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Apoptotic Effects of Co-Treatment with a Chios Gum Mastic and Eugenol on G361 Human Melanoma Cells

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We investigated the synergistic apoptotic effects of co-treatments with Chios gum mastic (CGM) and eugenol on G361 human melanoma cells. An MTT assay was conducted to investigate whether this co-treatment efficiently reduces the viability of G361 cells compared with each single treatment. The induction and augmentation of apoptosis were confirmed by DNA electrophoresis, Hoechst staining, and analyses of DNA hypoploidy. Western blot analysis and immunofluorescent staining were also performed to evaluate expression and translocation of apoptosis-related proteins following CGM and eugenol co-treatment. Proteasome activity and mitochondrial membrane potential (MMP) changes were also assayed.The results indicated that the co-treatment of CGM and eugenol induces multiple pathways and processes associated with an apoptotic response in G361 cells. These include nuclear condensation, DNA fragmentation, a reduction in MMP and proteasome activity, an increase of Bax and decrease of Bcl-2, a decreased DNA content, cytochrome c release into the cytosol, the translocation of AIF and DFF40 (CAD) into the nucleus, and the activation of caspase-9, caspase-7, caspase-3, PARP and DFF45 (ICAD). In contrast, separate treatments of 40 μg/ml CGM or 300 μM eugenol for 24 hours did not induce apoptosis. Our present data thus suggest that a combination therapy of CGM and eugenol is a potential treatment strategy for human melanoma.

Key words: chios gum mastic, eugenol, apoptosis, human melanoma cells

Introduction

Cells undergoing apoptosis usually develop characteristic changes, including nuclear condensation and degradation of DNA into oligonucleosomal fragments [1]. Apoptotic cell death is thought to result ultimately from the proteolytic actions of caspase [2] and alterations in mitochondrial function play a key role in the regulation of apoptosis [3]. In addition, the proteasome system has been shown to be implicated as a negative or positive mediator of apoptosis. The proteasome pathway is known to work mostly in upstream of mitochondrial alterations and caspase activation [4].

Malignant melanoma is such one of the most important cutaneous malignancy that is responsible for the majority of mortality from skin disease. Numbers of patients suffering from malignant melanoma have increased in recent years. Furthermore, malignant melanoma cells have been reported to be highly resistant to chemotherapeutic agents [5].

The plant *Pistiacia lentiscus L var chia* grows particularly and almost exclusively in the southern region of Chios Island, Greece, and produces a resin, known as Chios gum

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mastic (CGM). It is obtained from the stem and leaves of *Pistacia lentiscus* trees and has been extensively used for centuries in mediterranean and middle eastern countries, both as a dietary supplement and herbal remedy [6,7].

Eugenol (4-allyl-2-methoxyphenol) is a natural phenolic substance that is the main component of clove oil and it is widely used not only in dentistry as a component of zinc oxide eugenol cement and is applied to the mouth environment, but also as a fragrant and flavoring agent in various cosmetics and food products [8]. Eugenol-related compounds not only act as antioxidant and also as prooxindant, which may be involved in the cytotoxicity induction. They enhanced the generation of tissue damaging free radicals [9-11] Recent studies have demonstrated that the co-treatment of an anti-tumor agent with an anti-cancer natural product can be one of the potential therapeutic strategies reducing the extent and severity of cancer treatment-related toxicity [12-16]. In spite that usefulness of cancer therapy combining already known anti-cancer natural products can be expected, there is few studies on evaluating the significance of a co-treatment CGM and eugenol on human melanoma cell line. Here, we have investigated the synergistic apoptotic effect of co-treatment with a natural herbal medicine, CGM, and one of natural phenolic compound, eugenol, on G361 human melanoma cell line.

Materials and Methods

Reagents

CGM was obtained from Mastic Korea (Seoul, Korea). The following reagents were obtained commercially: Mouse monoclonal anti-human caspase-3, caspase-7, caspase-9, poly (ADP-ribose) polymerase (PARP), cytochrome c, apoptosis-inducing factor (AIF) antibodies, and rabbit polyclonal anti-human DFF40 (CAD), DFF45 (ICAD), β-actin antibodies, and FITC-conjugated goat anti-mouse and anti-rabbit IgGs were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); HRP-conjugated sheep anti-mouse and anti-rabbit IgGs were from Amersham GE Healthcare (Little Chalfront, UK). 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazol carbocyanine iodide (JC-1) was from Molecular Probes (Eugene, OR, USA). ApoScreen™ Annexin V-FITC Apoptosis Kit was from Beckman coulter (Fullerton, CA, USA). Suc-LLVY-AMC was from Calbiochem (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM) and FBS were from Gibco (Gaithersburg, MD, USA). Dimethyl sulfoxide (DMSO), eugenol, Hoechst 33342, RNase A, proteinase K, aprotinin, leupeptin, PMSF, thiazolyl blue tetrazolium bromide and propidium iodide (PI) were from Sigma (St. Louis, MO, USA); SuperSignal West Pico enhanced chemiluminescence Western blotting detection reagent was from Pierce (Rockford, IL, USA).

Cell culture

The G361 human melanoma cell line was purchased from ATCC (Rockville, USA). Cells were maintained at 37℃ with 5% CO_2 in air atmosphere in RPMI 1640 with 4 mM L-glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 1.0 mM sodium pyruvate supplemented with 10% FBS.

Treatment of CGM and eugenol for co-treatment

Stock solutions of the CGM (100 mg/ml) and eugenol (10 mM) made by dissolve in DMSO and distilled water were kept frozen at -20°C until use. Twenty four hours after G361 cells were subcultured, the original medium was removed. The cells were washed with PBS and then incubated in the same fresh medium. Since single treatment of 40 μg/ml CGM and 300 μM eugenol for 24 h showed slight induction of cell death in MTT assay, this single concentration was utilized for further assessment of apoptosis for co-treatment. The concentration of DMSO used in this study had no effect on G361 cell proliferation in the preliminary studies.

MTT assay

G361 Cells were placed in a 96-well plate and were incubated for 24 h. The cells were treated with 40 μg/ml of CGM, 300 μM of eugenol or co-treated with CGM and eugenol for 24 h. Then cells were treated with 500 μg/ml of thiazolyl blue tetrazolium bromide (MTT solution), and incubated at 37°C with 5% CO_2 for 4 h. After aspirating the medium, formed formazan crystals were dissolved in DMSO. Cell viability was measured by an ELISA reader (Tecan, Männedorf, Switzerland) at 570 nm excitatory emission wavelength. The concentration of DMSO used in this study had no effect on G361 cell proliferation in a preliminary study.

Hoechst staining

Cells were centrifuged onto a clean, fat-free glass slide with a cytocentrifuge. The samples were stained in 4 μg/ml Hoechst 33342 for 30 min at 37℃ and fixed for 10 min with 4% paraformaldehyde.

DNA electrophoresis

2 x 10^6 cells were resuspended in 1.5 mL of lysis buffer [10 mM Tris (pH 7.5), 10 mM EDTA (pH 8.0), 10 mM NaCl and 0.5% SDS] into which proteinase K (200 μg/ml) was added. After samples were incubated overnight at 48℃, 200 μl of ice cold 5 M NaCl was added and the supernatant containing fragmented DNA was collected after centrifugation. The DNA was then precipitated overnight at -20℃ in 50% isopropanol and RNase A-treated for 1 h at 37℃. The DNA from 10^6 cells (15 μl) was equally loaded on each lane of 2% agarose gels in Tris-acetic acid/EDTA buffer containing 0.5 μg/ml ethidium bromide at 50 mA for 1.5 h.

Proteasome activity

Cells were collected and lysed in proteasome buffer [10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2 mM ATP, 20% glycerol, and 4 mM dithiothreitol (DTT)], sonicated, and then centrifuged at 13,000 g at 4℃ for 10 min. The supernatant (20 μg of protein) was incubated with proteasome activity buffer [0.05 M Tris-HCl, pH 8.0, 0.5 mM EDTA, 50 μM Suc-LLVY-AMC] for 1 h at 37℃. The fluorescence intensity of each solution was measured by a modular fluorimetric system (Spex Edison, NJ, USA) at 380 nm excitatory and 460 nm emission wavelengths. All readings were standardized based on the fluorescence intensity of an equal volume of free AMC solution (50 μM).

Western blot analysis

Cells (2 x 10^6) were washed twice in ice-cold PBS, resuspended in 200 μl ice-cold solubilizing buffer [300 mM NaCl, 50 mM Tris-Cl (pH 7.6), 0.5% Triton X-100, 2 mM PMSF, 2 μl/ml aprotinin and 2 μl/ml leupeptin] and incubated at 4℃ for 30 min. The lysates were centrifuged at 14,000 revolutions per min for 15 min at 4℃. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, Richmond, CA, USA) and 50 μg of proteins were loaded onto 7.5-15% SDS/PAGE. The gels were transferred to Nitrocellulose membrane (Amersham GE Healthcare, Little Chalfont, UK) and reacted with each antibody. Immunostaining with antibodies was performed using SuperSignal West Pico enhanced chemiluminescence substrate and detected with Alpha Imager HP (Alpha Innotech, Santa Clara, USA).

Immunofluorescent staining

Cells were cytocentrifuged and fixed for 10 min in 4% paraformaldehyde, incubated with each primary antibody for 1 h, washed 3 times for 5 min, and then incubated with FITC-conjugated secondary antibody for 1 h at room temperature. Cells were mounted with PBS. Fluorescent images were observed and analyzed under Zeiss LSM 510 laser-scanning confocal microscope (Göettingen, Germany).

Assay of mitochondrial membrane potential (MMP)

JC-1 was added directly to the cell culture medium (1 μM final concentration) and incubated for 15 min. The medium was then replaced with PBS. Flow cytometry to measure MMP was performed on a CYTOMICS FC500 flow cytometry (Beckman Coulter, Brea, CA, USA). Data were acquired and analyzed using CXP software version 2.2.

Quantification of DNA hypoploidy and cell cycle phase by flow cytometry

After treatment for 24 h, cells were harvested by trypsinization and ice cold 95% ethanol with 0.5% Tween 20 was added to the cell suspensions to a final concentration of 70% ethanol. Fixed cells were pelleted, and washed in 1% BSA-PBS solution. Cells were resuspended in 1 ml PBS containing 20 μg/ml RNase A, incubated at 4℃ for 30 min, washed once with BSA-PBS, and resuspended in PI solution (10 μg/ml). After cells were incubated at 4℃ for 5 min in the dark, DNA content were measured on a CYTOMICS FC500 flow cytometry system (Beckman Coulter, FL, CA, USA) and data was analyzed using the Multicycle software which allowed a simultaneous estimation of cell-cycle parameters and apoptosis.

Annexin V-FITC/PI assay

After treatment for 24 h, cells were collected and washed twice in ice-cold PBS and resuspended in 300 μl of binding buffer at 2×10^5 cells/ml. The samples were incubated with 5 μl of Annexin V-FITC and 5 μl propidium iodide in the dark for 15 min at room temperature. Finally, samples were analyzed by flow cytometry and evaluated based on the percentage of cells for Annexin V positive.

Statistical analysis

Three independent experiments were performed for each experimental group and each experiment was performed in triplicate. The results of the experimental and control groups were compared for statistical significance ($p<0.01$ and 0.05) using paired T-test statistical method by SPSS for Win 12.0 for summary data.

Results

Co-treatment with CGM and eugenol augmented the reduction in the viability of G361 cells.

To investigate whether the co-treatment with CGM and eugenol reduced the viability of G361 cells, MTT assay was conducted. Single treatment of CGM at 40 μg/ml or eugenol at 300 μM for 24 h reduced the viability of G361 cells slightly (CGM, 78.5%; eugenol, 86.7%). Co-treatment with CGM and eugenol significantly reduced the to 39.5% cell viability compared to the treatment with single treatment (Fig. 1).

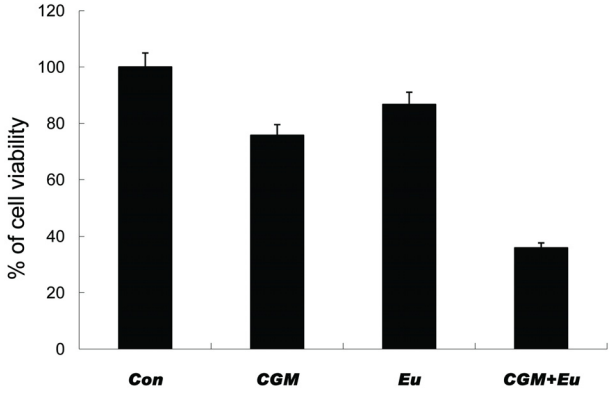

Fig. 1. Co-treatment with CGM and eugenol significantly reduced the cell viability in G361 cells. Cell viability was determined by MTT assay (CGM+Eu, $p<0.01$). Three independent assays were performed. Values are means ± SD of triplicates of each experiment (CGM, cells treated with 40 μg/ml CGM for 24 h; Eu, cells treated with 300 μM eugenol for 24 h; CGM+Eu, cells treated with 40 μg/ml CGM plus 300 μM eugenol for 24 h).

Co-treatment with CGM and eugenol augmented the nuclear condensation in G361 cells.

To explore whether the nuclear condensation and fragmentation a hallmark of apoptosis were induced by the co-treatment, Hoechst staining was conducted. Hoechst staining showed slight nuclear condensation in the single treatment with either CGM or eugenol. However, co-treatment with CGM and eugenol showed a variety of condensed and fragmented nuclei (Fig. 2).

Co-treatment with CGM and eugenol showed the DNA fragmentation in G361 cells.

To assess that DNA fragmentation was induced by the co-treatment, DNA electrophoresis was conducted. DNA electrophoresis did not show a ladder pattern of DNA fragments in the single treatment with either CGM or eugenol whereas a ladder pattern of DNA fragments was observed in cells with co-treatment. (Fig. 3).

Augmentation of apoptosis by the co-treatment with CGM and eugenol was demonstrated by proteasome activity in G361 cells.

Although single treatment with either CGM or eugenol reduced proteasome activity compared to the control group, the difference was not so significant. The co-treatment with CGM and eugenol remarkably reduced proteasome activity to the single treatment (Fig. 4).

Augmentation of apoptosis by the co-treatment with CGM and eugenol was demonstrated by reduction of mitochondrial membrane potential (MMP) in G361 cells.

Single treatment with either CGM or eugenol slightly reduced

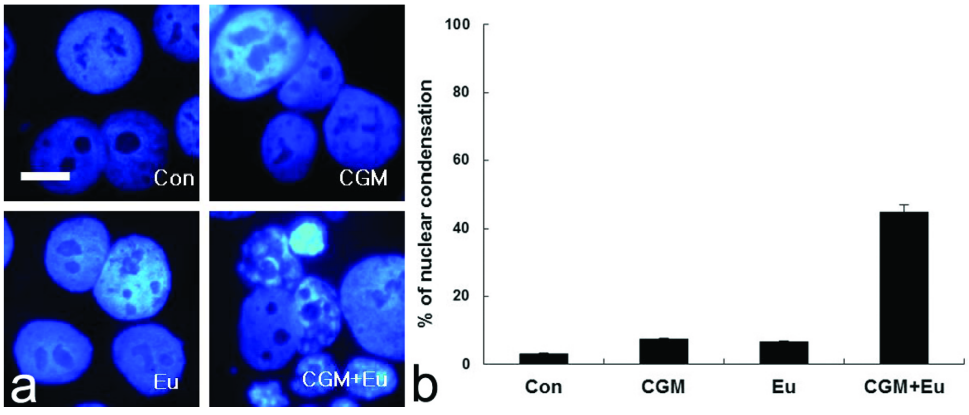

Fig. 2. Co-treatment with CGM and eugenol showed numerous condensed and fragmented nuclei in G361 cells compared to the single treatment. (A) Immunofluorescent micrographs showing nuclear morphology after Hoechst staining. Scale bar, 10 μm. (B) The values below micrographs are represented as the mean ± SD of the means of apoptotic cells as determined by Hoechst staining. The results presented are representative of three independent experiments (CGM+Eu, $p<0.01$).

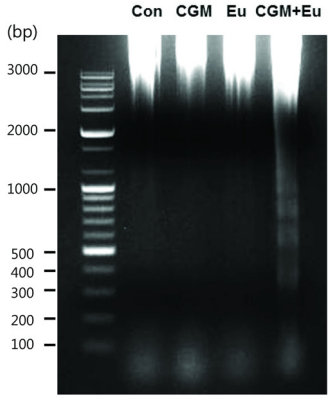

Fig. 3. Co-treatment with CGM and eugenol efficiently showed DNA fragmentation in G361 cells. DNA fragmentation analysis was determined by the agarose gel electrophoresis. Whereas single reagent treated cells showed no DNA fragmentation, co-treated cells exhibited DNA degradation characteristic of apoptosis with a ladder pattern of DNA fragments.

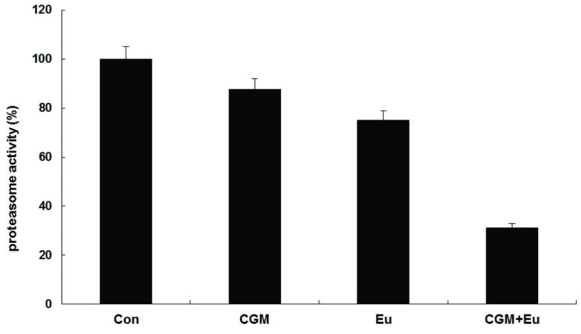

Fig. 4. Co-treatment with CGM and eugenol remarkably showed the reduction of proteasome activity in G361 cells compared to the single treatment (CGM+Eu, $p<0.01$). Three independent assays were performed. Values are means ± SD of triplicates of each experiment.

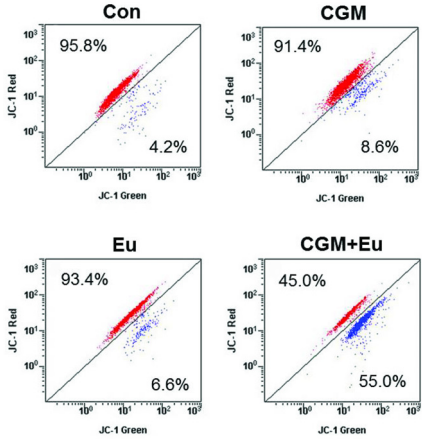

Fig. 5. Co-treatment with CGM and eugenol showed significantly the loss of MMP ($\triangle \Psi m$) compared to the single treatment (CGM+Eu, $p<0.01$). MMP was measured by JC-1 with flow cytometry.

the MMP compared to control group. The co-treatment with CGM and eugenol significantly showed the abolishment of MMP compared to the single treatment (Fig. 5).

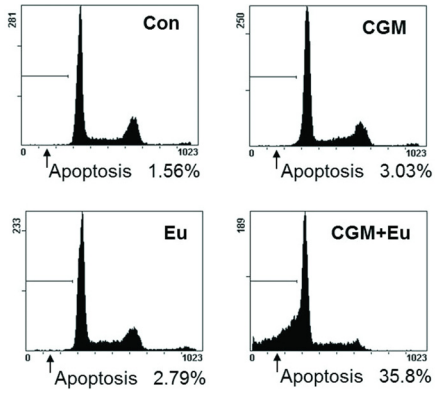

Fig. 6. The kinetic analysis of the effect of co-treatment on the G361 cell cycle progression and the induction of apoptosis. Co-treatment significantly showed the increase of apoptotic cells with DNA hypoploidy compared to the single treatment (CGM+Eu, $p<0.05$).

Table 1. Summary of the fractions of cells in each stage of apoptosis

	Normal	Early apoptosis	Late apoptosis	Others
Con	90.50	3.55	4.94	1.04
CGM	69.76	17.00	12.76	0.48
Eu	89.52	2.94	6.12	1.42
CGM + Eu	46.06	10.32	42.16	1.46

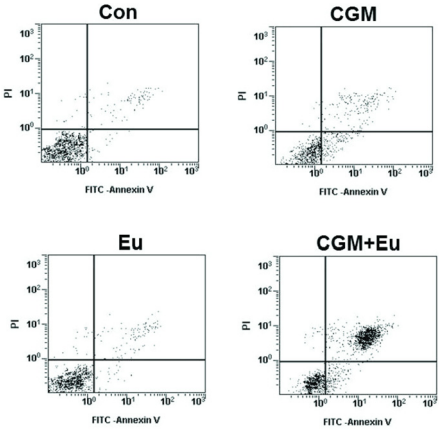

Fig. 7. Annexin V-FITC/PI assay. Cells were subjected to Annexin V-FITC/PI staining and analyzed by flow cytometry. Co-treatment showed remarkably the increase of apoptosis in late stage cells compared to the single treatment (Late stage; CGM+Eu, $p<0.01$).

Augmentation of apoptosis by the co-treatment with CGM and eugenol was demonstrated by the decrease in a DNA content of G361 cells.

The flow cytometry showed that co-treatment with CGM and eugenol significantly increased apoptotic cells with DNA hypoploidy compared to the single treatment (Fig. 6)

Augmentation of the late stage apoptosis by the co-treatment with CGM and eugenol was demonstrated by Annexin V-FITC/PI assay in G361 cells.

Annexin V-FITC/PI assay was performed to quantitate the apoptotic cells induced by the reagents. As shown in Fig. 7, the co-treatment with CGM and eugenol remarkably increased the percentage of the late stage apopotic cells compared to the single treatment (Table 1, Fig. 7).

Co-treatment with CGM and eugenol caused the translocation of AIF from mitochondria onto the nucleus.

The confocal microscopy showed that AIF was located at mitochondria by the single treatment with either CGM or eugenol whereas AIF was evidently translocated onto nuclei by the co-treatment (Fig. 8).

Co-treatment with CGM and eugenol caused the release of cytochrome c from mitochondria into the cytosol.

The confocal microscopy showed that cytochrome c was located at mitochondria by the single treatment with either CGM or eugenol whereas cytochrome c was evidently released into the cytosol by the co-treatment (Fig. 9).

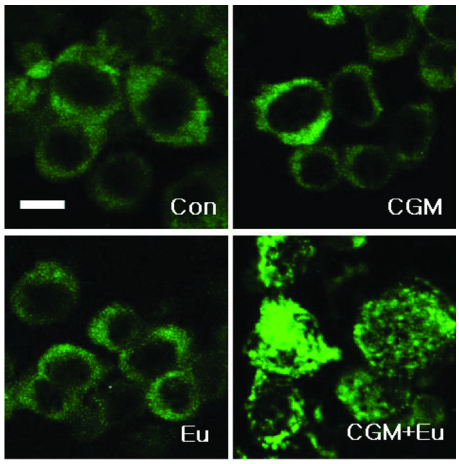

Fig. 9. The confocal microscopy showed that cytochrome c was evidently released to the cytosol in G361 cells when co-treated with CGM and eugenol. Scale bar, 10 μm.

Augmentation of apoptosis by co-treatment with CGM and eugenol was demonstrated by Western blot assay.

Through the co-treatment, the level of Bcl-2 anti-apoptotic protein a little decreased and the level of Bax pro-apoptotic protein significantly increased (Fig. 10a). The co-

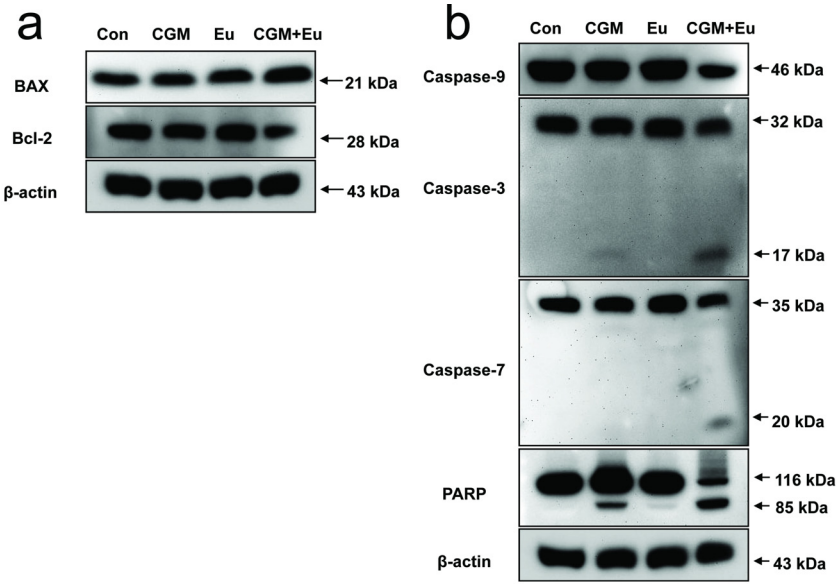

Fig 10. Western blot analysis showing that co-treatment with CGM and eugenol induced up-regulation of the Bax and down-regulation of the Bcl-2 (a). The levels of β-actin were used as an internal standard for quantifying Bcl-2 and Bax expression. The co-treatment with CGM and eugenol in G361 cells remarkably induced caspase-9 and PARP degradations and produced the processed caspase-3 17 kDa, caspase-7 20 kDa, and PARP 85 kDa cleaved products. β-actin, a loading control.

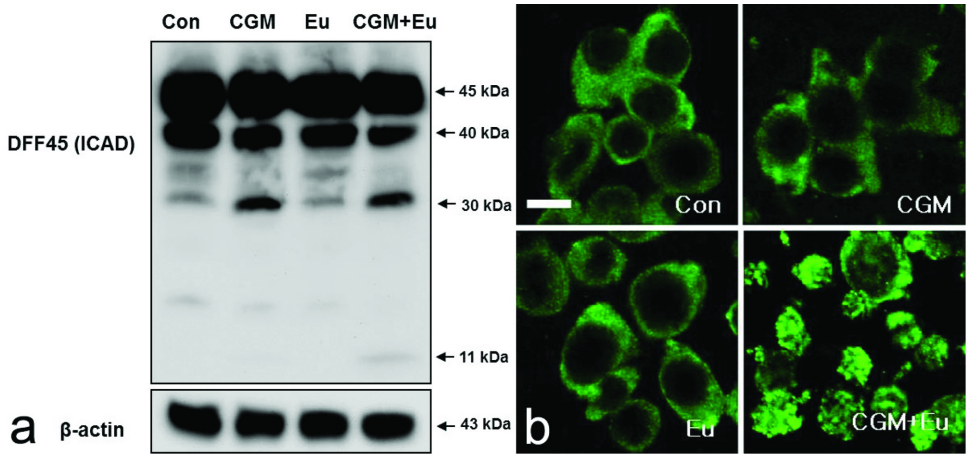

Fig. 11. Western blot analysis and confocal microscopy showing the efficient apoptotic effects in G361 cells co-treated with CGM and eugenol. (a) The co-treatment remarkably produced the processed DFF45 30 kDa and 11 kDa cleaved products. β-actin, a loading control. (b) The confocal microscopy showing that DFF40 (CAD) was evidently translocated onto the nuclei by the co-treatment of CGM and eugenol. Scale bar, 10 μm.

treatment with CGM and eugenol induced the degradation of caspase-9 and PARP, and produced the processed caspase-3 17 kDa, caspase-7 20 kDa, PARP 85 kDa, and DFF45 (ICAD) 30 kDa and 11 kDa cleaved products (Fig. 10b).

Co-treatment with CGM and eugenol caused the translocation of DFF40 (CAD) from cytosol onto the nuclei.

The co-treatment with CGM and eugenol induced DFF45 (ICAD) 30 kDa and 11 kDa cleaved products (Fig. 11a), and confocal microscopy showed that DFF40 (CAD) was located in cytosol by the single treatment with either CGM or eugenol whereas DFF40 (CAD) was clearly translocated into the nuclei by the co-treatment (Fig. 11b).

Discussion

Public attention on natural products as herbal remedies continues to grow. Moreover, as the pharmacological mechanism of herbal compound become known, herbal medicine is more and more [17] herbal medicines were extracted from herbal plants have a number of pharmacological activities, e.g. anti-allergic, anti-pyretic, analgesic, anti-inflammatory and anticancer effects [18-22]. Furthermore, recent studies have focused on inhibition of melanogenesis by herbal medicines [23-25]. In spite of numerous in vitro and in vivo studies, the mode of action of most herbal medicines remains elusive.

Eugenol is a naturally occurring phenolic compound used as a food flavor and fragrance agent and the main component of oil of clove and the essential oils or extracts of numerous plants [26,27]. It has been identified that eugenol inhibited immediate hypersensitivity by inhibiting the release of histamine and induced apoptosis from mast cells *in vivo* and *in vitro* [28,29]. CGM is a resinous exudate obtained from the stem and the main leaves of *Pistacia lenticulus* tree native to mediterranean areas. Previous studies have demonstrated that CGM is effective in the treatment of benign gastric and duodenal ulcers and has definite antibacterial activity against Helicobacter pylori [30-33]. It has also been shown that CGM has antimicrobial properties [34]. Recently it was reported that CGM induces cell cycle arrest and apoptosis in human prostate and colon cancer cells [6,7]. We also demonstrated that CGM induces G1 the cell cycle arrest via the modulation of cell cycle-related proteins, and also induces apoptosis via mitochondria and caspase pathway in oral squamous cell carcinoma cells [35]. Up to date, there were no reports about apoptotic effect of the co-treatment with eugenol and CGM on a variety of cancer cells. If the co-treatment with eugenol and CGM shows a synergistic anti-tumor effect, it could be a more fundamental therapeutic strategy for cancer chemotherapy.

Proteasome is a fundamental non-lysosomal tool that cells use to process or degrade a variety of short-living proteins. It was reported that proteolysis mediated by ubiquitin-proteasome system implicated in the regulation of apoptosis [36]. The proteasome pathway is mostly known to work at the upstream of the mitochondrial alterations and caspase activation [4]. In this study, the co-treatment with CGM and

eugenol on G361 melanoma cells causes the remarkable reduction of proteasome acitivity compared to the single treatment.

Mitochondria plays an important role in the induction of the mitochondrial permeability transition and also plays a key part in the regulation of apoptosis [3,37,38]. Outer mitochondrial membrane becomes permeable to intermembrane space proteins such as cytochrome c and AIF (apoptosis inducing factor) during apoptosis [39]. Release of cytochrome c and disruption of mitochondrial membrane potential (MMP) are known as characteristics in apoptosis triggered by proteasome inhibition [40,41]. When apoptosis was induced, AIF translocates to the nucleus, resulting in chromatin condensation and large-scale DNA fragmentation [42]. This study evidently showed that co-treatment with CGM and eugenol in G361 cells results in significant decrease of MMP, increase and decrease of Bax and Bcl-2, the release of cytochrome c into cytosol and translocation of AIF onto nuclei whereas the each single treatment does not show these patterns.

A common final event of apoptosis is the nuclear condensation, which is controlled by caspases, DFF, and PARP. Caspases, the cysteinyl aspartate-specific intracellular proteinase, play an essential role during apoptotic cell death [43]. Once activated, the effector caspases (caspase-3, caspase-6 or caspase-7) are responsible for the proteolytic cleavage of a broad spectrum of cellular targets, ultimately leading to cell death. The known cellular substrates include structural components (such as actin and nuclear lamin), inhibitors of deoxyribonuclease (such as DFF45 or ICAD) and DNA repair proteins (such as PARP) [44,45]. In apoptotic cells, activation of DFF40 (CAD), also a substrate of caspase-3, occurs with the cleavage of DFF45 (ICAD). Once DFF40 is activated and released from the complex of DFF45 and DFF40, it can translocate to the nucleus and then degrade chromosomal DNA and produce DNA fragmentation [46]. This study demonstrated that co-treatment with CGM and eugenol in G361 cells results in the degradation and the activation of caspase-3, caspase-7, PARP and DFF45 (ICAD), and also the translocation of DFF40 (CAD) onto nuclei whereas the each single treatment does not.

Interestingly, Annexin V-FITC/PI assay showed that the percentage of late stage apoptotic cells was remarkably increased in the co-treatment with CGM and eugenol. In the present study, co-treatment with CGM and eugenol clearly demonstrated that several lines of apoptotic manifestation were augmented in G361 cells. This result indicated that the low concentrations of CGM or eugenol single treatment dose not cause DNA damage, but the co-treatment with CGM and eugenol showed the synergistic apoptotic effect.

The results obtained in this study also suggest a combination therapy of CGM and eugenol is a potential treatment strategy for human melanoma.

References

1. Williams GT, Programmed cell death: apoptosis and oncogenesis. Cell, 1991;65:1097-8.
2. Yuan J, Evolutionary conservation of a genetic pathway of programmed cell death. J Cell Biochem, 1996;60:4-11.
3. Susin SA, HK Lorenzo, N Zamzami, I Marzo, BE Snow, GM Brothers, J Mangion, E Jacotot, P Costantini, M Loeffler, N Larochette, DR Goodlett, R Aebersold, DP Siderovski, JM Penninger, and G Kroemer, Molecular characterization of mitochondrial apoptosis-inducing factor. Nature, 1999;397:441-6.
4. Orlowski RZ, The role of the ubiquitin-proteasome pathway in apoptosis. Cell Death Differ, 1999;6:303-13.
5. Shibuya H, Y Kato, M Saito, T Isobe, R Tsuboi, M Koga, H Toyota, and J Mizuguchi, Induction of apoptosis and/or necrosis following exposure to antitumour agents in a melanoma cell line, probably through modulation of Bcl-2 family proteins. Melanoma Res, 2003;13:457-64.
6. Balan KV, J Prince, Z Han, K Dimas, M Cladaras, JH Wyche, NM Sitaras, and P Pantazis, Antiproliferative activity and induction of apoptosis in human colon cancer cells treated in vitro with constituents of a product derived from Pistacia lentiscus L. var. chia. Phytomedicine, 2007;14: 263-72.
7. He ML, A Li, CS Xu, SL Wang, MJ Zhang, H Gu, YQ Yang, and HH Tao, Mechanisms of antiprostate cancer by gum mastic: NF-kappaB signal as target. Acta Pharmacol Sin, 2007;28:446-52.
8. Markowitz K, M Moynihan, M Liu, and S Kim, Biologic properties of eugenol and zinc oxide-eugenol. A clinically oriented review. Oral Surg Oral Med Oral Pathol, 1992;73: 729-37.
9. Suzuki Y, K Sugiyama, and H Furuta, Eugenol-mediated superoxide generation and cytotoxicity in guinea pig neutrophils. Jpn J Pharmacol, 1985;39:381-6.
10. Wright SE, DA Baron, and JE Heffner, Intravenous eugenol causes hemorrhagic lung edema in rats: proposed oxidant mechanisms. J Lab Clin Med, 1995;125:257-64.
11. Satoh K, H Sakagami, I Yokoe, M Kochi, and S Fujisawa, Interaction between eugenol-related compounds and radicals. Anticancer Res, 1998;18:425-8.
12. Adhami VM, A Malik, N Zaman, S Sarfaraz, IA Siddiqui, DN Syed, F Afaq, FS Pasha, M Saleem, and H Mukhtar, Combined inhibitory effects of green tea polyphenols and selective cyclooxygenase-2 inhibitors on the growth of

human prostate cancer cells both in vitro and in vivo. Clin Cancer Res, 2007;13:1611-9.
13. Mai Z, GL Blackburn, and JR Zhou, Genistein sensitizes inhibitory effect of tamoxifen on the growth of estrogen receptor-positive and HER2-overexpressing human breast cancer cells. Mol Carcinog, 2007;46:534-42.
14. Song MQ, JS Zhu, JL Chen, L Wang, W Da, L Zhu, and WP Zhang, Synergistic effect of oxymatrine and angiogenesis inhibitor NM-3 on modulating apoptosis in human gastric cancer cells. World J Gastroenterol, 2007;13:1788-93.
15. Vinall RL, K Hwa, P Ghosh, CX Pan, PN Lara, Jr., and RW de Vere White, Combination treatment of prostate cancer cell lines with bioactive soy isoflavones and perifosine causes increased growth arrest and/or apoptosis. Clin Cancer Res, 2007;13:6204-16.
16. Lee SH, JK Ryu, KY Lee, SM Woo, JK Park, JW Yoo, YT Kim, and YB Yoon, Enhanced anti-tumor effect of combination therapy with gemcitabine and apigenin in pancreatic cancer. Cancer Lett, 2008;259:39-49.
17. Zhu J, L Miao, C Xu, Y Wang, and T Chen, [Analysis of 822 infants with limb reduction defect in China]. Hua Xi Yi Ke Da Xue Xue Bao, 1996;27:400-3.
18. Kim HM, EH Lee, SH Hong, HJ Song, MK Shin, SH Kim, and TY Shin, Effect of Syzygium aromaticum extract on immediate hypersensitivity in rats. J Ethnopharmacol, 1998;60:125-31.
19. Kim HM, JM Yi, and KS Lim, Magnoliae flos inhibits mast cell-dependent immediate-type allergic reactions. Pharmacol Res, 1999;39:107-11.
20. Park HI, MH Jeong, YJ Lim, BS Park, GC Kim, YM Lee, HM Kim, KS Yoo, and YH Yoo, Szygium aromaticum (L.) Merr. Et Perry (Myrtaceae) flower bud induces apoptosis of p815 mastocytoma cell line. Life Sci, 2001;69:553-66.
21. Na HJ, HJ Jeong, H Bae, YB Kim, ST Park, YG Yun, and HM Kim, Tongkyutang inhibits mast cell-dependent allergic reactions and inflammatory cytokines secretion. Clin Chim Acta, 2002;319:35-41.
22. Kim JM, HR Bae, BS Park, JM Lee, HB Ahn, JH Rho, KW Yoo, WC Park, SH Rho, HS Yoon, and YH Yoo, Early mitochondrial hyperpolarization and intracellular alkalinization in lactacystin-induced apoptosis of retinal pigment epithelial cells. J Pharmacol Exp Ther, 2003;305:474-81.
23. Im SJ, KN Kim, YG Yun, JC Lee, YJ Mun, JH Kim, and WH Woo, Effect of Radix Ginseng and Radix Trichosanthis on the melanogenesis. Biol Pharm Bull, 2003;26: 849-53.
24. Dattner AM, Herbal and complementary medicine in dermatology. Dermatol Clin, 2004;22:325-32, vii.
25. Lee KJ, JY Kim, JH Choi, HG Kim, YC Chung, SH Roh, and HG Jeong, Inhibition of tumor invasion and metastasis by aqueous extract of the radix of Platycodon grandiflorum. Food Chem Toxicol, 2006;44:1890-6.
26. IARC, Monographs on the Evaluation of Carcinogenic Risks to Humans. Allyl Compounds, Aldehydes, Epoxides and Peroxides 1985;36:75.
27. Zhu YP, Materia Medica: Cemistry, Pharmacology and Application, 1st edi., Amsterdam. Harwood Academic Publishers, 1996;1-3.
28. Kim HM, EH Lee, CY Kim, JG Chung, SH Kim, JP Lim, and TY Shin, Antianaphylactic properties of eugenol. Pharmacol Res, 1997;36:475-80.
29. Shin BK, EH Lee, and HM Kim, Suppression of L-histidine decarboxylase mRNA expression by methyleugenol. Biochem Biophys Res Commun, 1997;232:188-91.
30. Al-Habbal MJ, Z Al-Habbal, and FU Huwez, A double-blind controlled clinical trial of mastic and placebo in the treatment of duodenal ulcer. Clin Exp Pharmacol Physiol, 1984;11:541-4.
31. Al-Said MS, AM Ageel, NS Parmar, and M Tariq, Evaluation of mastic, a crude drug obtained from Pistacia lentiscus for gastric and duodenal anti-ulcer activity. J Ethnopharmacol, 1986;15:271-8.
32. Huwez FU and MJ Al-Habbal, Mastic in treatment of benign gastric ulcers. Gastroenterol Jpn, 1986;21:273-4.
33. Correction: Mastic Gum Kills Helicobacter pylori. N Engl J Med, 1999;340:576.
34. Aksoy A, N Duran, and F Koksal, In vitro and in vivo antimicrobial effects of mastic chewing gum against Streptococcus mutans and mutans streptococci. Arch Oral Biol, 2006;51:476-81.
35. Park JH, Kim GC, Kwak HH, Kim IR, Lee SE, Chung J, Park HR, Shin SH, Choi SH, Kim CH, Nam CO, Park BS, Chios Gum Mastic Induces Cell Cycle Arrest and Apoptosis in YD9 Human Oral Squamous Carcinoma Cells. Korean J Phys Anthropol 2008;21:55-68.
36. Drexler HC, W Risau, and MA Konerding, Inhibition of proteasome function induces programmed cell death in proliferating endothelial cells. FASEB J, 2000;14:65-77.
37. Kroemer G, N Zamzami, and SA Susin, Mitochondrial control of apoptosis. Immunol Today, 1997;18:44-51.
38. Green DR and JC Reed, Mitochondria and apoptosis. Science, 1998;281:1309-12.
39. Golab J, T Stoklosa, A Czajka, A Dabrowska, M Jakobisiak, R Zagozdzon, C Wojcik, M Marczak, and S Wilk, Synergistic antitumor effects of a selective proteasome inhibitor and TNF in mice. Anticancer Res, 2000;20:1717-21.
40. Wagenknecht B, M Hermisson, P Groscurth, P Liston, PH Krammer, and M Weller, Proteasome inhibitor-induced apoptosis of glioma cells involves the processing of multiple caspases and cytochrome c release. J Neurochem, 2000;75:2288-97.
41. Marshansky V, X Wang, R Bertrand, H Luo, W Duguid, G Chinnadurai, N Kanaan, MD Vu, and J Wu, Proteasomes modulate balance among proapoptotic and antiapoptotic Bcl-2 family members and compromise functioning of the electron transport chain in leukemic cells. J Immunol, 2001;166:3130-42.
42. Daugas E, SA Susin, N Zamzami, KF Ferri, T Irinopoulou, N Larochette, MC Prevost, B Leber, D Andrews, J Penninger, and G Kroemer, Mitochondrio-nuclear translocation of AIF in apoptosis and necrosis. FASEB J, 2000; 14:729-39.
43. Acehan D, X Jiang, DG Morgan, JE Heuser, X Wang, and CW Akey, Three-dimensional structure of the apoptosome: implications for assembly, procaspase-9 binding, and activation. Mol Cell, 2002;9:423-32.

44. Gross A, JM McDonnell, and SJ Korsmeyer, BCL-2 family members and the mitochondria in apoptosis. Genes Dev, 1999;13:1899-911.
45. Porter AG, Protein translocation in apoptosis. Trends Cell Biol, 1999;9:394-401.
46. Cheng AC, CB Jian, YT Huang, CS Lai, PC Hsu, and MH Pan, Induction of apoptosis by Uncaria tomentosa through reactive oxygen species production, cytochrome c release, and caspases activation in human leukemia cells. Food Chem Toxicol, 2007;45:2206-18.