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Mechanism underlying Chios gum mastic-induced cell cycle arrest and apoptosis of the G361 human melanoma cell line

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Chios gum mastic (CGM) is a resin extracted from the stem and leaves of *Pistacia lentiscus L. var. chia*. It has been used as a traditional medicine in many Mediterranean countries. Recently, numerous researches have demonstrated that CGM induces cell cycle arrest and apoptosis in many cancer cells. In the present study, an alteration of the cell cycle and induction of apoptosis by CGM treatment on malignant melanoma was investigated.

CGM treatment showed the inhibition of cell viability in a dose- and time-dependent manner on the G631 melanoma cell line. Apoptotic hallmarks, such as nuclear condensation and DNA fragmentation, were also identified in CGM-treated cells. Several lines of apoptotic manifestation were demonstrated. The proapoptotic factor Bax was increased in a time-dependent manner, leading to MMP loss, proteasome activity reduction, AIF translocation, and cytochrome c release. Activation of caspases, such as caspase-9, -7, and -3, led to the cleavage of PARP and DFF45 (ICAD). DFF40 (CAD) was translocated into the nucleus from the cytoplasm of the CGM-treated G361 cells. CGM halted the cell cycle progression by G1 arrest and the reduction in the level of cyclin-dependent kinases (CDKs). Accumulation of p53 protein was not observed in cells treated with CGM for 24–72 h. The level of $p27^{KIP1}$ was increased by CGM treatment only for an initial 24-h period. Cyclin D1, D3, and E protein levels were diminished in CGM-treated cells. Therefore, it is possible that CGM treatment could serve as a novel therapeutic strategy against human melanoma.

Keywords: Chios gum mastic, Cell cycle arrest, Apoptosis, Melanoma

Introduction

Chios gum mastic (CGM) is produced from Pistacia lentiscus L var chia, which grows only on Chios Island in Greece. CGM, a kind of resin extracted from the stem and leaves, has been used for many centuries as a dietary supplement and folk medicine for stomach and duodenal ulcers in many Mediterranean countries. Recently, He and co-workers demonstrated that CGM inhibited the prolifera-

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tion of androgen-independent prostate cancer. apparently through modulation of the NF-κB target gene [1]. In addition, CGM has also been shown to contain compounds that can induce apoptosis of human colon cancer cells through caspase-dependent pathways *in vitro* [2].

Malignant melanoma is the most serious cutaneous malignancy because it has aggressive metastatic potential. Numerous studies have demonstrated that the primary therapeutic approach for melanoma is often surgical excision, which is almost 100% effective on treating an early tumor. In contrast, once the melanoma has spread, particularly to lymph nodes, lung, and brain, only about 45% of these patients survive for 5 years. On the other hand, the 5-year survival rate for patients with distant metastases falls to approximately 15% [3, 4]. Malignant melanoma is an aggressive malignancy often resistant to standard chemotherapy and immunotherapy. Even though various chemotherapeutic agents, including dacarbazine (DTIC), cisplatin and temozolomide (TMZ), have been used for the treatment of melanoma, they have all failed to significantly improve the average survival rate of 6-10 months [5-9]. Moreover, the use of these conventional chemotherapy agents is associated with adverse side-effects such as myelosuppression, nausea and emesis. And their efficacy is limited by intrinsic or acquired drug resistance. The poor response rate to chemotherapy of (less than 15%), as well as the observation that the lifetime risk of melanoma is rapidly increasing, emphasizes the need for more effective therapies[10].

Apoptosis is an essential physiological process required for embryonic development, regulation of immune responses and maintenance of tissue homeostasis. However, apoptosis is also implicated in a wide range of pathological conditions, including immunological diseases, allergy and cancer [11, 12]. The induction of apoptosis leads to specific morphological and biochemical changes, including cell blebbing, exposure of cell surface phosphatidylserine, cell size reduction including cell shrinkage, chromatin condensation and internucleosomal cleavage of genomic DNA[13, 14]. These contrasts with necrotic cells, which are characterized by an overall increase in cellular size, with little initial change in the chromatin or nuclear morphology, accompanied by electron-lucent cytoplasm, mitochondrial swelling, shrinking of the inner mitochondrial membrane and loss of plasma membrane integrity[13]. Also, cell death occurs in the cells arrested in a postmitotic phase [15]. G1 arrest may be related to transcription factor as p53 tumor suppressor protein which regulates expression of several components implicated in pathways for cell cycle progression and apoptosis induction[16, 17].

Recently, it has been reported that some natural medicinal products extracted from herbal plants may act by significantly enhancing apoptosis induction and mitosis reduction in a variety of cancer cells [18-24]. A number of studies have been pursued on the targeted induction of apoptosis to control the unlimited cell growth and proliferation. In addition, induction of apoptosis in the activated cancer cells may be an effective strategic approach for cancer therapy.

The effect of CGM on melanoma suggests that regulation of the cell cycle via through cyclin/Cdk mechanisms and apoptotic mechanisms may be operating. This research therefore deals with the modulation of cell cycle and apoptosis induction in G361 human melanoma cell line treated with CGM.

Materials and Methods

Reagents

Chios gum mastic resin was obtained from Mastic Korea (Seoul, Korea). The following reagents were obtained commercially: TUNEL reaction mixture was from Boehringer Mannheim (Mannheim, Germany); Suc-LLVY-AMC were from Calbiochem (EMD Biosciences, Germany); 5,5',6,6'-tetrachloro-1,1',3,3-tetraethyl- benzimidazol carbocyanine iodide (JC-1) was from Molecular Probes (Eugene, OR, USA); RPMI 1640 and fetal bovine serum (FBS) were from Gibco (Gaithersburg, MD, USA); Dimethyl sulfoxide (DMSO), Hoechst 33342, RNase A, proteinase K, aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), thiazolyl blue tetrazolium bromide, crystal violet, collagenase 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).

Antibodies

Mouse monoclonal anti-human p53 was from BD biosciences (San Diego, CA, USA); rabbit polyclonal anti-human AIF, cytochrome c and caspase-9 antibodies were from Upstate (NY, USA); mouse monoclonal anti-human $p27^{KIP1}$, caspase-3, caspase-7, Bax, Bcl-2, Bcl-xL, DFF45 (ICAD), cyclin D1, Cdk2, Cdk4, poly(ADP-ribose) polymerase (PARP) antibodies, and rabbit polyclonal anti-human β-actin antibody, and FITC-conjugated goat anti-mouse and anti-rabbit IgGs were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Mouse monoclonal anti-human cyclin D3 was from Cell Signaling (Danver, MA, USA); rabbit polyclonal anti-human DFF40 (CAD) antibody was from Stressgen (San Diego, USA); HRP-conjugated sheep anti-mouse and anti-rabbit IgGs were from Amershan GE Healthcare (Little Chalfont, UK);

Cell line and cell culture condition

G361 Human melanoma cell line was purchased from the ATCC (Rockville, MD, USA). G361 cells were cultured in RPMI 1640 medium 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% fetal bovine serum (FBS). The cell cultures were maintained and propagated in a humidified 37°C incubator with a 5% CO_2 in air atmosphere.

In vitro treatment of CGM

Cells were cultured on culture dishes and/or several type of wells for 24 h. The original medium was removed and that washed with phosphate-buffered saline (PBS). It was changed that the fresh medium on the plates. CGM (100 mg/ml) stock solution was added to the medium to obtain 5, 10, 25, 50, 100 μg/ml concentrations of the drug. CGM was dissolved in DMSO and it was kept frozen at -20°C until use. The concentrations of DMSO, 0.002-0.1% (vol/vol) used in this study, both as a vehicle for CGM and as a control, had no effect on G361 cells proliferation in my preliminary studies.

Cell viability test

The viability of the cultured cells was estimated using the MTT method. The cells were cultured in a 96-well plate and incubated for 24 h. The cells treated with various concentrations and time points of

CGM. And then cells were treated with 500 μg/ml of MTT stock solution. After the cells were incubated at 37°C with 5% CO_2 for 4 h. The medium was aspirated and formed formazan crystals were dissolved in the mixture solution of DMSO and absolute ethanol (1 : 1). Cell viability was monitored on a ELISA reader (Sunrise Remote Control, Tecan, Austria) at 570 nm excitatory emission wavelength. Since viability assays demonstrated evident induction of G361 cell death at 50 μg/ml CGM for 48 h, this concentration was utilized for further assessment of apoptosis and alternation of the cell cycle.

Colony-forming assay

Cells were seeded at 2.5×10^2 per well (6-well culture plate) and incubated overnight. The cells were treated with CGM from 0 to 25 μg/ml. After treatment, cells were washed and allowed to grow for 7 days. The colonies were then fixed 100% methanol and stained with a filtrated solution of 0.5% (w/v) crystal violet for 10 min. And after washing cells with tap water, they were dried at room temperature. The colonies, defined as groups of ≥ 50 cells, were scored manually and photographed under an IMT-2 inverted microscope (Olympus, Japan). Standard value was determined to value of control in 100 percentage. And then, the value of CGM treated cells were compared with control. Three independent experiments were conducted.

Hoechst staining

Hoechst staining method was employed for the identification of apoptosis nuclei. After cells were treated with CGM, they were harvested and cytocentrifuged onto a clean fat-free glass slide with a cytocentrifuge. Cells were stained in 4 μg/ml Hoechst 33342 solution for 10 min at 37°C in the dark and washed twice in PBS. The slides were mounted with glycerol. The samples were observed and photographed under an epifluorescence microscope (Carl Zeiss, German). The number of cells that showed condensed or fragmented nuclei was determined by a blinded observer from a random sampling of 3×10^2 cells per slide. Three independent experiments were done.

TUNEL technique

To identify apoptotic cells by terminal deoxynucleotidyl transferase (TDT) - mediated dUTP nick and labelling (TUNEL), an In Situ Cell Death Detection Kit, was used as recommended by the manufacturer. Cells were harvested after treatment of CGM on 60 mm dishes The cell suspension was centrifuged onto a clean fat-free glass slide with a cytocentrifuge. After fixing with 4% paraformaldehyde for 1 h, the slides were washed with PBS and permeabilized with 0.1% TritonX-100 solution for 2 min on ice. Cells were added with reaction mixture for 1 h at 37°C. Total cell number, at least 300 cells from each group, was counted under DIC optics and the percentage of TUNEL positive cells were calculated and photographed under epifluorescence microscope (Carl Zeiss, German).

DNA fragmentation assay

Cells were seeded at the density of 2 x 10^6cells /100 mm culture dishes. The cells were sub-cultured for 24 h and incubated for 24 to 72 h after being

treated with CGM (50 μg/ml), After treatment, both the attached and unattached cells were harvested. The cells were washed in PBS, and lysed with a 0.5 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 55°C, 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°C in 50% isopropanol and RNase A-treated for 1 h at 37°C. 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 100 mA for 0.5 h.

Proteasome activity

After cells were treated with 50 μg/ml CGM for various time points, they were 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°C 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 37°C. The fluorescenic intensity of each solution was measured by a modular fluorimetric system (Spex Edison, USA) at 380 nm excitatory and 460 nm emission wavelengths. All readings were standardized using the fluorescenic intensity of an equal volume of free AMC solution (50 μM).

Western blot analysis

Cells were plated at a density of 2 x 10^6 cells in 100 mm culture dishes. CGM treated cells were washed twice with ice-cold PBS and centrifuged at 2,000 rpm for 10 min. Total protein of cell was lysed with a RIPA buffer [300 mM NaCl, 50 mM Tris-HCl (pH 7.6), 0.5% TritonX-100, 2 mM PMSF, 2 μl/ml aprotinin and 2 μl/ml leupeptin] and incubated at 4°C for 1 h. The lysates were centrifuged at 14,000 rpm for 15 min at 4°C, and sodium dodecyl sulfate (SDS) and sodium deoxycholic acid (0.2% final concentration) were added. Protein concentrations of cell lysates were determined with Bradford protein assay (Bio-Rad, USA) and BSA was used as a protein standard. A sample of 25 μg protein in each well was separated and it were loaded onto 7.5-15% SDS/PAGE. The gels were transferred to Nitrocellulose membrane (Amersham Pharmacia Biotech, 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, San Leandro, USA). Equivalent protein loading was confirmed by Ponceau S staining.

Assay of mitochondrial membrane potential (MMP)

Cells were plated in a standard 6-well plate at a density of 5 × 10^5/ml. CGM treated cells were incubated for various time points. The cells were harvested and then JC-1 was added directly (1 μM final concentration) and incubated for 15 min at 37°C in the dark. Flow cytometry to measure MMP(△Ψm) was performed on a CYTOMICS FC500 flow cytometry system (Beckman Coulter, USA). The fluorescence emission were collected through a 530/30 band pass

filter (FL-1) and through a 585/42 band pass filter (FL-2), both on a log scale. Data were acquired and analyzed using CXP software version 2.2. The analyzer threshold was adjusted on the FSC channel to exclude noise and most of the subcellular debris.

Immunofluorescent staining

Cells were placed on slides by cytocentrifuge and fixed for 10 min in 4% paraformaldehyde. After blocking nonspecific binding with 3% bovine serum albumin, the cells were incubated with a primary antibody at a dilution of 1:100 for 1 h. After the incubation, the cells were washed 3 times for 5 min, and then incubated with FITC-conjugated secondary antibody at a dilution of 1 : 100 for 1 h at room temperature. Fluorescent images were observed and analyzed under Zeiss LSM 510 laser-scanning confocal microscope (Göettingen, Germany).

Flow cytometry analysis

Cells were seeded into a 6-well plate at 1×10^6 cells/ml and incubated overnight. CGM treated cells were incubated for various time points. In each time, The harvested cells were washed with PBS containing 1% bovine serum albumn and centrifuged at 2,000 rpm for 10 min. The cells were resuspended ice-cold 95% ethanol with 0.5% Tween 20 to a final concentration of 70% ethanol. Fixed cells were pelleted, and washed in 1% BSA-PBS solution. They were resuspended in 1 ml PBS containing 20 μg/ml RNase A, incubated at 4°C for 30 min, washed once with BSA-PBS, and resuspended in PI solution (10 μg/ml). After cells were incubated at 4°C for 5 min in the dark, DNA content were measured on a CYTOMICS FC500 flow cytometry system (Beckman Coulter, USA). The data were analyzed using the Multicycle software which allowed a simultaneous estimation of cell-cycle parameters and apoptosis. The percentage of sub-G1 in each population was resolved from at least 1×10^4 cells.

Statistical Analysis

Three independent experiments were performed triplicately. The results of treated and control groups were compared for statistical significance ($p<0.001$, 0.01 and 0.05) using paired T-test statistical method by SPSS for Win 12.0 for summary data.

Results

Effect on the viability and proliferation in G361 melanoma cells

The cytotoxic effect of CGM was performed to measure the viability of G361 cells by MTT assay. After CGM treatment on G361 cells (0 to 100 μg/ml) at 24 h, the cell viability was reduced the concentrations of 50 (64.8%) to 100 μg/ml (38.2%) of CGM (Fig. 1). Also, after treatment of 50 μg/ml CGM to the cells, the decreasing viability was undertaken at 24, 48 and 72 h. At 50 μg/ml CGM, cell viability in a time-dependent manner (24 h, 64.8%; 48 h, 55.1%; 72 h, 19.1%) was contineuasly (Fig. 2). Hence, the half maximal inhibitory concentration (IC_{50}) of CGM was at the 50 μg/ml for 48 h. This concentration was utilized for further assessment of apoptosis and alternation of the cell cycle.

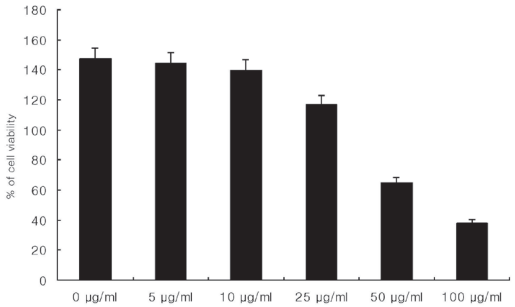

Fig. 1. Reduction of cell viability on G361 cells were treated with CGM, and measured by MTT assay. G361 cells were treated with CGM(0~100 μg/ml) for 24 h. The viability of G361 cells were decreased significantly does-dependent treatment with CGM (50~100 μg/ml, $p<0.05$).

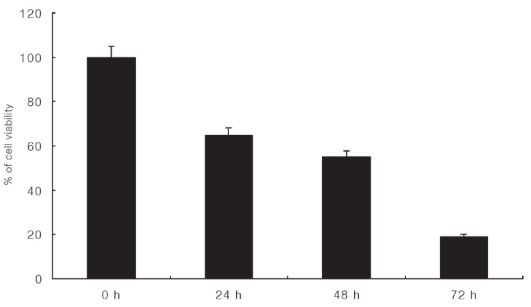

Fig. 2. Reduction of cell viability on G361 cells were treated with CGM in a time-dependent manner, and measured by MTT assay. After treatment with 50 μg/ml CGM, viability was decreased from at 24 to at 72 h continuously (24 h, $p<0.05$; 72 h, $p<0.01$).

After exposure to low level CGM concentrations (0 to 25 μg/ml) on G361 cells for 7 days, the inhibition of colony formation was shown in Fig. 3. The growth of CGM treated group was determined by percentage of control. The values of colony formation were 78.0% in 5 μg/ml CGM treated cells, 39.8% (10 μg/ml CGM treated cells), 12.4% (15 μg/ml CGM treated cells), 2.7% (20 μg/ml CGM treated cells), 1.1% (25 μg/ml CGM treated cells). It means that the growth of G361 cells are remarkably inhibited from 10 μg/ml CGM until high level concentration of CGM continuously.

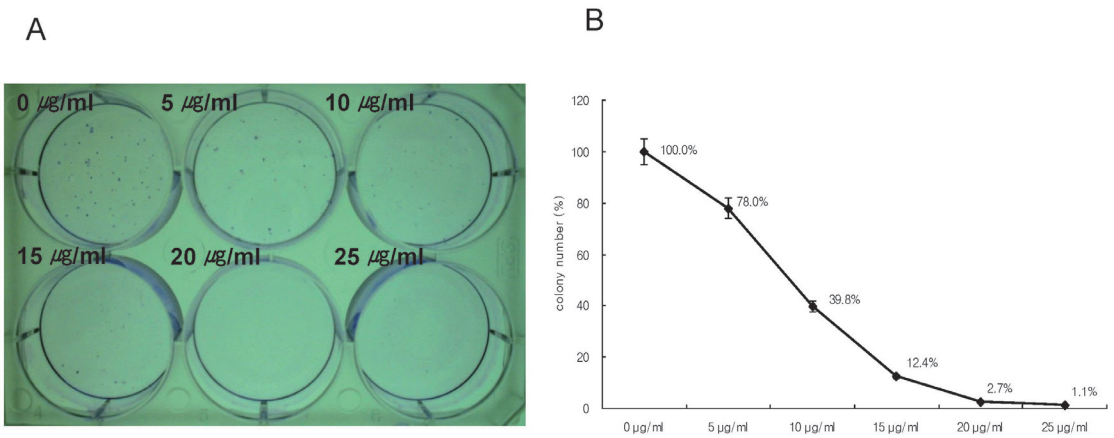

Fig. 3. Cell proliferation was examined by colony forming assay. CGM treatment was performed from 0 to 25 μg/ml on G361 cells. (A) Cells were allowed to grow for 7 days before staining with Crystal violet. Colonies were decreased gradually according to medium-concentration as above photographs. (B) Percentage of colony formation efficiency in different concentrations were displayed. And the growth of CGM treated groups was more decreased than control group (5~10 μg/ml, $p<0.01$; 15 μg/ml, $p<0.001$; 20 μg/ml, $p<0.05$). Values are means ± SD of triplicates of each experiment.

Morphological and biochemical changes in G361

To test whether CGM can change the nuclear morphology of G361 cells by Hoechst staining method. Non-treated control cells was round shaped nuclei, on the other side, 50 μg/ml CGM for 48 h treated group investigated that the nuclei of G361 were observed shrink and condense in shape (Fig. 4A). The conformation value in shape was detected more higher (about 59.0%) than control group (2.0%) (Fig. 4B). The cell death induced by CGM was examined DNA fragmentation which is the biochemical hallmark of apoptosis, and it was demonstrated by DNA electrophoresis and TUNEL technique.

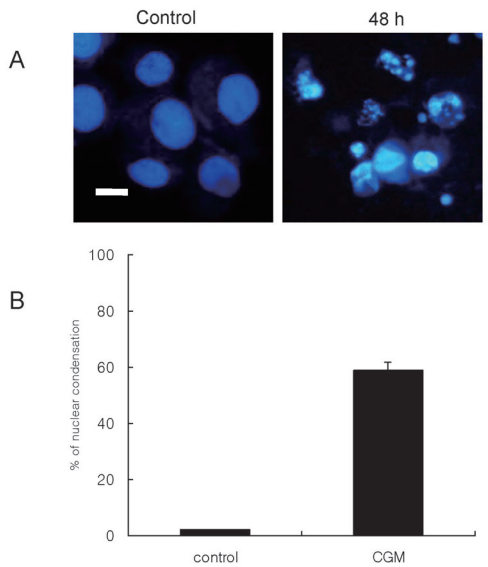

Fig. 4. Apoptotic condensed nuclei were investigated by Hoechst staining in G361 cells treatment with CGM. (A) Control cells showing round-shape nuclei (left panel). Treated cells with 50 μg/ml CGM for 48 h show the production of nuclear condensation or fragmentation (right panel). (B) The values of apoptotic nuclear condensation (N=300 cells) were designated as graph. The each result was obtained by three times experiments. $p<0.01$ (compared with control group).

In the result of DNA electrophoresis, whereas cells treated with 50 μg/ml CGM for 24 h had no evidence of DNA fragmentation. In the cells were treated with the same concentration of CGM for 48 h and 72 h, characteristic DNA degradation of apoptosis was clearly observed with a ladder pattern of DNA fragments (Fig. 5). To detect oligonucleosomal DNA fragmentation, TUNEL technique was also employed. The TUNEL positive cells in the control cells did not show but the numerous TUNEL positive cells treated with 50 μg/ml CGM for 48 h were shown (Fig. 6).

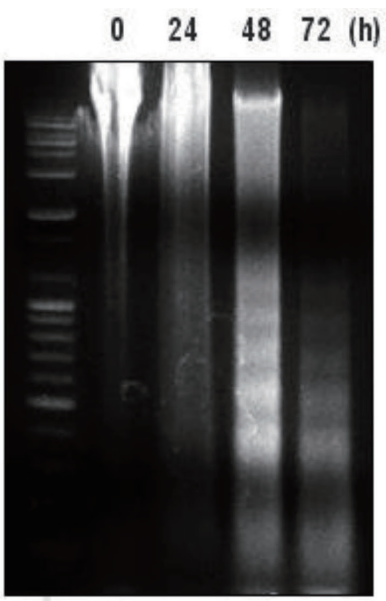

Fig. 5. DNA fragmentation was demonstrated by DNA electrophoresis. DNA fragmentation was not detected in treatment with 50 μg/ml CGM for 24h, but for 48 and 72 h groups, they were obtained DNA degradation which had characteristic of apoptosis with a ladder pattern of DNA fragments.

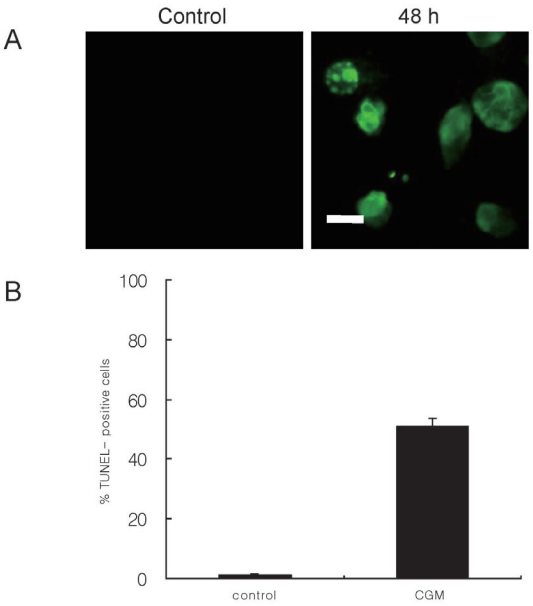

Fig. 6. Examination of apoptosis by TUNEL assay. G361 cells were treated with 50 μg/ml CGM for 48 h. (A) TUNEL positive cells in control group were not shown (left panel). Numerous TUNEL positive cells in experimental group were shown (right panel). Scale bar, 10 μm. (B) The values below micrographs are the mean ± SD of the means of TUNEL positive cells as determined by TUNEL method. The each result was obtained by three times experiments. $p<0.01$ (compared with control group).

Inhibition of proteasome activity

In order to inhibited effect of proteasome activity at 50 μg/ml CGM, proteasome activity assay was employed. in this assay, CGM remarkably abolished proteasome activity in a time-dependent manner (Fig. 7).

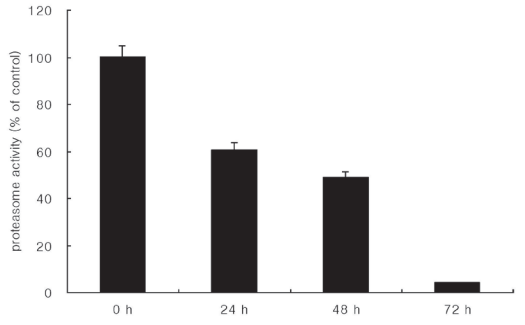

Fig. 7. The proteasome activity which was occurred in the cytoplasmic extract of un-treated or treated with CGM (50 μg/ml) on G361 cells was measured by fluoro-count. Proteasome activity was remarkably according to decreased a time-dependent manner (24 h, $p<0.01$; 72 h, $p<0.01$).

Effect on mitochondrial events and caspase activation

Induction of apoptosis is regulated by Bcl-2 family members. Bcl-2 has a function of antiapoptosis, whereas BAX promotes apoptosis. And also, proapoptotic Bcl-2 family such as Bax , Bad and Bid induces loss of mitochondrial membrane potential (△Ψm) and released cytochrome c. This results in induced AIF releasing. To examine the role of Bcl-2 family proteins in CGM-induced apoptosis, it was performed by western blot assay. The expression of Bax was up-regulated in a time-dependent manner but the expression of Bcl-2 was not occured down-regulation (Fig. 8). The mitochondria were stained with JC-1 dye, and the mitochondria membrane potential (△Ψm) was measured by flow cytometry. G361 cells treated for 0, 24, 48 and 72 h each with 50 μg/ml CGM that induced the loss of mitochondrial membrane potential (△Ψm) in a time-dependent manner

(Fig. 9). Western blot assay and confocal microscopy were used to examine whether having released function or not, such as fuction of AIF and cytochrome in mitochondrial intermembrane protein. The expression of AIF slightly increased over a period of 48 h after treatment of the CGM (Fig. 10A). And AIF was translocated to from mitochondria to nuclei in G361 cells (Fig. 10B). Also, cytochrome c was released from mitochondria into the cytosol after treatment of the CGM (Fig. 11). These investigation suggests

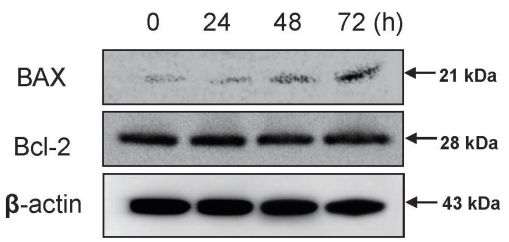

Fig. 8. Bax and Bcl-2 in G361 cells treatment CGM 50 μg/ml were detected by western blot analysis. Pro-apoptotic factor, Bax was significantly up-regulated in a time-dependent manner, but anti-apoptotic factor, Bcl-2 was not changed in level. The levels of β-actin were used as an internal standard for quantifying Bax and Bcl-2 expression.

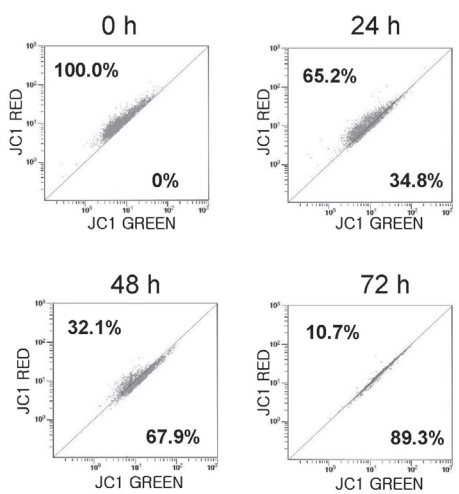

Fig. 9. Reduction of MMP (△Ψm) by CGM treatment. MMP (△Ψm) was reduced after treatment with the 50 μg/ml CGM in G361 cells by time dependently (24~72 h, $p<0.01$). MMP (△Ψm) was measured by flow cytometry. Each experiment were performed as three independent assays.

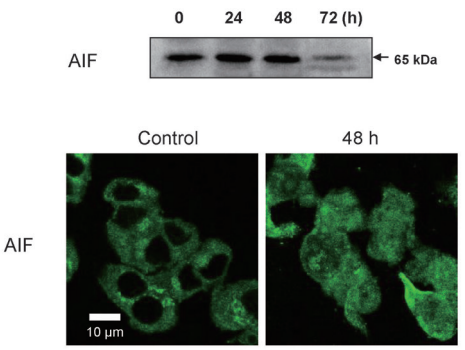

Fig. 10. AIF by western blot analysis and confocal micrographs. (A) Western blot analysis showed that the expression level of AIF was increased compared to the control over a period of 48 h. (B) AIF was released from mitochondria into the cytosol in G361 cells treated with CGM at 50 μg/ml. Scale bar, 10 μm.

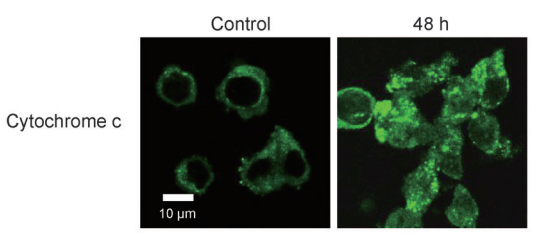

Fig. 11. Cytochrome c was released from mitochondria and then, it was into the cytosol of G361 cells treated with CGM 50 μg/ml. Scale bar, 10 μm.

that CGM-induced apoptosis was accompanied by modulation of proapoptotic Bcl-2 family protein, Bax, resulting in loss of MMP, AIF translocation and cytochrome c release. The mitochondrial damage causes the release of cytochrome c, thereby initiating the activation of caspase machinery that is executed during apoptosis. Therefore, as shown by the western blot analysis, CGM treatment at 50 μg/ml gave the inducing of active form of caspase-9, caspase-3 and caspase-7, and the cleavages of PARP and DFF45 (ICAD) in tim-dependent fashion (Fig. 12 and Fig. 13A). Also, as shown by data in Fig. 13B, CGM treated cells were found to result in that the DFF40 (CAD) was translocated into nuclei.

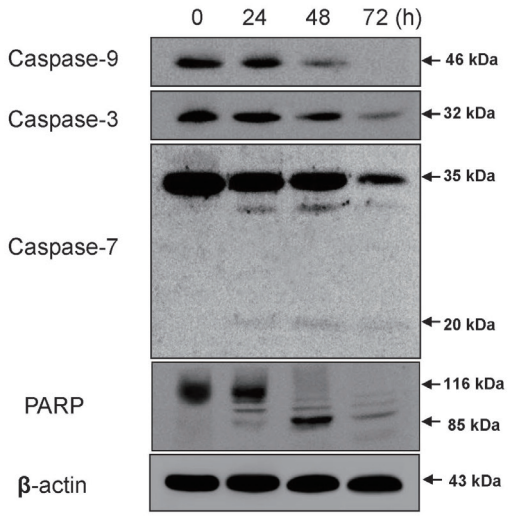

Fig. 12. Apoptotic enzymes were detected such as caspase-9, caspase-3, caspase-7, and PARP in CGM treated cells. CGM leaded to degrade caspase-9, caspase-3 and PARP. But caspase-9, caspase-3 and PARP were produced cleaved products (48 h and 72 h). The levels of β-actin were used as an internal standard for quantifying caspase-9, caspase-3, caspase-7, and PARP expression.

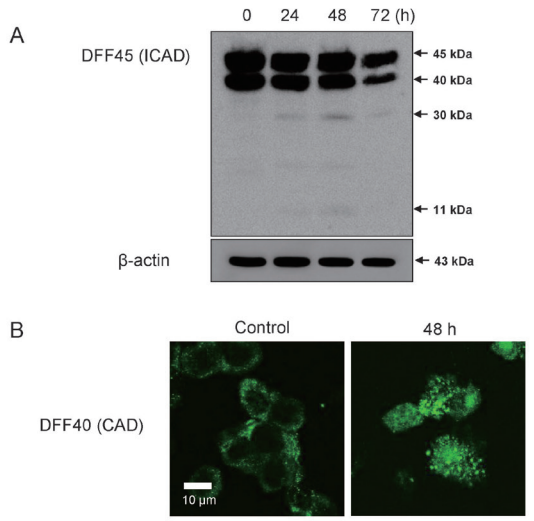

Fig. 13. Alteration of DFF45 (ICAD) and DFF40 (CAD) in CGM treated cells. (A) Western blot analysis showed the degradation of DFF45 (ICAD) after treatment of CGM (50 μg/ml). And DFF45 (ICAD) induced cleaved products, mainly 30 kDa or small 11 kDa. (B) DFF40 (CAD) was translocated from cytosol into nuclei. The levels of β-actin were used as an internal standard for quantifying DFF45 (ICAD) expression.

Effect of CGM on cell cycle

The evaluation of cell cycle and apoptotic percentages were confirmed with flow cytometry analysis. G_0/G_1 phase of cell cycle was increased in percentage 50 μg/ml CGM-treatment onto cells from for 24 h to 72 h. In contrary, the percentage of either G2/M or S phase were decreased (Fig. 14A). The apoptosis was observed increasing peak according to increasing of G_0/G_1 phase in cell cycle arrest. In S phase cells, although apoptosis was increased, but A phase was decreased in percentage (Fig. 14B). From the G_1 to S phase of the cell cycle, Cdk4 and Cdk2 are bound with cyclin D and cyclin E, respectively. In G361 cells, accumulation of p53 protein was not

observed following treatment with at 50 μg/ml CGM from for 24 h to 72 h. Especially, the protein level of $p27^{KIP1}$ was increased at 24 h. CGM treatment led to diminish cyclin D1, cyclin D3 and cyclin E protein expression in time dependently. The protein levels of Cdk4 and Cdk2 were also decreased (Fig. 15).

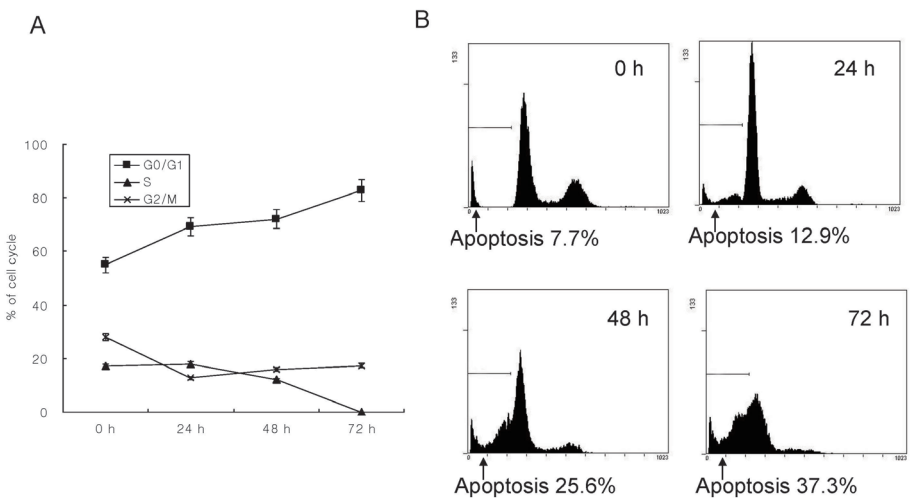

Fig. 14. CGM-induced cells were confirmed cell cycle arrest and apoptosis using flow cytometry. (A) G_0/G_1 were increased in percentages. S and G_2/M were decreased after treatment with the 50 μg/ml CGM in G361 cells by time dependently. (B) The percentage of apoptotic cells were also increased after treatment with CGM by time dependently (48 h, $p<0.01$; 72 h, $p<0.001$). Data were shown representative of three independent experiments.

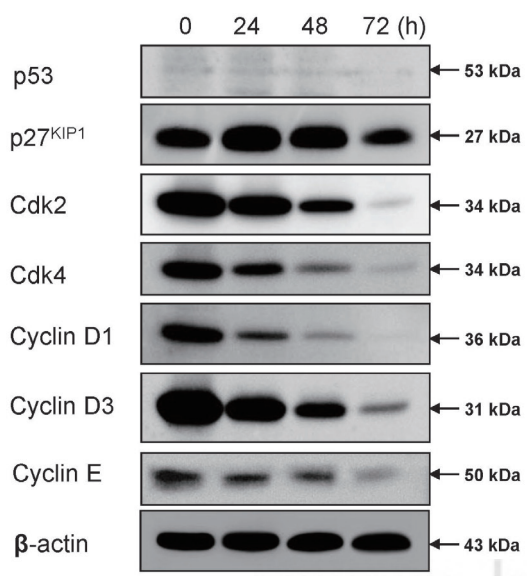

Fig. 15. Western blot analysis of total protein lysate showing effect of CGM on cell cycle-related proteins in the G1 transition. The levels of Cdk2, Cdk4, Cyclin D1, Cyclin D3 and Cyclin E was down-regulated in time dependently. The level of the KIP1 family of Cdk inhibitors, $p27^{KIP1}$, was up-regulated at 48 h. But p53 expression was not detected. The levels of β-actin were used as an internal standard for quantifying p53, $p27^{KIP1}$, Cdk2, Cdk4, Cyclin D1, Cyclin D3, and Cyclin E expression.

Discussion

Public attention on natural products as herbal remedies continues to grow. Moreover, the revelation of the pharmacological mechanisms of herbal plant compounds is contributing to their acceptance by healthcare professionals and the public. A number of studies has elucidated that individual 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 [25-29]. Furthermore, recent studies have focused on inhibition of melanogenesis by herbal medicines [30-32]. In spite of numerous *in vitro* and *in vivo* studies, the mode of action of most herbal medicines remains elusive.

CGM is an herbal extract derived from *Pistacia lenticulus var. chia* that has been traditionally used as a medical agent for hepatic inflammation, and for disorders of the stomach and intestine. It is also regarded as an anti-cancer agent for tumors of breast, liver stomach, spleen and uterus [33]. These traditional beliefs are in line with recent studies that report antimicrobial, antioxidant and hepatoprotective properties of CGM, and also that CGM induces cell cycle arrest and apoptosis in prostate [2] and colon cancer cells [1] by as yet unknown mechanisms. Thus, the present work investigating the effects of CGM on cell viability and cell cycle in G361 human melanoma cells showed that CGM produced a dose- and time-dependent reduction in viability of G361 cells, as assessed by MTT assay. Moreover, the colony forming assay confirmed that CGM at 5 to 25 μg/ml remarkably inhibited the growth of G361 cells. Surprisingly, CGM did not reduce the cell viability and growth at the same concentrations and treatment times on HaCaT human keratinocyte and HGF-1 human gingival fibroblast (data not shown). These data indicate that CGM exerts a specific cytotoxic effect on human melanoma cells but not on normal cells and CGM has the possibility of acting as an anti-cancer therapeutic for human melanoma.

Apoptosis and necrosis are conceptually distinct forms of cell death and can be distinguished by their specific morphological changes. During apoptosis, cells were shown the cell blebbing, reduction of cell size, cell shrinkage, chromatin condensation and DNA fragmentation [13, 14]. In this study, G361 cell treated with CGM induced apoptotic hallmarks such as internucleosomal DNA fragmentation, the formation of apoptotic bodies and the increase of TUNEL-positive cells. These results indicate that CGM inhibited the growth of G361 cells by activating the apoptosis.

Mitochondrial plays an important role in the induction of the mitochondrial permeability transition and also plays a key part in the regulation of apoptosis [34-36]. Outer mitochondrial membrane becomes permeable to intermembrane space proteins such as cytochrome c and AIF (apoptosis inducing factor) during apoptosis [37]. Release of cytochrome c and disruption of mitochondrial membrane potential (MMP) are known as features in apoptosis triggered by proteasome inhibition [38]. On induction of apoptosis, AIF translocates to the nucleus, resulting in chromatin condensation and large-scale DNA fragmentation [39]. This study evidently showed that treatment of CGM in G361 cells results in remarkable 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 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 death [40]. 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) [41, 42]. 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 [43].

Various studies on the molecular analyses of cancers have revealed that cell cycle regulators are frequently mutated in most common malignance. Therefore, control of cell cycle progression in tumor cells is considered to be a potentially effective strategy for the control of tumor growth. In the case of Cdks, cyclins and Cdk inhibitors, these play critical roles in the regulation of cell cycle progression [44]. Cdk inhibitors inhibit the active Cdk-cyclin complex, which is a target of p53 [45]. The importance of p53 in maintaining the genomic stability is well established and it is known that p53 has critical role for G1/S checkpoint arrest pathway in response to DNA damage. $p27^{KIP1}$,a Cdk inhibitor,has been demonstrated to play an important role in regulating progression from the G1/Sphase, by binding to and preventing premature activation of Cdk4/cyclinD and Cdk2/cyclinE complexes[46, 47]. However, other studies have found that, when abnormalities are found in Cdks, cyclins and Cdk inhibitors, including those induced by the overexpression of any cyclin or Cdk, the system of protein degradation of cyclins and Cdk, mediated by the ubiquitin-proteasome pathway, can also induce apoptosis. Cdk inhibitors therefore function not only as suppressors, but also as activators of Cdk, depending on expression levels in several kinds of malignant tumors [48, 49]. The present work demonstrated that CGM strongly inhibited G361 cells growth by G1 cell cycle arrest and apoptosis. A correlation between G1 cell cycle arrest and the induction of apoptosis could be assumed, based on the fact that the increased percentage of G1 phase and the concomitant fall in the percentage of S phase cells paralleled an increase in the proportion of apoptotic cells. CGM induced the arrest of the G1 phase in G361 cells in this study. Thus, it can be postulated that the arresting mechanism may be associated with down-regulation of overexpressed cyclinD1, cyclinD3, cyclinE, Cdk2 and Cdk4 mediated by the ubiquitin-proteasome pathway. The protein level of $p27^{KIP1}$was up-regulated and p53 was not detected.

Therefore, these findings suggest that $p27^{KIP1}$ may play a key role as a suppressor, further investigation is needed and that accumulation of p53 is a cause of, not response to, DNA damage, in CGM-induced G1phase arrest.

In conclusion, the results of the present work demonstrated that CGM strongly inhibits cell proliferation via the cell cycle-related proteins including cyclins, Cdks and Cdk inhibitors. In addition, CGM induces apoptosis via mitochondrial and caspase

pathways in G361 cells. Thus, CGM treatment could be a novel therapeutic strategy for human melanoma.

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한글초록

사람흑색종세포에서 Chios Gum Mastic에 의해 유도된 세포주기정지와 세포자멸사의 기전에 대한 연구

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Chios gum mastic (CGM)은 그리이스 키오스 섬에서만 자생하는 *Pistacia lentiscus L. var. Chia.* 의 잎과 줄기로부터 얻어지는 식물성 수지 이며, 과거 수세기 동안 지중해와 중동 지역 국가들에서 음식 첨가물과 위궤양, 십이지장궤양 등의 민간 치료약재로서 사용되어져 왔다. 최근 CGM이 암세포에서 세포주기 정지와 세포자멸사 (apoptosis) 를 유도한다는 몇 가지 연구가 발표되었으나, 정확한 기전은 밝혀지지 않았다. 본 연구는 항암치료에 높은 내성을 가지는 사람흑색종에서 CGM이 세포주기의 변형과 세포자멸사에 대한 분자생물학적 기전을 밝히기 위해 실험을 시행하였다.

CGM으로 처리된 G361 세포는 MTT와 colony formation assay를 통해 농도와 시간의존적으로 세포의 성장이 억제되었고, 생존율 역시 감소 함을 확인 하였다. 세포자멸사의 분자생물학적 특징인 핵의 농축과 DNA 분절 등은 hoechst 염색법, DNA 전기영동법 그리고 TUNEL 염색법을 통해 확인되었다. 공초점레이저주사현미경 검경 과 western blot 법등을 시행하여 CGM이 G361 세포에서 apoptosis의 기전 중 proteasome pathway를 통해 사립체의 변화와 caspase의 활성유도를 확인 하였다. CGM 처리 후 시간 의존적으로 proteasome 활성도는 현저히 감소하고 proapoptotic factor인 Bax는 증가하였다. Bax의 증가로 인해 사립체막전위는 감소하고 apoptosis-inducing factor 인 AIF의 핵으로의 위치이동과 사립체 내막에 존재하는 단백질인 cytochrome c의 세포질로의 유리 등이 관찰되었다. caspase의 경우 caspase-9, caspase-3 그리고 caspase-7 등이 활성화 되었고, 이들의 활성화로 인해 PARP와 DFF45 (ICAD)의 분절, 그리고 DFF40 (CAD)가 핵으로 위치이동과 같은 다양한 세포자멸사의 증거를 보였다. Flow cytometry 분석에서는 G1 세포주기정지를 확인 하였고, G1주기와 연관된 단백질인 cyclin D1, cyclin D3, cyclin E, Cdk2 그리고 Cdk4의 발현이 감소를 보였고, Cdk inhibitor인 $p27^{KIP1}$ 은 증가하였다. 반면에 p53 단백질의 축적은 보이지 않았다.

본 연구는 G361 세포에서 CGM이 세포주기 관련 단백질들의 변형에 의한 G1 세포주기 정지와 proteasome, 사립체 그리고 caspase 경로를 통해 세포자멸사를 유도하므로 천연물질인 CGM이 새로운 항암치료재로서 가능성을 보여 준다고 생각한다.

주제어: Chios gum mastic, 세포주기정지, 세포자멸사, 흑색종