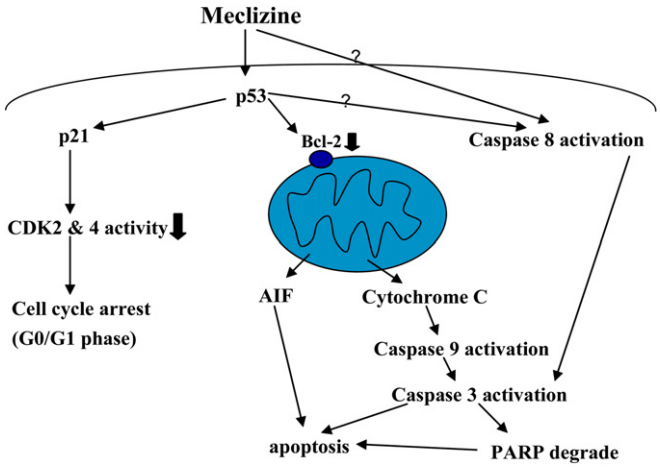

Fig. 13. Summary for MEC-induced anticancer activity in COLO 205 cells. In response to MEC treatment, p53 and p21 were upregulated and CDK2 and CDK4 kinase activity was suppressed, so that the cell-cycle was arrested in the G0/G1 phase. P53 upregulation also induced cytochrome C released from mitochondria to the cytosol and AIF from mitochondria to the nucleus. Caspase 3, 8, and 9 were activated, resulting in apoptosis.

These findings show for the first time that MEC can induce apoptosis and cell-cycle arrest in cultured human cancer cells. Based on results of the present study, we summarize the molecular mechanism in Fig. 13 to explain how MEC executes its anticancer activity in a human colon cancer cell line.

Cancer is a disease of deregulated cell proliferation together with suppressed apoptosis (Evan and Vousden, 2001). Most chemotherapeutic drugs exert their cytotoxic action through inhibiting cancer cell growth and inducing apoptosis (Johnstone et al., 2002). But the toxicities of these drugs limit their use at high doses. Combination with other agents may reduce those undesirable toxic effects and help maintain or enhance their anticancer activities. MEC is a safe drug with a low incidence of side effects and produced no harm to cultured normal human colon epithelial cells. Our results show the molecular basis of in vitro MEC-induced cancer cell growth inhibition, and further animal experiments will be important to demonstrate the potential anticancer effect of MEC in vivo.

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