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Anti-invasive Effect of a Rare Mushroom, *Ganoderma colossum*, on Human Hepatoma Cells

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Ganoderma colossum is a rare species of the Ganodermataceae family with biological activity but has not been widely used to date. Because of its rareness and hard availability, the literature regarding the bioactivity of *G. colossum* is very limited and the bioactive components in *G. colossum* have never been explored. In the present study, an ethanol extract was prepared from the fruiting body of a *G. colossum* strain (EEGC) and then fractionated by reverese-phase high-performance liquid chromatography (HPLC). The inhibitory effects and molecular mechanisms of the EEGC on the phorbol-12-myristate-13-acetate (PMA)-induced invasion of HepG2 cells were investigated. The fractions of the EEGC cannot be totally identified, but the lucidenic acids were considered as the major component. When HepG2 cells were treated with the EEGC, the PMA-induced invasion was reduced in a dose-dependent manner and the PMA-induced matrix metalloproteinase (MMP)-9 was also suppressed at the transcriptional level. The EEGC also showed an inhibitory effect on the PMA-induced phosphorylation of extracellular signal-regulated kinase (ERK1/2) and protein kinase B (Akt) in cytosol, as well as the activator protein-1 (AP-1) and nuclear factor-κB (NF-κB) levels in the nucleus of HepG2 cells. This study provides the first evidence demonstrating that the EEGC is an effective inhibitior on the PMA-induced invasion of hepatoma cell. The EEGC could be further tested by an *in vivo* model to verify whether it is effective for the prevention of hepatoma invasion or metastasis.

KEYWORDS: *Ganoderma colossum*; hepatocellular carcinoma; invasion; MAPK pathway; MMP-9 activity

INTRODUCTION

Ganodermataceae is a large fungal family with more than 200 species, and some members of Ganodermataceae, such as *Ganoderma lucidum*, *Ganoderma tsugae*, and *Ganoderma applanatum*, have been used as Chinese herbs and folk dietary supplements to prevent various diseases for many years (*1*,*2*). However, numerous species of the Ganodermataceae family have not been widely used with different biological activities. *Ganoderma colossum* (Fr.) C. F. Baker (synonymous: *Polyporus colossus* and *Dendrophagus colossus*) is a rare species of the Ganodermataceae family that is capable of causing selective delignification of wood to obtain nutrients and reproduces asextually with the production of chlamydospores in culture (*3*,*4*). In addition, *G. colossum* was reported to have the highest antimicrobial activity among four species of *Ganoderma* from Nigeria (*5*). Recently, lanostane triterpenes isolated from *G. colossum* with the inhibitory effect on human immunodeficiency virus type-1 (HIV-1) protease activity was also revealed (*6*). Although a number of biological activities of *G. colossum* have been found; however, this mushroom has not been widely used because of its rareness and hard availability. Thus, the literature regarding the bioactivity of *G. colossum* is still limited, and the bioactive components in *G. colossum* have never been explored.

Hepatocellular carcinoma (HCC) is one of the most common malignancies in the world as a whole, as well as in Taiwan specifically. HCC is a hypervascular tumor, in which venous invasion is a common and important risk factor for tumor metastasis (*7*). Metastasis is responsible for the majority of failures in cancer treatment and is also the major cause of death in various cancer patients (*8*). Therefore, in addition to minimizing the growth of existing tumors, treatments that limit their spread to new sites and blockade their invasions have been considered to enhance the survival of cancer patients (*9*). The invasion and metastasis of cancer cells involve degradation of environmental barriers, such as the extracellular matrix (ECM) and basement membrane, by various proteolytic enzymes, leading to enhanced mobility and metastasis (*10*). The matrix metalloproteinase (MMP)-2 and MMP-9 are both enzymes capable of degrading type-IV collagen, which is a major constituent of the basement

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membrane. These enzymes are highly expressed in various malignant tumors, and the expression levels are closely related to the invasion and metastasis of cancer cells (*11*, *12*). Secretion of MMP-9 has been reported in lung, colon, breast, and liver cancers (*13*), and MMP-2 expression has been found in human lung cancer cell line A549 (*14*). In recent years, some *in vitro* and *in vivo* studies have demonstrated the relationship between MMP-9 expression and HCC invasion (*15*, *16*). Therefore, several inhibitors against MMPs have been tested in clinical trials for the prevention of tumor invasion and metastasis (*17*, *18*).

In the present study, an ethanol extract of *G. colossum* (EEGC) was prepared and further fractionated by means of reverse-phase high-performance liquid chromatography (HPLC). The human hepatocarcinoma HepG2 cell line is a common cell model for cancer research, which secretes both MMP-2 and MMP-9 simultaneously and enhances invasive activity with induction (*15*). The aims of this study were to evaluate the inhibitory effect of the EEGC on the phorbol-12-myristate-13-acetate (PMA)-induced invasion of HepG2 cells and to explore the underlying molecular mechanisms.

MATERIALS AND METHODS

Materials and Chemicals. The *G. colossum* strain, which was preserved in the Biotechnology Research and Development Institute (Double Crane, Tainan Hsien, Taiwan), was grown to the fruiting body stage according to the protocol of Chen et al. (*19*). It was identified by Dr. Shen-Hwa Wu (National Museum of Natural Science, Taiwan) using morphological characterization and DNA profiles. PMA and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO). Methanol, ethanol, acetone, ethyl acetate, acetonitrile (HPLC grade), and dimethylsulfoxide (DMSO) were purchased from Tedia Co. (Fairfield, OH). Dulbecco's modified Eagle's medium (DMEM) was purchased from HyClone, Inc. (Logan, UT). Fetal bovine serum (FBS) was purchased from Gibco BRL Co. (Grand Island, NY). The antibodies of total and phosphorylated mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK)1/2, p38 MAPK, stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), Akt (PKB), c-Jun, c-Fos, and nuclear factor-κB (NF-κB) (p65) were purchased from Cell Signaling Technology, Inc. (Boston, MA). The β-actin and lamin B monoclonal antibodies were purchased from BioVision, Inc. (Moutain View, CA). Goat anti-rabbit IgG (H&L) horseradish-peroxidase-conjugated antibody and goat anti-mouse IgG (H&L) horseradish-peroxidase-conjugated antibody were purchased from Chemicon International, Inc. (Billerica, MA).

Preparation of the EEGC. Dried fruiting bodies of *G. colossum* were ground to powders and extracted with absolute ethanol (1:10, w/v) in a shaking incubator at room temperature for 8 h. After cooling, the extract was filtered and the filtrate was evaporated to dryness under vacuum using a rotary evaporator to be the EEGC and then stored at −20 °C until use. The EEGC was redissolved in DMSO for subsequent experiments. The final concentration of DMSO was controlled under 0.5% in control and each test group.

Fractionation of the EEGC. The fractionation and identification of the EEGC was performed as the method described by Chen and Chen (*20*). Briefly, an acidic ethyl-acetate-soluble material containing fraction was obtained after extraction and purification according to the previous report (*15*), further dissolved with 50% ethanol, and then subjected to semi-preparative reverse-phase HPLC (Hitachi, Tokyo, Japan). Semi-preparative HPLC was performed on a HITACHI 6050 pump equipped with a Hitachi L-4200 UV−vis detector and a Hitachi D2500 integrator. The detection wavelength was set at 252 nm. A column of Lichrosorb RP-18 (Merck Hibar, 7 μm, 250 × 25 mm) was used. The mobile phase is acetonitrile/2% acetic acid = 1:3 for the first 80 min and then changed to 1:2. The flow rate was set at 7.8 mL/min. The HPLC profile was initially compared to the HPLC chromatogram of triterpenoid standards. The eluted peaks were collected and concentrated in a rotary evaporator and stood for 4−8 days for crystallization and identification.

Cell Culture. HepG2 cells were obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsin Chu, Taiwan). Cells were grown in DMEM, supplemented with 10% (v/v) FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, 0.37% (w/v) $NaHCO_3$, 0.1 mM non-essential amino acids, and 1 mM sodium pyruvate at 37 °C, in a humidified atmosphere of 95% air and 5% CO_2. In the invasive experiments, cells were cultured in a serum-free medium.

Cell Viability Assay. Cell viability was determined with a MTT assay. HepG2 cells were seeded onto 96-well plates at a concentration of 5×10^4 cells/well in DMEM without FBS. After 24 h of incubation, the cells were treated with various concentrations of EEGC and further incubated for 24−72 h. The controls were treated with 0.5% DMSO alone. The dye solution [(10 μL; 5 mg/mL dye in phosphate-buffered saline (PBS)] was added to each well for an additional 60 min of incubation at 37 °C. After the addition of DMSO (100 μL/well), the reaction solution was incubated 30 min in the dark. The absorbances at 570 and 630 nm (reference) were recorded with a Fluostar Galaxy plate reader. The percent viability of the treated cells was calculated as follows:

$$[(A_{570\ \mathrm{nm}} - A_{630\ \mathrm{nm}})_{\mathrm{sample}}/(A_{570\ \mathrm{nm}} - A_{630\ \mathrm{nm}})_{\mathrm{control}}] \times 100$$

Cell Invasion Assay. HepG2 cells were detached from the tissue culture plates, washed with PBS, resuspended in serum-free DMEM (5×10^4 cells/200 μL) in the presence or absence of the compound (PMA and EEGC), and then seeded onto the upper chamber of Matrigel-coated filter inserts (8 μm pore size) purchased from BD Biosciences (San Jose, CA). Serum-free DMEM (500 μL) was added to the lower chamber. After 24 h of incubation, filter inserts were removed from the wells and the cells on the upper surface of the filter were wiped with a cotton swab. Filters were fixed with methanol for 10 min and stained with Giemsa dye for 1 h, and then the cells that had invaded the lower surface of the filter were counted under a microscope.

Gelatin Zymography. HepG2 cells were incubated in a serum-free DMEM with or without EEGC and 200 nM PMA for 24 h, and the conditioned media were collected as samples. The unboiled samples were separated by electrophoresis on 8% sodium dodecyl sulfate (SDS) polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were washed twice in washing buffer (2.5% Triton X-100 in dH_2O) for 30 min at room temperature and were then incubated in reaction buffer (10 mM $CaCl_2$, 0.01% NaN_3, and 40 mM Tris-HCl at pH 8.0) at 37 °C for 12 h. Bands corresponding to activity were visualized by negative staining using Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, CA).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was prepared from HepG2 cells using the 3-Zol (Trizol) reagent (MDBio, Inc., Piscataway, NJ) and purified by following the instructions of the manufacturer. For RT-PCR, 4 μg of total cellular RNA was used as a template in a 20 μL reaction solution, which contained 4 μL of dNTPs (2.5 mM), 2.5 μL of oligo dT (10 pmol/μL), and RTase (200 units/μL). This reaction was performed at 42 °C for 1 h. The resulting cDNA (5 μL) was amplified by PCR with the following primers: MMP-9 (269 bp), 5′-CACTGTCCACCCCTCAGAGC-3′ (sense) and 5′-GCCA-CTTGTCGGCGATAAGG-3′ (anti-sense); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; 309 bp), 5′-TCCCTCAAGATTGTCAGCAA-3′ (sense) and 5′-AGATCCACAACGGATACATT-3′ (anti-sense). PCR amplification was performed under the following conditions: 25 cycles of 94 °C for 1 min, 63 °C for 1 min, and 72 °C for 2 min, followed by a final incubation at 72 °C for 10 min. PCR products were analyzed with 1.8% agarose gel electrophoresis and visualized by ethidium bromide staining.

Preparation of Cell Lysates and Nuclear Fractions. Cell lysates and nuclear fractions were prepared using a nuclear extraction kit (Panomics, Redwood City, CA). Briefly, harvested cells (1×10^6 cells/6 cm plate) were washed twice with 5 mL of cold 1× PBS. A 0.5 mL aliquot of buffer A solution was added to each plate. The plate was transferred to an ice bucket on a rocking platform at 150 rpm for 10 min. The treated cell suspension was transferred to a sterilized eppendorf tube and centrifuged at 14000*g* for 3 min at 4 °C. The supernatant (cytosolic fraction) was removed, and the pellet was kept on ice. A 75 μL aliquot of buffer B solution was added to each pellet. Pellets were vortexed at maximum speed for 10 s, and the eppendorf tubes were shook on a rocking platform at 150 rpm for 2 h. After centrifugation at 14000*g* for 5 min at 4 °C, the supernatant was nuclear extract and was stored at −80 °C until use.

Western Blotting. Samples (10 μg) of total cell lysates or nuclear fractions were size-fractionated by a SDS−polyacrylamide gel electrophoresis

(PAGE) of 10% polyacrylamide gels and electrophoretically transferred onto a polyvinylidene fluoride (PVDF) membrane using the Bio-Rad Mini Protean electrotransfer system. The blot was subsequently incubated with 5% skim milk in PBST for 1 h and probed with the total and phosphorylated MAPK/ERK1/2, p38 MAPK, SAPK/JNK, and Akt (PKB), and β-actin, NF-κB (p65), c-Jun, c-Fos, and lamin B were detected with each specific antibody overnight at 4 °C. Detection was performed with an appropriate peroxidase-conjugated secondary antibody at room temperature for 1 h. Intensive PBS washing was performed after each incubation. After the final PBS washing, the signal was developed by an enhanced chemiluminescence (ECL) detection system and Kodak X-OMAT blue autoradiography film. The protein content was determined by the Bradford assay (*21*), with bovine serum albumin as a standard.

Statistical Analysis. Data are presented as the mean ± standard deviation (SD) of three independent measurements. Differences between variants were analyzed using the Student's *t* test for unpaired data. Values of $p < 0.05$ (*) or $p < 0.01$ (**) were regarded as statistically significant.

RESULTS

EEGC Contains Lucidenic Acids, but Some Fractions of the EEGC Are Unidentifiable. The EEGC was prepared and further fractionated by means of a reverse-phase HPLC as described in the Materials and Methods. On the basis of the HPLC chromatogram, three major peaks a, b, and c with retention times of 35.18, 26.37, and 30.06 min were obtained (**Figure 1A**). The peak fractions a and b were initially recognized as lucidenic acids A and B as compared to the spectroscopic values of HPLC of those of triterpenoid standards (**Figure 1B**). The structures and identification of lucidenic acids were published in a previous study (*15*). Even though the peak fraction c was unable to be identified by a comparison to various triterpenoid standards, at least the lucidenic acids were affirmed as the major component in the EEGC. Because the lucidenic acids on the inhibition of HCC invasion had been evaluated in our previous study (*15*), we therefore inferred that the EEGC might exert an anti-invasion effect on HCC.

Effect of the EEGC on the Viability of HepG2 Cells. The cytotoxicity of the EEGC on HepG2 cells was first determined by a MTT assay, which is used for measuring the metabolic activity of a cell. The absorbance decrease in this assay could be either a consequence of cell death or a reduction of cell proliferation. The HepG2 cells were treated with the EEGC at various concentrations (0, 0.01, 0.1, and 1 mg/mL) for 24, 48, and 72 h. The results showed that HepG2 cells treated with the EEGC at a concentration of 1 mg/mL for 72 h still retained over 90% viability as compared to the control (**Figure 2**). Hence, the noncytotoxic concentrations (0−1 mg/mL) and treatment time (24 h) of the EEGC were used for the subsequent experiments on hepatoma cells.

EEGC Inhibits PMA-Induced Invasion of HepG2 Cells. To examine the potential anti-invasive effect of EEGC, the invasive activity of HepG2 cells was examined with the cell invasion assay. Results indicated that the invasive activities of HepG2 cells without induction were not affected by treatment with the EEGC (data not shown). We have previously shown that the invasive and MMP-9 activities of HepG2 cells can be significantly ($p < 0.01$) induced by co-incubation with 200 nM PMA for 24 h (*15*); hence, HepG2 cells were treated with PMA first for the subsequent tests, and the outcome was in the same manner as what we obtained before. After treatment with the EEGC at a concentration higher than 0.5 mg/mL on PMA-treated HepG2 cells for 24 h, the invasive ability was significantly ($p < 0.05$) reduced by at least 24% relative to the PMA treatment only (**Figure 3**).

EEGC Suppresses PMA-Induced MMP-9 Activity of HepG2 Cells at a Transcriptional Level. It is known that ECM degradation is a crucial step to cell invasion. To clarify the MMP-2 and MMP-9 activities involved in the EEGC-inhibited invasion of

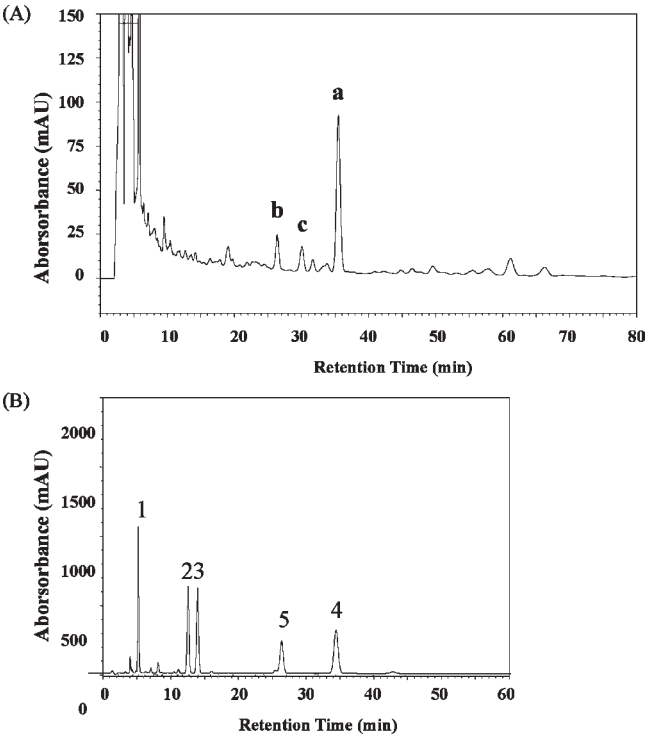

Figure 1. (A) Reverse-phase HPLC profile of the EEGC. The retention times of three major peaks a, b, and c are 35.18, 26.37, and 30.06 min, respectively. (B) Reverse-phase HPLC chromatogrphy of the lucidenic acid standard. Peaks 1, 2, 3, 4, and 5 with retention times of 6.71, 13.84, 14.93, 34.73, and 26.23 min are lucidenic acids H, C, N, A, and B, respectively.

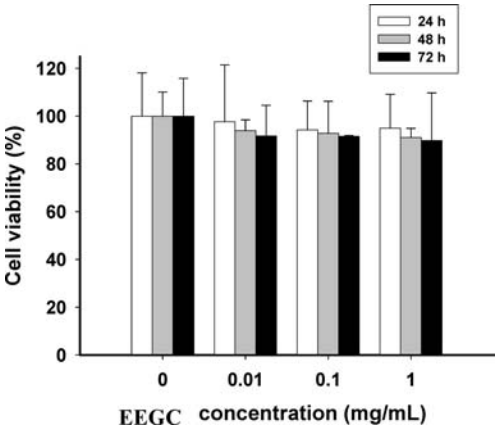

Figure 2. Effects of the EEGC on the viability of HepG2 cells by a MTT assay. Cells were incubated in a serum-free medium with 0−1 mg/mL EEGC for 24, 48, and 72 h, and the cell in a serum-free medium without EEGC was used as the control. The bar graphs represent the percentage of cell viability of the control.

HepG2 cells or not, the dosages of the EEGC, which we used in an anti-invasive assay (0, 0.01, 0.1, 0.5, and 1 mg/mL), were applied to analyze their effects on MMP-2 and MMP-9 activities by gelatin zymography. As shown in **Figure 4A**, the MMP-9 activity was suppressed in a dose-dependent manner but the MMP-2 activity was not significantly affected during treatment with the EEGC on HepG2 cells. The data of **Figures 3** and **4A** suggest that the EEGC inhibits PMA-induced invasion of HepG2 cells through the suppression of MMP-9 activity. A semi-quantitative RT-PCR was further employed to analyze the effect of the EEGC on the mRNA level of MMP-9 in HepG2 cells. After treatment

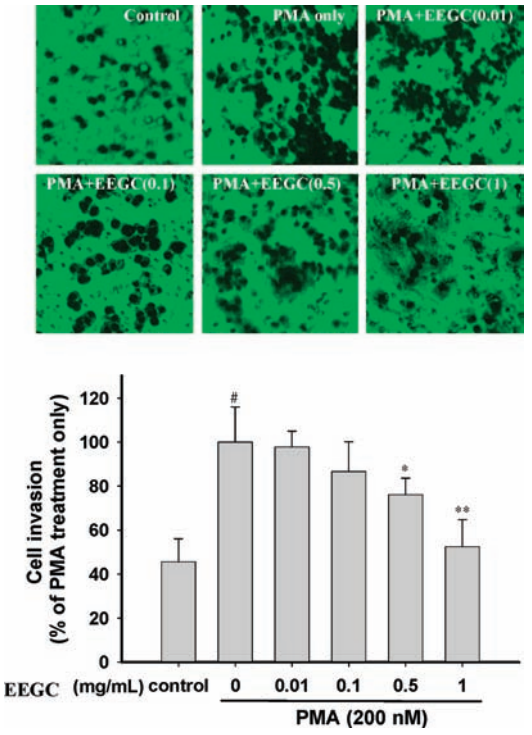

Figure 3. Effect of the EEGC on PMA-induced invasion of HepG2 cells by a Matrigel invasion assay. HepG2 cells were incubated in a serum-free medium containing 0−1 mg/mL EEGC and 200 nM PMA for 24 h, and the cell in a serum-free medium without treatment was used as the control. The bar graphs represent the percentage of the invasive cells of the PMA treatment only. (#) $p < 0.01$ compared to the control. (∗) $p < 0.05$ and (∗∗) $p < 0.01$ compared to the PMA treatment only.

with various concentrations (0, 0.01, 0.1, 0.5, and 1 mg/mL) of the EEGC on the PMA-induced HepG2 cells for 24 h, the mRNA expression of MMP-9 was significantly reduced in a dose-dependent manner, while the internal control (GAPDH) remained unchanged (**Figure 4B**). These results indicate that the regulation of MMP-9 activity in HepG2 cells is, at least partly, on the transcriptional level.

EEGC Inhibits PMA-Induced MMP-9 Expression through Suppressing Phosphorylation of MAPK/ERK1/2 and Akt. Previous reports have revealed that the regulation of MMP-9 expression is partly through MAPK and phosphoinositide-3 kinase/protein kinase B (PI3K/Akt) pathways. Because the bioactivity of the EEGC on the suppression of the PMA-induced MMP-9 expression in HepG2 cells was demonstrated, the effects of PMA and EEGC on the MAPK and PI3K/Akt pathways were investigated by western blotting to clarify the underlying mechanisms. The results showed that PMA induced but EEGC reduced the phosphorylation of ERK1/2 and Akt in HepG2 cells (**Figure 5**). The quantitative densitometric data of western blots after correcting by the corresponding internal control (β-actin) of cytoplasma indicated that the treatment of 0.5 mg/mL EEGC decreased the phosphorylation of ERK1/2 and Akt to 50 and 60%, respectively, relative to the PMA treatment only. Neither PMA nor EEGC affected SAPK/JNK and p38 MAPK significantly (**Figure 5**). The results suggest that the EEGC inhibits PMA-induced MMP-9 expression of HepG2 cells through the inactivation of MAPK/ERK1/2 and Akt signaling pathways.

EEGC Inhibits Transcriptional Activation of the *MMP-9* Gene through the Suppression of PMA-Stimulated NF-κB and Activator Protein-1 (AP-1) Activities. It is known that the AP-1 and NF-κB proteins are the downstream targets of MAPK signaling and the

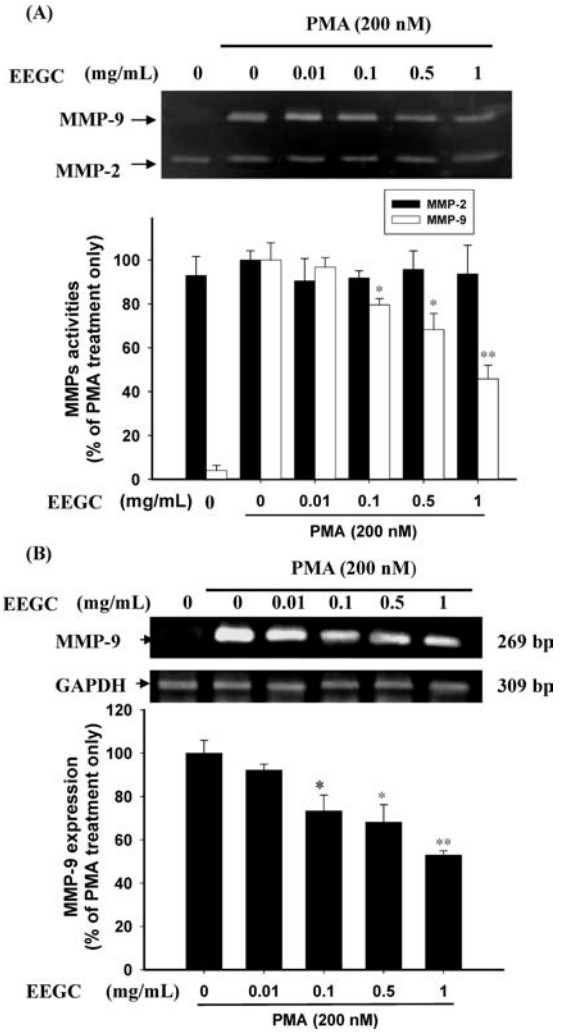

Figure 4. Concentration-dependent inhibitory effect of the EEGC on PMA-induced MMP-9 activity and mRNA expression of HepG2 cells. HepG2 cells were incubated in a serum-free medium containing 200 nM PMA and 0−1 mg/mL EEGC for 24 h, and the cell in a serum-free medium without treatment was used as the control. (A) The medium was used for MMP-9 activity assays by gelatin zymography. (B) The RNA extract was used for mRNA expression assays by semi-quantitative RT-PCR, and GAPDH was used as an internal control. The bands on the gels were quantified by densitometric analyses, with the density of PMA treated only being 100%. (∗) $p < 0.05$ and (∗∗) $p < 0.01$ compared to the PMA treatment only.

expression of the *MMP-9* gene is regulated by the interaction of AP-1 and NF-κB with their binding sequences on the *MMP-9* gene promoter in the nucleus. Because the results showed that EEGC could inhibit transcription of MMP-9 and affect the phosphorylation state of MAPK signaling proteins, the effects of PMA and EEGC on the protein levels of NF-κB and AP-1 in the nucleus of PMA-induced HepG2 cells were examined. HepG2 cells were treated with various non-cytotoxic concentrations of EEGC (0, 0.01, 0.1, 0.5, and 1 mg/mL) in the presence of PMA (200 nM) for 24 h, and the nuclear extracts were analyzed by western blotting to measure the levels of NF-κB, c-Jun, and c-Fos. As shown in **Figure 6**, the levels of PMA-induced NF-κB, c-Jun, and c-Fos after correcting by the corresponding internal control (lamin B) in the nucleus were decreased by EEGC treatment in a dose-dependent manner.

DISCUSSION

Some dietary nutraceuticals might have preventive and/or therapeutic effects against cancer. *G. lucidum* has been investigated

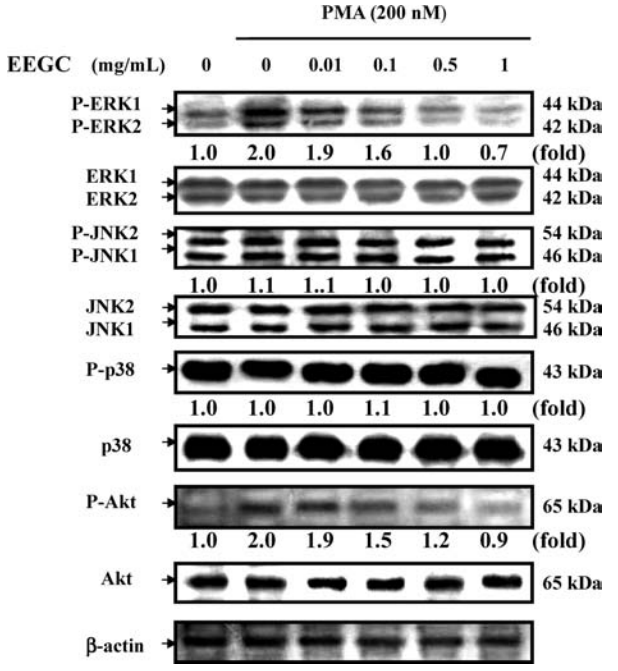

Figure 5. Inhibitory effect of the EEGC on the phosphorylation of ERK1/2 and Akt at the protein level. HepG2 cells were cultured in serum-free media containing 200 nM PMA and 0−1 mg/mL EEGC for 24 h, and then the cell lysates were subjected to western blot analysis. Levels of proteins were subsequently quantified by densitometric analyses, and the relative density was compared to that of the untreated group being 100%.

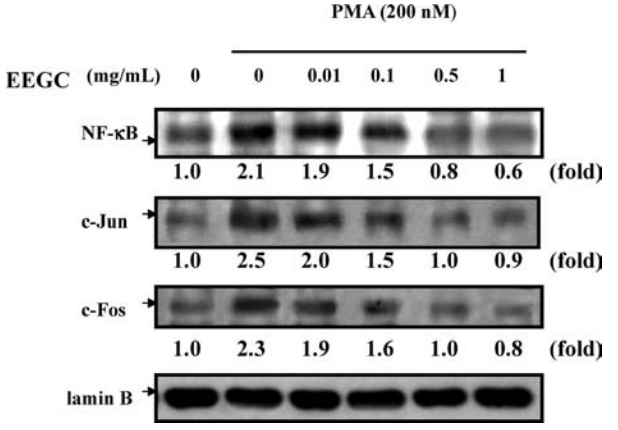

Figure 6. Inhibitory effect of EEGC on the expression levels of NF-κB, c-Jun, and c-Fos. HepG2 cells were cultured in serum-free media containing 200 nM PMA and 0−1 mg/mL EEGC for 24 h, and then the nuclear extracts were subjected to western blot analysis. Levels of protein were subsequently quantified by densitometric analyses, and the relative density was compared to that of the untreated group being 100%.

extensively for its anti-tumor, anti-virus, and anti-invasion effects, as well as the inhibition of proliferation and apoptosis of leukemia cells; *G. colossum* has been known thus far to have anti-inflammatory, cytotoxic (*22*), and antimicrobial activities (*5*), even though the related literature is still insufficient to date. *G. colossum* and *G. lucidum* reproduce asexually via the production of chlamydospores and grow in culture at an optimum temperature above 30 °C, and both are capable of causing selective delignification of oak wood (*4*, *23*). Because many characteristics of *G. colossum* and *G. lucidum* are so close, some aspects of the biological or medicinal properties between these two species might also be resembled. Previously, the ethanol extract of *G. lucidum* (GLE) was demonstrated to serve as a chemopreventive agent for the tumorigenesis and metastasis of highly invasive hepatoma cells *in vitro* and *in vivo*, and the treatment of GLE on hepatoma cells can reduce the PMA-induced invasion through suppressing the MMP-9 expression (*16*). The EEGC was therefore presumed to have an anti-invasive activity on hepatoma cells as that of GLE.

The triterpenoid component has also been recorded in many *Ganoderma* species, such as *G. lucidum*, *G. colossum*, *G. tsugae*, and *G. applanatum* (*24*). Seven triterpenoid metabolites, colossolactones A−G, have been isolated from the fruiting body of *G. colossum* by silica gel chromatography/thin-layer chromatography (TLC) methods (*22*). Colossolactones have been reported with moderate cytotoxicity against L-929, K-562, and HeLa cells and with anti-inflammatory properties and exhibit an inhibitory effect on 3α-hydroxysteroid dehydrogenase (*25*). Recently, eight new lanostane triterpenes, colossolactones I−VIII, were isolated from the fruiting bodies of *G. colossum* by silica gel chromatography/TLC/silica gel chromatography/preparative HPLC methods (*6*, *26*), which exhibited anti-HIV-1 protease activity. It is thus evident that some functional triterpenoids truly exist in *G. colossum*. In the present study, we provided a novel result on the component fractionation of *G. colossum* by semi-preparative reverse-phase HPLC and found that the triterpenoid pattern of HPLC of *G. colossum* (**Figure 1**) is very different from those of *G. tsugae* (*19*) and *G. lucidum* (*15*). One of the limitations of our study is that the identification of the whole EEGC was not available because of the technical problem and the lack of sufficient information. However, our analysis, at least, revealed that lucidenic acids are one of the components in EEGC. Lucidenic acids have been found in *G. lucidum* (*15*) and *G. tsugae* (*19*) strains and were further extrapolated that it might be the bioactive component in *G. lucidum* that possessed anti-invasive activity on HepG2 cells (*15*). Hence, we hypothesized that the anti-invasive properties of *Ganoderma* might be derived from triterpenoid components. In comparison of EEGC to GLE, an approximate amount of lucidenic acids A and B can be derived from their HPLC profiles. In a previous study (*16*), the proposed daily intake of 1.32 g of GLE for an adult human with a body weight of 60 kg on the inhibition of cancer invasion was obtained. The dosage might be also applied to the EEGC and could be available via human dietary supplementation. Meanwhile, 1 mg/mL of EEGC used in the experiments of this study approximately contained 16 and 6.7 μg/mL (34.9 and 14.1 μM) lucidenic acids A and B, respectively.

It is known that the activation of one or more MAPK pathways (e.g., ERK1/2, JNK, and p38) is important for the MMP-9 induction by PMA in various cell types (*27*, *28*), and PMA treatment is also able to direct activation of PI3K/Akt (*29*). PI3K might play an important role in facilitating co-localization between AFAP-110 and cSrc in response to PMA, and the loss of PI3K/Akt protein expression or activity could prevent PMA-induced activation of cSrc and subsequent migration potential (*29*). In our previous (*30*) and present studies on HepG2 cells, PMA-induced MMP-9 expression and a stimulation of phosphorylation of ERK1/2 and Akt were observed. According to the findings of signaling pathways of PMA-induced MMP-9 activity, we presumed the inhibitory effect of the EEGC on PMA-induced MMP-9 activity of HepG2 cells through the inactivation of signaling pathways of PMA induction. This hypothesis was then supported by the results shown in **Figures 4** and **5**, which indicated that the PMA-induced MMP-9 activity and ERK1/2 and Akt signalings of HepG2 cells are all suppressed by the treatment of the EEGC.

The transcription factors AP-1 and NF-κB are involved in many pathological processes, such as angiogenesis, inflammation,

metastasis, and invasion (*31*−*33*). The AP-1 and NF-κB elements of the MMP-9 promoter are centrally involved in the induction of the *MMP-9* gene associated with the invasion of tumor cells by PMA and cytokines. Cheng et al. (*34*) indicated that NF-κB modulates the radiation-enhanced MMP-9 activity in HepG2 cells. Huang et al. (*35*) showed that the inhibitory effect of carnosol on melanoma cell migration and invasion by reducing MMP-9 expression is mediated through suppressing the ERK1/2, Akt, p38, and JNK pathways, as well as inhibiting NF-κB and AP-1 DNA-binding activities. Chung et al. (*36*) also found that caffeic acid (CA) and caffeic acid phenyl ester (CAPE) inhibit the transcriptional activity of MMP-9 in the PMA-induced HepG2 cells by blocking NF-κB activation. Our findings demonstrated that the treatment of the EEGC on PMA-induced HepG2 cells resulted in the reduction of nuclear translocation of c-Jun, c-Fos, and NF-κB proteins (**Figure 6**). Thus, the regulation of AP-1 and NF-κB as well as the downstream of the MAPK and PI3K/Akt pathways might be involved in PMA-induced and EEGC-inhibited MMP-9 expression. These molecular targets were also observed in lucidenic acids against PMA-induced invasion of HepG2 cells (*30*).

In conclusion, this study is the first evidence to demonstrate the EEGC is an effective inhibitor on the PMA-induced MMP-9 expression, which contributes to the inhibition of HepG2 cell invasion. The inhibitory activity inactivates the phosphorylation of ERK1/2 and Akt in cytosol as well as reduces the AP-1 (c-Jun and c-Fos) and NF-κB protein expressions in the nucleus of HepG2 cells. With the clarification of signal transduction mediators and transcriptional factors involved in the anti-invasive process of the EEGC on a human hepatoma cell line, it might be possible to develop specific mediators to inhibit undesired cell invasion.

ABBREVIATIONS USED

AP-1, activator protein-1; DMEM, Dulbecco's modified Eagle's medium; EEGC, ethanol extract of *G. colossum*; ERK, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PI3K/Akt, phosphoinositide-3 kinase/protein kinase B; PMA, phorbol-12-myristate-13-acetate; RT-PCR, reverse transcription-polymerase chain reaction; SAPK/JNK, stress-activated protein kinase/c-Jun N-terminal kinase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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