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Mechanism Underlying Curcumin-induced Apoptosis and Cell Cycle Arrest on SCC25 Human Tongue Squamous Cell Carcinoma Cell Line

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Several studies have shown that curcumin, which is derived from the rhizomes of turmeric, possesses antimicrobial, antioxidant and anti-inflammatory properties. The antitumor properties of curcumin have also now been demonstrated more recently in different cancers. This study was undertaken to investigate the modulation of cell cycle-related proteins and the mechanisms underlying apoptosis induction by curcumin in the SCC25 human tongue squamous cell carcinoma cell line. Curcumin treatment of the SCC25 cells resulted in a time- and dose-dependent reduction in cell viability and cell growth, and onset of apoptotic cell death. The curcumin-treated SCC25 cells showed several types of apoptotic manifestations, such as nuclear condensation, DNA fragmentation, reduced MMP and proteasome activity, and a decreased DNA content. In addition, the treated SCC25 cells showed a release of cytochrome c into the cytosol, translocation of AIF and DFF40/CAD into the nuclei, a significant shift in the Bax/Bcl-2 ratio, and the activation of caspase-9, caspase-7, caspase-6, caspase-3, PARP, lamin A/C, and DFF45/ICAD. Furthermore, curcumin exposure resulted in a downregulation of G1 cell cycle-related proteins and upregulation of $p27^{KIP1}$. Taken together, our findings demonstrate that curcumin strongly inhibits cell proliferation by modulating the expression of G1 cell cycle-related proteins and inducing apoptosis via proteasomal, mitochondrial, and caspase cascades in SCC25 cells.

Key words: curcumin, apoptosis, cell cycle arrest, oral squamous cell carcinoma

Introduction

Curcumin, a yellow pigment derived from the rhizomes of turmeric (*Curcuma longa*), is used as an aromatic and coloring in food, as well as having a significant role in Asian medicine. It has been shown to play critical roles in cellular proliferation, apoptosis, migration, and metastasis [1-3]. Several studies have demonstrated that curcumin possesses antimicrobial and antioxidant activities and anti-inflammatory properties. More recently, antitumor properties of curcumin were reported in many cancers [4-6]. Several mechanisms by which curcumin exerts its anticancer effect have been reported [7-9].

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, allergies, and cancer [10, 11]. The induction of apoptosis leads to specific morphological

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and biochemical changes, such as cell blebbing, exposure of cell surface phosphatidylserine, and cell size reduction, including cell shrinkage, chromatin condensation, and internucleosomal cleavage of genomic DNA [12,13].

Oral squamous cell carcinoma (OSCC) is the most common type of oral cancer. It is responsible for nearly 500,000 cancer-related deaths annually worldwide [14]. OSCC is a major malignancy, which remains incurable with current therapies [15]. OSCC patients are treated with classical modalities of treatment consisting of surgery, radiotherapy, and/or chemotherapy. As OSCC still results in high mortality rates [16-18], new therapeutic approaches are being investigated [19,20]. The use of natural agents has been suggested as one of the most promising approaches in anticancer treatments [21-23].

Although a few studies have explored the apoptosis-inducing efficacy of curcumin on cancer cells *in vitro* [24-26], there are no reports on the apoptotic effect of curcumin on a human tongue squamous cell carcinoma cell line. The present study was conducted to examine the cytotoxicity and cell growth inhibition, in addition to the molecular mechanism underlying alterations in the expression of cell cycle-related proteins and apoptosis induction, of an SCC25 human tongue squamous carcinoma cell line treated with curcumin *in vitro*.

Materials and Methods

Reagents

The following reagents were obtained commercially: curcumin, Dimethyl sulfoxide (DMSO), Hoechst 33342, RNase A, proteinase K, aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), thiazolyl blue tetrazolium bromide, crystal violet and propidium iodide (PI) were from Sigma (St. Louis, MO, USA); TUNEL reaction mixture was from Roche (Mannheim, Germany); Suc-LLVY-AMC was from Calbiochem (Darmstadt, Germany); 5,5',6,6'-tetrachloro-1,1',3,3-tetraethyl-benzimidazolcarbocyanine iodide (JC-1) was from Molecular Probes (Eugene, OR, USA); Dulbecco's Modified Eagle Medium : Nutrient Mixture F-12 (1 : 1) (DMEM/F12) and fetal bovine serum (FBS) were from Gibco (Gaithersburg, MD, USA); SuperSignal West Pico enhanced chemiluminescence Western blotting detection reagent was from Pierce (Rockford, IL, USA).

Antibodies

Rabbit polyclonal anti-human AIF antibody was from Upstate (NY, USA); mouse monoclonal anti-human caspase-9, caspase-7, caspase-6, caspase-3, Bax, Bcl-2, cytochrome c, Lamin A/C, DFF45 (ICAD), $p27^{KIP1}$, Cyclin D1, Cyclin D3, Cdk2, Cdk4, poly (ADP-ribose) polymerase (PARP) antibodies, and mouse monoclonal anti-human GAPDH antibody, and FITC-conjugated goat anti-mouse and anti-rabbit IgGs were from Santa Cruz Biotechnology (Santa Cruz, CA, USA); Rabbit polyclonal anti-human DFF40 (CAD) antibody was from Stressgen (Ann Arbor, MI, USA); HRP-conjugated sheep anti-mouse and anti-rabbit IgGs were from Amersham GE Healthcare (Little Chalfont, UK).

Cell culture and treatment of curcumin

SCC25 human tongue squamous cell carcinoma cell line was purchased from the ATCC (Rockville, MD, USA). The cells were maintained at 37℃ with 5% CO_2 in air atmosphere in DMEM/F12 medium with 4 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose and 1.0 mM sodiumpyruvate supplemented with 10% fetal bovine serum (FBS). Cells were cultured on culture dishes and/or several types of wells for 24 h. The original medium was removed and then changed that the fresh medium on the plates. Curcumin (100 mM) stock solution was added to the medium to obtain 5 ~ 50 μM concentrations of the drug.

MTT assay

The cells were cultured in a 96-well plate and incubated for 24 h. The cells treated with various concentrations and time points of curcumin. And then cells were treated with 500 μg/ml of MTT stock solution. After the cells were incubated at 37℃ 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 (Tecan, Männedorf, Switzerland) at 570 nm excitatory emission wavelength. Since viability assays demonstrated evident induction of SCC25 cell death at 25 μM curcumin for 24 h, this concentration was utilized for further assessment of apoptosis induced by curcumin.

Clonogenic (Colony-forming) assay

Cells were seeded at 2.5×10^2 per well (6-well culture plate) and incubated overnight. The cells were next treated

with curcumin 0, 0.1, 0.5, 1, 5 and 10 μM and allowed to grow (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. The wells were then washed with tap water and dried at room temperature. The colonies, defined as groups of ≥ 50 cells, were scored manually and photographed under an IMT-2 inverted microscope (Olympus, Tokyo, Japan). Clonogenic survival was expressed as the percentage of colonies formed in curcumin-treated cells with respect to control cells. Three independent experiments were conducted.

Hoechst staining

After curcumin treatment, cells were harvested and cytocentrifuged onto a clean, fat-free glass slide with a cytocentrifuge. Cells were stained in 4 μg/ml Hoechst 33342 for 10 min at 37℃ 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, Göettingen, Germany). 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 experiment. Three independent experiments were conducted.

DNA electrophoresis

2×10^6 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 assay

1×10^6 cells 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℃ for 10 min. The supernatant (20 μg of protein) were incubated with proteasome activity buffer [0.05 M Tris-HCl, pH 8.0, 0.5 mM EDTA and 50 μM Suc-LLVY-AMC] for 1 h 37℃. The intensity of fluorescence 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 using the fluorescence intensity of an equal volume of free AMC solution (50 μM).

Western blot analysis

Cells were plated at a density of 2×10^6 cells in 100 mm culture dishes. Cells treated with curcumin were washed twice with ice-cold PBS and centrifuged at 2,000 rpm for 10 min. Total cell proteins were lysed with a RIPA buffer [300 mM NaCl, 50 mM Tris-HCl (pH 7.6), 0.5% TritonX-100, 2 mM PMSF, 2 μg/ml aprotinin and 2 μg/ml leupeptin] and incubated at 4°C for 1 h. The lysates were centrifuged at 14,000 revolutions per min 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, Richmond, CA, USA) and BSA was used as a protein standard. A sample of 50 μg protein in each well was separated and it was 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 Femto enhanced chemiluminescence substrate and detected with Alpha Imager HP (Alpha Innotech, Santa Clara, USA). Equivalent protein loading was confirmed by Ponceau S staining.

Measurement of mitochondrial membrane potential (MMP)

JC-1 was added directly to the cell culture medium (1 μM final concentration) and incubated for 15 min. Flow cytometry to measure MMP was performed on a CYTOMICS FC500 flow cytometry system (Beckman Coulter, Brea, CA, USA). 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

SCC25 cells were plated on coverslips for 1 days and then used for stimulation or staining with Mitotracker red (50 nM). After washing two times with PBS, the cells were fixed with PFA 4% in PBS for 15 min and then washed three times with PBST. After permeabilization with

Triton X-100 and blocking with 10% goat serum in PBS, cells were incubated with primary antibodies (in 3% BSA) overnight at 4°C. After washing with PBS, cells were incubated with FITC-conjugated secondary antibodies in 3% BSA-PBS for 60 min and rinsed in PBS. Fluorescent images were observed and analyzed under Zeiss LSM 750 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. Cells treated with curcumin were incubated for various time points. In each time point, the harvested cells were washed with PBS containing 1% bovine serum albumin 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. 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.

Results

Effects of curcumin on the viability and proliferation of the SCC25 cells

The viability of the SCC25 cells after curcumin treatment was measured in an MTT assay to determine its cytotoxic effect. After curcumin treatment of the SCC25 cells (0 to 50 μM) for 24 h, the cell viability was reduced at concentrations of 5 μM (96.6%) to 50 μM (15.2%) of curcumin (Fig. 1A). After treatment with 25 μM of curcumin, the cell viability decreased in a time-dependent manner (12 h, 91.5%; 24 h, 52.1%; 48 h, 26.1%) (Fig. 1B). Hence, the half-maximal inhibitory concentration (IC_{50}) of curcumin was 25 μM for 24 h. This concentration was utilized to assess apoptosis and alternation of the expression of cell cycle-related proteins.

To investigate whether curcumin inhibited the growth of

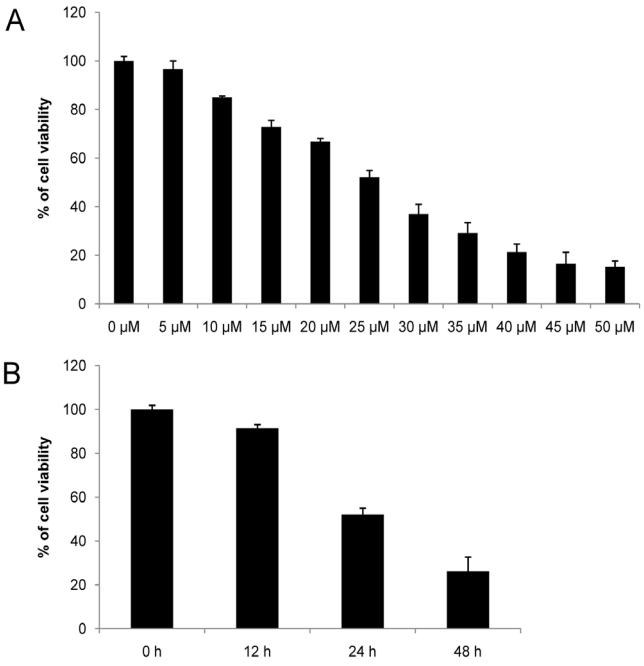

Fig. 1. Effect of curcumintreatment on viability of SCC25. (A) Cells were treated with curcumin (0～50 μM) for 24 h. SCC25 cells show the remarkable reduction of viability in a dose-dependent manner. (B) After treatment with 25 μM curcumin, viability was undertaken over a period of 48 h. Curcumin remarkably reduced viability of SCC25 cells in a time-dependent manner.

SCC25 cells, a clonogenic assay was performed. After exposure of the SCC25 cells to curcumin concentrations (0 to 10 μM) for 7 days, colony formation was inhibited, as shown in Figure 2. The growth of cells in the curcumin-treated group was compared with that of a control. The values for colony formation were 94.6% (0.1 μM curcumin treated cells), 70.3% (0.5 μM curcumin treated cells), 35.6% (1 μM curcumin treated cells), 9.1% (5 μM curcumin treated cells), and 1.1 % (10 μM curcumin treated cells).

Morphological and biochemical changes in the curcumin-treated SCC25 cells

The SCC25 cells treated with 25 μM of curcumin showed morphological and biochemical changes associated with apoptosis. A Hoechst stain demonstrated that curcumin induced a change in nuclear morphology. Compared with the typical round nuclei of the control cells, the SCC25 cells treated with 25 μM of curcumin for 24 h displayed condensed and fragmented nuclei (Fig. 3A & 3B). DNA fragmentation, which is a biochemical hallmark of apoptosis, was demonstrated by DNA electrophoresis. The SCC25 cells

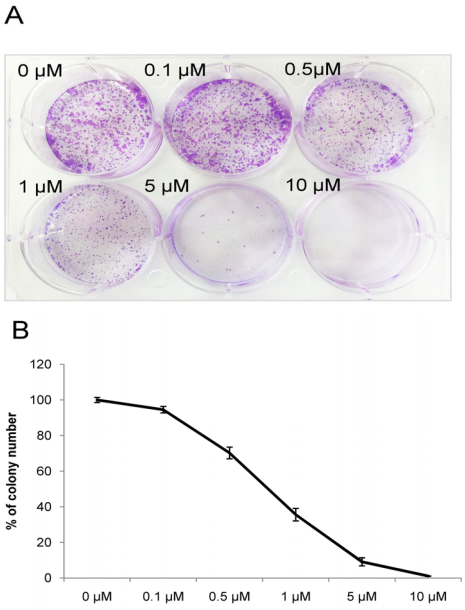

Fig. 2. The effect of growth inhibition on SCC25 cells was examined by clonogenic assay. SCC25 cells were cultured in the presence of the indicated concentrations (0 to 10 μM) of curcumin for 7 days. (A) The photograph showing colony formation in SCC25 cells. (B) The growth of curcumin treated groups is expressed as percentage of the control. Note that curcumin significantly inhibited the growth of SCC25 cells. Values are means ± SD of triplicates of each experiment.

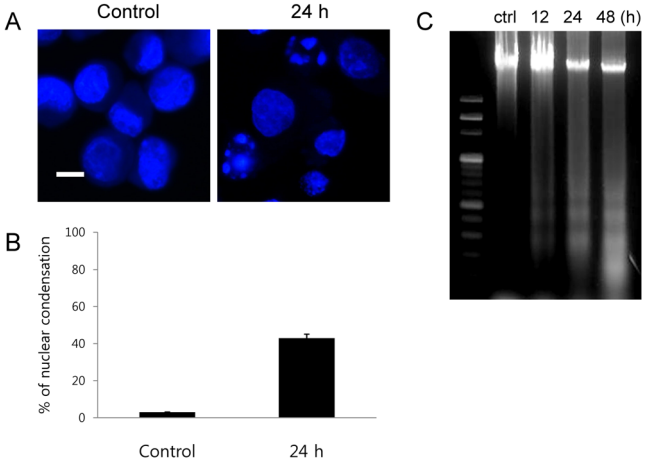

Fig. 3. Demonstration of apoptosis in SCC25 cells treated with 25 μM curcumin for 24 h. (A) Immun of luorescent micrographs after Hoechst staining. Control cells showing round-shape nuclei (left panel). Cells treated with curcumin for 24 h show the production of nuclear condensation (right panel). Scale bar, 10 μm. (B) The values below micrographs are the mean ± SD of apoptotic cells as determined by Hoechst staining. (C) The results presented are representatives of three independent experiments. DNA fragmentation was demonstrated by DNA electrophoresis. Cells treated with 25 μM curcumin for 12, 24, 48 hr clearly showed DNA degradation characteristic of apoptosis with a ladder pattern of DNA fragments.

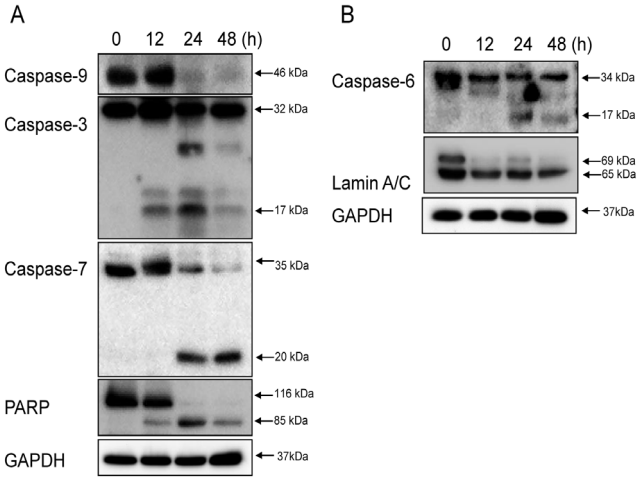

Fig. 4. Curcumin activates apoptosis-related proteins. (A) Curcumin treatment induced caspase-9, caspase-7 and PARP degradation, and produced the processed caspase-3 17 kDa, caspase-7 20 kDa and PARP 85 kDa cleaved products. (B) Curcumin treatment induced caspase-6 and Lamin A/C degradation, and produced the processed caspase-6 17 kDa cleaved products.The levels of GAPDH were used as an internal standard

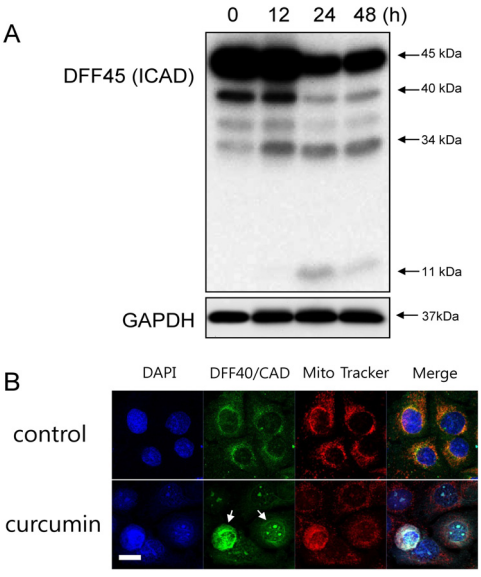

Fig. 5. Curcumin induced activation of CAD and ICAD. (A) Western blot analysis showed the activation of DFF45/ICAD after treatment of 25 μM curcumin. DFF45 /ICAD induced cleaved products (34 kDa and 11 kDa). The levels of GAPDH were used as an internal standard for quantifying DFF45/ICAD expression. (B) Confocal microscopy showing that DFF40/CAD translocated from cytosol into nuclei. Scale bar, 10 μm.

treated with 25 μM of curcumin at various time points showed DNA ladders by DNA electrophoresis (Fig. 3C). The Western blot results showed that curcumin treatment at various time points induced degradation of caspase-9, caspase

-6, PARP, and lamin A/C and that it produced caspase-3 17 kDa, DFF45/ICAD 30 kDa, and 11 kDa cleaved products (Fig. 4A, Fig, 4B & Fig. 5A). Confocal microscopy showed that curcumin led to the translocation of DFF40/CAD from the cytosol into the nuclei (Fig. 5B).

Proteasome activity in the SCC25 cells treated with curcumin

To investigate the inhibition of proteasome activity at a concentration of 25 μM of curcumin, a proteasome activity assay was employed. In this assay, curcumin gradually abolished proteasome activity in a time-dependent manner (Fig. 6).

Mitochondrial events associated with curcumin-induced apoptosis of the SCC25 cells

The induction of apoptosis is regulated by Bcl-2 family members. Bcl-2 functions in antiapoptosis, whereas Bax promotes apoptosis. The proapoptotic Bax, Bad, and Bid, induces loss of mitochondrial membrane potential (△Ψm) and releases cytochrome c and apoptosis inducing factor (AIF). To examine the role of Bcl-2 family proteins in curcumin-induced apoptosis, a Western blot assay was performed. Upregulation of Bax and downregulation of Bcl-2 occurred in a time-dependent manner (Fig. 7). The mitochondria were stained with JC-1 dye, and the mitochondrial membrane potential (△Ψm) was measured by flow cytometry. The SCC25 cells treated with 25 μM of curcumin showed loss of mitochondrial membrane potential (△Ψm) in a time-dependent manner (Fig. 8). Confocal microscopy was conducted to examine whether AIF and cytochrome c are released in the mitochondria. AIF was translocated from the mitochondria to nuclei, and cytochrome c was released from the mitochondria into the cytosol in the SCC25 cells treated with 25 μM of curcumin (Fig. 9 & 10).

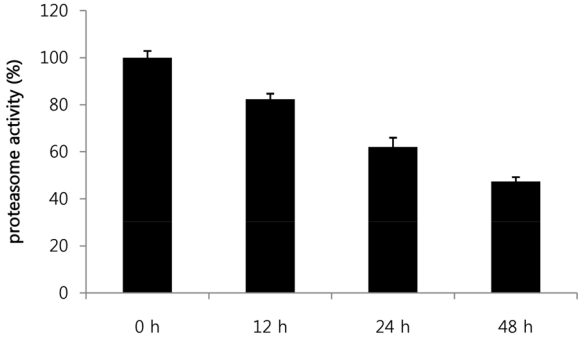

Fig. 6. The proteasome activity which was occurred in the cytoplasmic extract un-treated or treated with 25 μM curcumin on SCC25 cells was measured by fluoro-count. Proteasome activity decreased in a time-dependent manner. Three independent assays were performed.

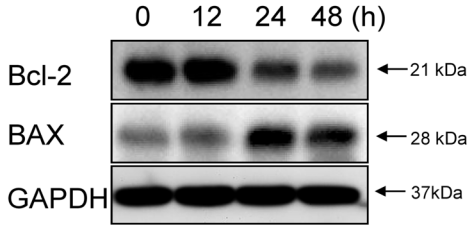

Fig. 7. Bax and Bcl-2 expression level in SCC25 cells treated with 25 μM curcumin was detected by Western blot analysis. Pro-apoptotic factor, Bax was significantly up-regulated in a time-dependent manner whereas anti-apoptotic factor, Bcl-2 was down-regulated. The levels of GAPDH were used as an internal standard for quantifying Bax and Bcl-2 expression.

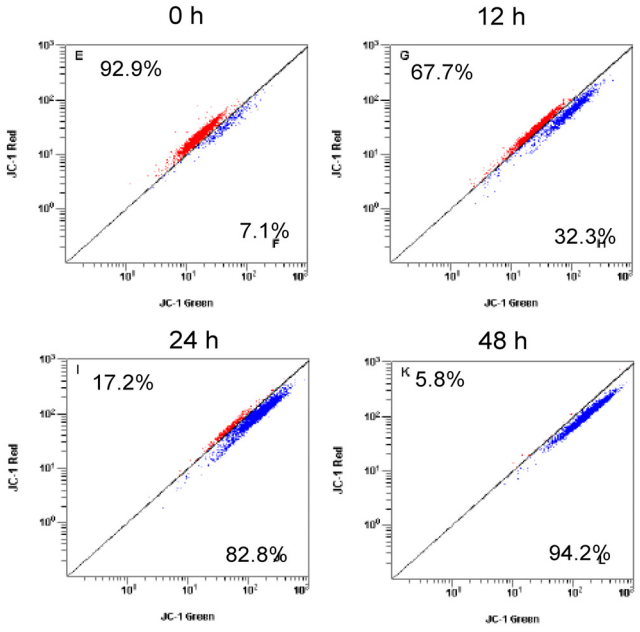

Fig. 8. Mitochondrial membrane potential (△Ψm) at various time points after 25 μM curcumin treatment. △Ψm was reduced in SCC25 cells in a time-dependent manner. △Ψm was measured by flow cytometry. Three independent assays were performed.

Quantification of DNA hypoploidy in the SCC25 cells treated with curcumin

The evaluation of the percentage of apoptosis was confirmed by flow cytometry analysis. The flow cytometry showed that treatment with 25 μM of curcumin significantly increased apoptotic cells with DNA hypoploidy compared to the control group (Fig. 11).

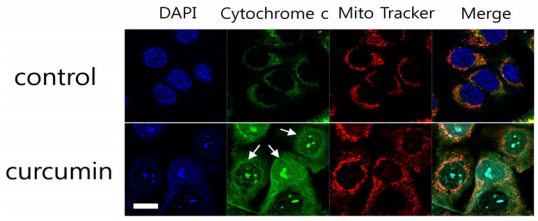

Fig. 9. Confocal microscopy showed that cytochrome c was released from mitochondria into the cytosol in SCC25 cells treated with 25 μM curcumin. Scale bar, 10 μm.

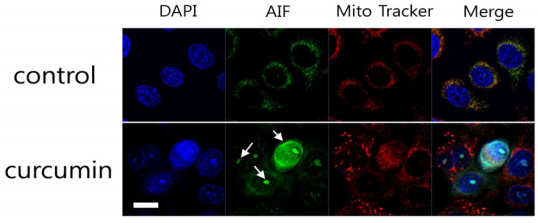

Fig. 10. Confocal microscopy showed that AIF was released from mitochondria into the cytosol, and that translocation onto nuclei was evident in SCC25 cells treated with 25 μM curcumin. Scale bar, 10 μm.

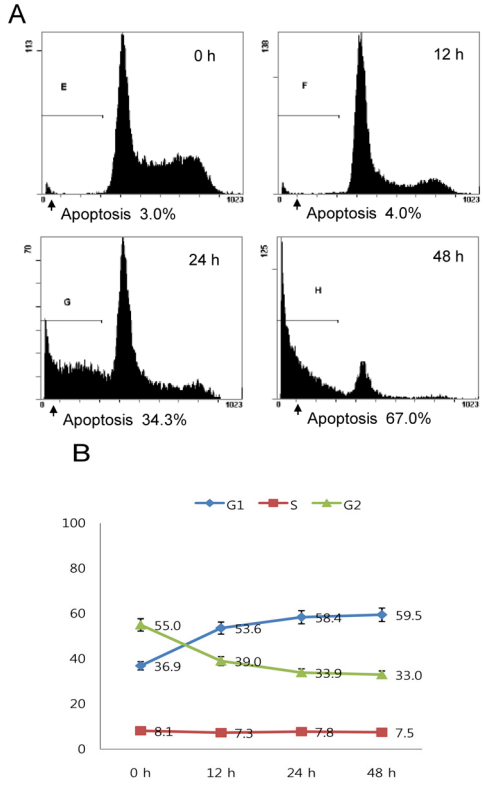

Fig. 11. The kinetic analysis of curcumin treatment effect on SCC25 cell cycle progression and induction of apoptosis using flow cytometry. Representative DNA histograms are shown. curcumin treatment significantly showed the increase of apoptotic cells with DNA hypoploidy in a time-dependent manner. The percentages of G1, S and G2 are depicted. Data shown are representative of three independent experiments.

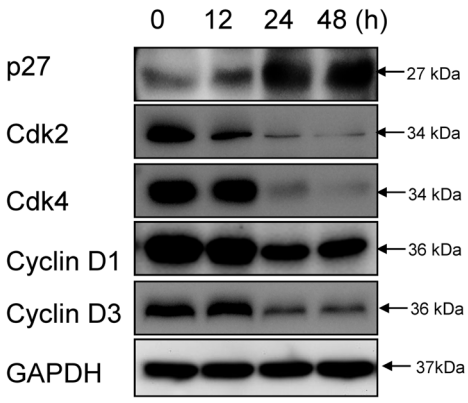

Fig. 12. Western blot analysis of cell cycle-related proteins. Cdk2, Cdk4, Cyclin D1 and Cyclin D3 were down-regulated in a time dependent manner. $p27^{KIP1}$ was remarkably up-regulated in a time-dependent manner. The levels of GAPDH were used as an internal standard.

Alteration of the expression of cell cycle-related proteins in the SCC25 cells treated with curcumin

To investigate the alteration of cell cycle-related proteins, a Western blot assay was conducted. Western blotting data showed that the expression level of cyclin D1, cyclin D3, Cdk2, and Cdk4 regulating the G0/G1 phase decreased in a time-dependent manner. In addition, the cdk inhibitor, $p27^{KIP1}$, was remarkably upregulated (Fig. 12).

Discussion

The use of natural products as herbal remedies is attracting growing public interest. Moreover, as the pharmacological mechanism of herbal compounds becomes known, the popularity of herbal medicine is increasing among health care professionals and the public. A number of studies have elucidated the pharmacological activities (e.g. antiallergic, antipyretic, analgesic, anti-inflammatory, and anticancer effects) of extracts from herbal plants [27-30]. Curcumin derived from the rhizomes of turmeric has been reported to inhibit the growth and proliferation of various tumor cells [31-33].

The present study of the effects of curcumin on the cell viability of SCC25 human tongue squamous carcinoma cells revealed that it reduced the viability of these cells in a dose- and time-dependent manner in an MTT assay. In addition, the clonogenic assay (colony forming assay) confirmed that curcumin at 0.025 to 0.5 mM markedly inhibited the growth of the SCC25 cells. These data

indicate that curcumin exerts a specific cytotoxic effect on SCC25 cells.

Apoptosis and necrosis are conceptually distinct forms of cell death and can be distinguished by specific morphological changes. The morphological changes that apoptotic cells undergo include cell blebbing, reduction of cell size, cell shrinkage, chromatin condensation, and DNA fragmentation [12,13]. The results of the Hoechst stain and DNA electrophoresis revealed that the SCC25 cells treated with curcumin showed apoptotic hallmarks, such as the formation of apoptotic bodies and a DNA ladder pattern. These results indicate that curcumin induced SCC25 cell death via the activation of apoptosis.

Apoptotic stimuli may induce apoptosis by inhibiting the proteasome activity of target cells [34]. One study reported that a proteasome inhibitor can induce apoptosis in certain cells [35]. Generally, the proteasome-mediated step(s) in apoptosis are located upstream of mitochondrial changes and caspase activation, and they can involve different systems, including various cyclins, p53, NF-κB, Bax, and Bcl-2 [36-38]. Thus, it is possible that curcumin may have affected the proteasome activity in the SCC25 cells and induced the mitochondrial pathway of apoptosis. In this study, the proteasome activity was reduced in a time- and concentration-dependent manner in the SCC25 cells treated with curcumin. These data suggest that curcumin induced apoptosis via the proteasome pathway.

As mitochondria play a crucial role in apoptosis, the induction of the mitochondrial permeability transition plays a key part in the regulation of apoptosis [39-43]. Various intracellular and extracellular stress signals can also trigger the mitochondrial pathway, resulting in the activation of proapoptotic proteins, including Bax and Bak, or inactivation of antiapoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL [44]. As a result of the activation/inactivation of Bcl-2 family proteins, changes in the mitochondrial membrane lead to the dissipation of inner membrane potential and permeabilization of the outer mitochondrial membrane. This, in turn, induces the release of various proapoptotic proteins, such as cytochrome c, Smac/Diablo, endonuclease G, and AIF [45,46]. The present study showed a significant shift in the ratio of Bax to Bcl-2 in the SCC25 cells treated with curcumin. The shift in the ratio may be the molecular mechanism by which curcumin induces apoptosis of SCC25 cells. Studies have reported that in isolated mitochondria, the proapoptotic Bcl-2 family induces cytochrome c release and loss of mitochondrial membrane potential (MMP), resulting in the release of AIF [47,48]. Cytochrome c release and disruption of the MMP are known to contribute to apoptosis triggered by proteasome inhibition [49,50]. Generally, cytochrome c is released into the cytosol during apoptosis, where it binds to Apaf-1. The cytochrome c/Apaf-1 complex (apoptosome) promotes the autoactivation of procaspase-9 to caspase-9. Caspase-9 then acts on procaspase-3 to initiate a caspase activation cascade [38,51]. Released AIF through proapoptotic Bcl-2 family activation induces its translocation to the nucleus, resulting in chromatin condensation and large-scale DNA fragmentation [52]. In the present study, curcumin treatment also induced the translocation of AIF from the mitochondria into the nuclei, cytochrome c release from the mitochondria into the cytosol, a significant loss of MMP, and the production of caspase-9 cleavage. These data clearly demonstrate that the curcumin-induced apoptosis in the SCC25 cells was involved with the mitochondrial events mentioned above.

A common final event of apoptosis is nuclear condensation, which is controlled by caspases, the DNA fragmentation factor (DFF), and PARP. Caspases, aspartate-specific intracellular cysteine proteases, play an essential role during apoptotic death [53]. Once activated, the effector caspases (caspase-3, caspase-6 or caspase-7) lead to the proteolytic cleavage of a broad spectrum of cellular targets, resulting ultimately in cell death. The known cellular substrates include structural components (such as actin and nuclear lamin), inhibitors of deoxyribonucleases (such as DFF45/ICAD), and DNA repair proteins (such as PARP) [54,55]. In apoptotic cells, the activation of DFF40/CAD, also a substrate of caspase-3, occurs with the cleavage of DFF45/ICAD. Once DFF40/CAD 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 [56]. Furthermore, in apoptotic cells, the degradation, a substrate of caspase-6, sometimes occurs [57,58]. In this study, cleavage of caspase-3 and DFF45 and degradation of PARP, caspase-6, and lamin A/C were observed in the curcumin-treated SCC25 cells. In addition, confocal microscopy showed that curcumin led to the translocation of DFF40/CAD from the cytosol into the nuclei in the SCC25 cells. These data demonstrate that curcumin-induced apoptosis in SCC25 cells is associated with

caspase-3 and caspase-6 activation. They further show that activated caspase-3 and caspase-6 lead to the activation of PARP, lamin A/C, and DFF4 and that the translocation of DFF40/CAD from the cytosol into the nuclei degrades the chromosomes into fragments.

Various molecular analyses of cancers have revealed that cell cycle regulators are frequently mutated in the majority of common malignancies [59,60]. Therefore, the control of cell cycle progression in tumor cells is considered a potentially effective strategy for the control of tumor growth. Cdks, cyclins, and Cdk inhibitors play critical roles in the regulation of cell cycle progression. Cdk inhibitors inhibit the active Cdk-cyclin complex [61]. $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ have been shown to play an important role in regulating progression from the G1/S phase by binding to and preventing premature activation of Cdk4/cyclin D and Cdk2/cyclin E complexes [62,63]. It is known that cell cycle G1 arrest may be related to the activation of the p53 tumor suppressor protein, which acts as a transcription factor and regulates the expression of several components implicated in pathways that regulate cell cycle progression and apoptosis induction [64,65]. In this study, Cdk2, Cdk4, cyclin D1, and cyclin D3 were remarkably downregulated, whereas $p27^{KIP1}$ was remarkably upregulated. These data demonstrate that the curcumin-induced apoptosis in the SCC25 cells resulted in alterations in the expression of G1 cell cycle-related proteins. Moreover, they indicate that $p27^{KIP1}$ may play a key role in curcumin-induced SCC25 cell death.

Taken collectively, this study demonstrates that curcumin strongly inhibits cell proliferation via modulation of the expression of G1 cell cycle-related proteins and induction of apoptosis via proteasomal, mitochondrial, and caspase cascades in SCC25 cells. Therefore, our data highlight the possibility that curcumin could be considered as a novel therapeutic strategy for human tongue squamous cell carcinoma.

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