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Mechanism Underlying NaF-induced Apoptosis and Cell Cycle Arrest on G361 Human Melanoma Cell Line

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Abstract : Fluoride is widely used in dentistry to prevent dental caries, even though the safety of fluoride is a controversial issue. There are no known adverse effects of long-term fluoride ingestion for caries prevention, but an overdose can cause serious acute toxicity. Nevertheless it is accepted that fluoride is an important material for oral health. This study was undertaken to investigate the modulation of cell cycle-related proteins and apoptosis induction underlying mechanism by NaF treatment on G361 human melanoma cell line.

The viability of G361 cells and the growth inhibition of G361 cells were assessed by MTT assay and clonogenic assay respectively. Hoechst staining, DNA electrophoresis and TUNEL staining were conducted to observe G361 cells undergoing apoptosis. G361 cells were treated with NaF, and Western blotting, immunocytochemistry, confocal microscopy, FACScan flow cytometry, MMP activity and proteasome activity were performed.

NaF treatment in G361 cells resulted in a time- and does-dependent decrease of cell viability and a does-dependent inhibition of cell growth, and induced apoptotic cell death. And tested G361 cells showed several lines of apoptotic manifestation such as nuclear condensation, DNA fragmentation, the reduction of MMP and proteasome activity, the decrease of DNA contents, the release of cytochrome c into cytosol, the translocation of AIF and DFF40 (CAD) onto nuclei, a significant shift of Bax/Bcl-2 ratio, and the activation of caspase-9, caspase-7, caspase-6, caspase-3, PARP, Lamin A/C and DFF45 (ICAD). Furthermore, NaF resulted in down-regulation of the G1 cell cycle-related proteins, and up-regulation of p53.

Taken collectively, our present findings demonstrate that NaF strongly inhibits cell proliferation by modulating the expression of the G1 cell cycle-related proteins and induces apoptosis via proteasome, mitochondria and caspase cascades in G361 cells.

Keywords : NaF, Apoptosis, Cell cycle arrest, Human melanoma cells

Introduction

Fluoride is extensively distributed in the water, soils, and the atmosphere. Human beings and animals are constantly exposed to fluoride compounds. The principal sources of fluoride ingestion are from the drinking water, fluoride-containing beverages and food (WHO 1994, David 2001). Fluoride is widely used in dentistry to prevent dental caries, even though the safety of fluoride is a controversial issue. there are no known adverse effects of long-term fluoride ingestion for caries prevention. but an overdose can cause serious acute toxicity (Li 1993, Lee et al. 2008). Nevertheless it is accepted that the important material for oral health.

Apoptosis is an essential physiological process required for embryonic development, regulation of immune responses and maintenance of tissue homeostasis. However,

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apoptosis is also implicated in a wide range of pathological conditions, including immunological diseases, allergy and cancer (Carson and Ribeiro 1993, Ohta and Yamashita 1999). 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 (Wyllie et al. 1980, Williams 1991).

Malignant melanoma is the most important cutaneous malignancy because it accounts for the majority of mortality due to skin disease. And malignant melanoma patients are increasing in recent years. Furthermore, it was reported that malignant melanoma cells showed high resistance to chemotherapeutic agents (Shibuya et al. 2003).

It was reported that fluoride induced apoptosis in epithelial lung cells, alveolar macrophages (Hirano and Ando 1996, Refsnes et al. 1999) and human gingival fibroblasts, MDPC-23 odontoblast-like cells (Lee et al. 2008, Karube et al. 2009). And it was also reported that fluoride induces apoptosis in such cancer cells as osteosarcoma cells (Hirano and Ando 1997) and human leukemia HL-60 cells (Anuradha et al. 2000, Song et al. 2002). These studies were 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.

Although few studies elicited the apoptosis-inducing efficacy of NaF on cancer cells *in vitro*, there is no report about the apoptotic effect of NaF on human melanoma cell line. The present study was conducted in order to examine the effects of cytotoxicity and cell growth inhibition, and the molecular mechanism underlying the expression alterations of cell cycle-related proteins and apoptosis induction in G361 human melanoma cell line treated with NaF *in vitro*.

Materials and Methods

1. Reagents

The following reagents were obtained commercially: Sodium fluoride (NaF), 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-benzimidazol carbocyanine 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).

2. 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), 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); Rabbit polyclonal anti-human DFF40 (CAD) antibody was from Stressgen (Ann Arbor, MI, USA); Mouse monoclonal anti-human p53 was from BD biosciences (San Diego, CA, USA); HRP-conjugated sheep anti-mouse and anti-rabbit IgGs were from Amersham GE Healthcare (Little Chalfont, UK).

3. Cell culture and treatment of NaF

G361 human melanoma cell line, HGF-1 human gingivl fibroblast and HaCaT human keratinocyte were purchased from the ATCC (Rockville, MD, USA). These cells were maintained at 37°C with 5% CO_2 in air atmosphere in RPMI1640 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). 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. NaF (100 mM) stock solution was added to the medium to obtain 1, 1.1, 1.2, 1.3, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, 2 mM concentrations of the drug.

4. 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 NaF. 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 (Tecan, Männedorf, Switzerland) at 570 nm excitatory emission wavelength. Since viability assays demonstrated evident induction of G361 cell death at 1.1 mM NaF for 48 h, this concentration was utilized for further assessment of apoptosis induced by NaF.

5. Hemacolor staining

Cells were fixed Hemacolor fixative solution and stained by dipping the slides 10 seconds in hemacolor red reagent, and then counterstained by dipping the slieds 10 seconds in hemacolor blue regent. Slides were rinsed off excess dye and allowed to air-dry. To quantify apoptosis, preparations were examined under inverted micro scope magnification ($\times 200$). A minimum of three fields containing around 100 cells were analysed.

6. 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 NaF 0, 0.025, 0.05, 0.1, 0.25 and 0.5 mM 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 NaF-treated cells with respect to control cells. Three independent experiments were conducted.

7. Hoechst staining

After NaF 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˚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, 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.

8. 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 NaF on 60 mm culture dishes. The cell suspension was centrifuged onto a clean fat-free glass slide with a cytocentrifuge. After fixing with 4% paraformaldehyde for 1 h, washing with PBS and permeabilizing with 0.1% Triton X-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, Göettingen, Germany).

9. 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˚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 50 mA for 1.5 h.

10. 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˚C 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, 50 μM Suc-LLVY-AMC] for 1 h 37˚C. The intensity of fluorescence of each solution was measured by a modular fluori-

metric 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).

11. Western blot analysis

Cells were plated at a density of 2×10^6 cells in 100 mm culture dishes. Cells treated with NaF 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 Pico enhanced chemiluminescence substrate and detected with Alpha Imager HP (Alpha Innotech, Santa Clara, USA). Equivalent protein loading was confirmed by Ponceau S staining.

12. 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.

13. 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 each 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).

14. Flow cytometry analysis

Cells were seeded into a 6-well plate at 1×10^6 cells/mL and incubated overnight. Cells treated with NaF 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˚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, FL, CA, USA) and data was analyzed using the Multicycle software which allowed a simultaneous estimation of cell-cycle parameters and apoptosis.

15. Statistical analysis

Three independent experiments were performed, and statistical significance between groups was determined using the paired t-test by SPSS for Win 12.0 for summary data. $p < 0.01$ and $p < 0.05$ were considered statistically significant.

Results

1. Effects of NaF on the viability and proliferation of the G361 cells

The cytotoxic effect of NaF was performed to measure the viability of G361 cells by MTT assay. After NaF treatment on G361 cells (0 to 2 mM) at 48 h, the cell viability was reduced at the concentrations of 1 mM (66.2%) to 2 mM (8.3%) of NaF. Furthermore, the cytotoxic effect of NaF was performed to measure the viability of HaCaT and HGF-1 cells compared with that of G361 cells by MTT

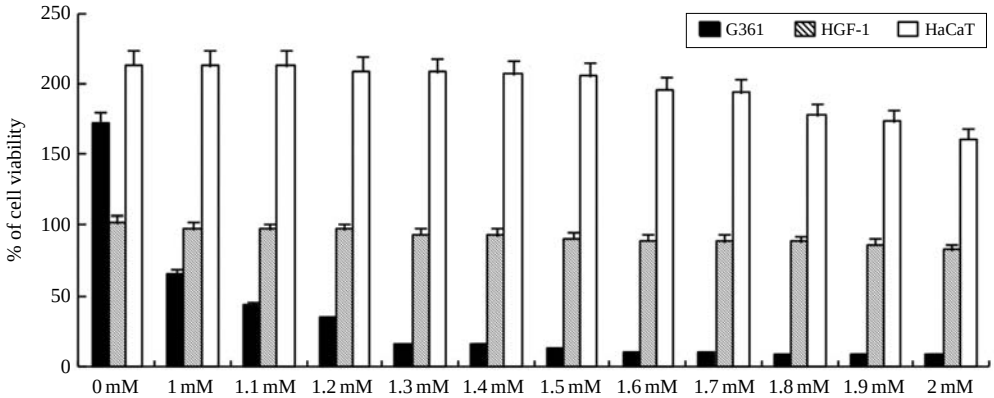

Fig. 1. MTT assay of G361, HGF-1 and HaCaT cells. Cells were treated with NaF (0～2 mM) for 48 h. G361 cells show the remarkable reduction of viability in a does-dependent manner (1～1.3 mM, $p < 0.01$). But the viability of HGF-1 and HaCaT cells were sustained or increased.

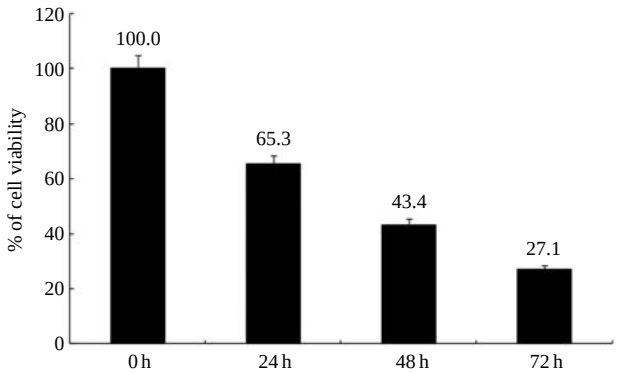

Fig. 2. Effect of NaF treatment on viability of G361 cells as determined by MTT assay. After treatment with 1.1 mM NaF, viability was undertaken over a period of 72 h. NaF remarkably reduced viability of G361 cells in a time-dependent manner (24～48 h, $p < 0.05$; 72 h, $p < 0.01$).

assay. The viability of G361 cells were decreased dose-dependently, but the viability of HGF-1 and HaCaT cells were sustained or increased (Fig. 1). After treatment 1.1 mM NaF, the cell viability was showed in a time-dependent manner (24 h, 65.3%; 48 h, 43.4%; 72 h, 27.1%) (Fig. 2). Hence, the half maximal inhibitory concentration (IC_{50}) of NaF was at the 1.1 mM for 48 h. This concentration was utilized for further assessment of apoptosis and alternation of the cell cycle-related proteins.

To investigate whether NaF inhibited the growth of G361 cells, clonogenic assay was performed. After exposure to NaF concentrations (0 to 0.5 mM) on G361 cells for 7 days, the inhibition of colony formation was determined and was shown in Fig. 3. The growth of NaF-treated group was determined by percentage of control. The values on colony

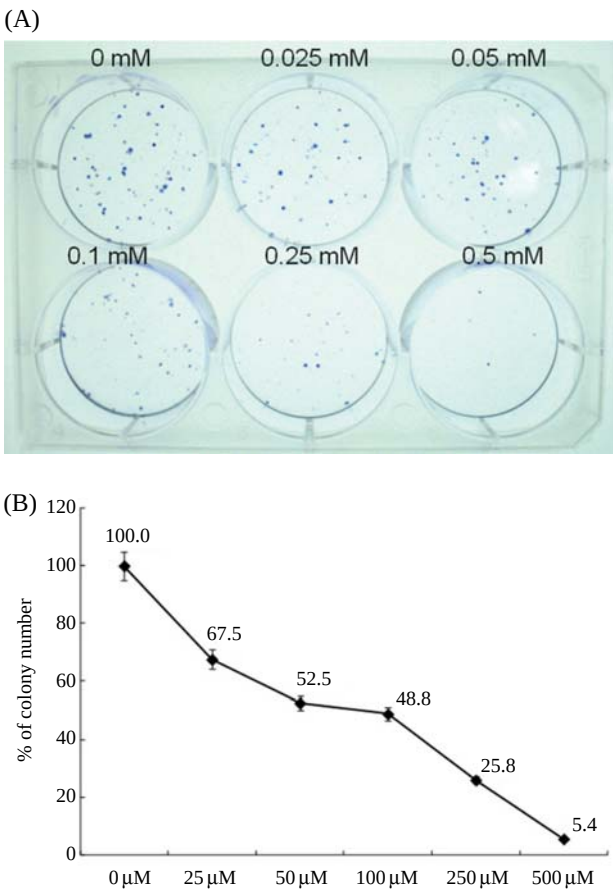

Fig. 3. The effect of growth inhibition on G361 cells was examined by clonogenic assay. G361 cells were cultured in the presence of the indicated concentrations (0 to 0.5 mM) of NaF for 7 days. (A) The photograph showing colony formation in G361 cells. (B) The growth of NaF treated groups is expressed as percentage of the control. Note that NaF significantly inhibited the growth of G361 cells (25～100 μm, $p < 0.05$; 250～500 μm, $p < 0.01$). Values are means ± SD of triplicates of each experiment.

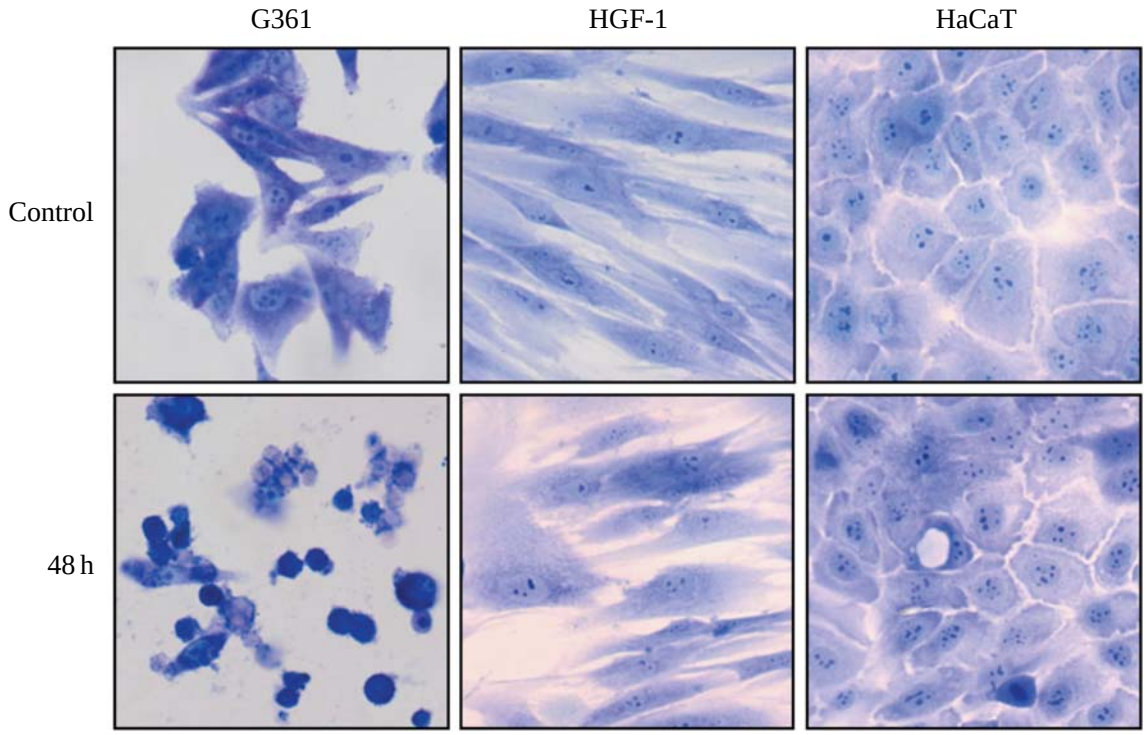

Fig. 4. Hemacolor staining of G361, HGF-1 and HaCaT cells treated with NaF at 1.1 mM for 48 h. In G361 cells treated with NaF, numerous apoptotic cells were shown but in HaCaT and HGF-1 cells treated with NaF, cells were shown normal morphology.

formation were 67.5% (0.025 mM NaF treated cells), 52.5% (0.05 mM NaF treated cells), 48.8% (0.1 mM NaF treated cells), 25.8% (0.25 mM NaF treated cells), 5.4% (0.5 mM NaF treated cells).

2. Morphological and biochemical changes in NaF treated G361 cells

G361 cells treated with NaF at 1.1 mM resulted in morphological and biochemical changes associated with apoptosis. To investigate whether NaF displays morphological changs on G361 cells and normal cells (HGF-1 and HaCaT cells) or not, hemacolor staining was conducted. G361 cells treated with 1.1 mM NaF for 48 h displayed condensed and dark stained nuclei whereas HGF-1 and HaCaT cells showed morphology of normal cells (Fig. 4). Hoechst stain demonstrated that NaF induced a change in nuclear morphology. Compared with the typical round nuclei of the control cells, G361 cells treated with 1.1 mM NaF for 48 h displayed condensed and fragmented nuclei (Fig. 5). DNA fragmentation which is the biochemical hallmark of apoptosis, was demonstrated by TUNEL technique and DNA electrophoresis. The TUNEL positive G361 cells in the control cells were not shown but the number of TUNEL positive G361 cells treated with 1.1 mM NaF were increased in a time-dependent manner (Fig. 6). And G361 cells treated with 1.1 mM NaF at various time points showed DNA ladder by DNA electrophoresis (Fig. 7). The Western blot assay showed that NaF treatment at various time points induced degradations of caspase-9, caspase-6, PARP and lamin A/C, and produced caspase-3 17 kDa, and DFF45 30 kDa and 11 kDa cleaved products (Figs. 8, 9, 10A). And confocal microscopy showed that NaF led to the translocation of DFF40 (CAD) from cytosol onto nuclei (Fig. 10B).

3. Proteasome activity in G361 cells treated with NaF

In order to investigate the inhibition effect of proteasome activity at 1.1 mM NaF, proteasome activity assay was employed. In this assay, NaF gradually abolished proteasome activity in a time-dependent manner (Fig. 11).

4. Mitochondrial events were closely associated with NaF-induced apoptosis of G361 cells

Induction of apoptosis is regulated by Bcl-2 family mem-

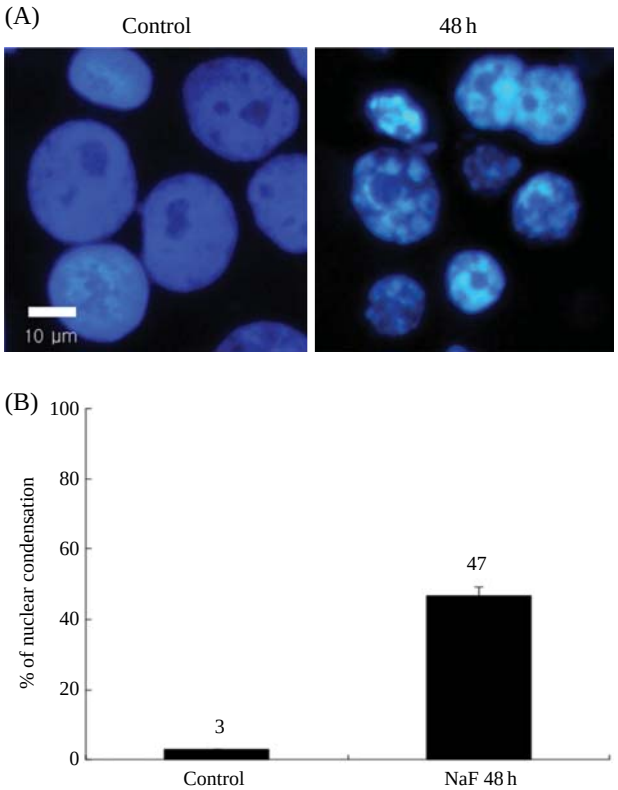

Fig. 5. Demonstration of apoptosis in G361 cells treated with 1.1 mM NaF for 48 h. (A) Immunofluorescent micrographs after Hoechst staining. Control cells showing round-shape nuclei (left panel). Cells treated with NaF for 48 h show the production of nuclear condensation (right panel). (B) The values below micrographs are the mean ± SD of the mean of apoptotic cells as determined by Hoechst staining. The results presented are representatives of three independent experiments (48 h, $p < 0.01$).

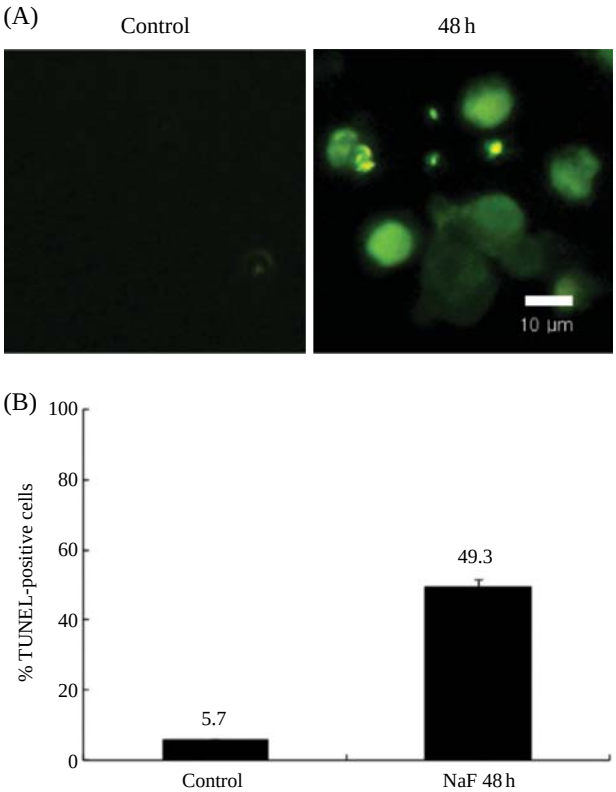

Fig. 6. Demonstration of apoptosis by TUNEL assay. G361 cells were treated with 1.1 mM NaF for 48 h. (A) TUNEL positive cells in control group (0 h) were not shown. Numerous TUNEL positive cells increased in a time-dependent manner. Scale bar, 10 μm. (B) The values below micrographs are the mean ± SD of the means of TUNEL positive cells as determined by TUNEL method. The each result was obtained by three times experiments (48 h, $p < 0.01$).

bers. Bcl-2 has a function of anti-apoptosis, whereas Bax promotes apoptosis. And also, pro-apoptotic Bcl-2 family such as Bax, Bad and Bid induces loss of mitochondrial membrane potential ($\Delta\Psi m$) and released cytochrome c and AIF. To examine the role of Bcl-2 family proteins in NaF-induced apoptosis, it was performed by Western blot assay. The up-regulation of Bax and the down-regulation of Bcl-2 were shown in a time-dependent manner (Fig. 12). The mitochondria were stained with JC-1 dye, and the mitochondrial membrane potential ($\Delta\Psi m$) was measured by flow cytometry. G361 cells treated with 1.1 mM NaF showed the loss of mitochondrial membrane potential ($\Delta\Psi m$) in a time-dependent manner (Fig. 13). The confocal microscopy was conducted to examine whether AIF and cytochrome c are released in the mitochondria or not, AIF was translocated to from mitochondria to nuclei and cytochrome c was released from mitochondria into the cytosol in G361 cells treated with 1.1 mM NaF (Figs. 14, 15).

5. Quantification of DNA hypoploidy in G361 cells treated with NaF

The evaluation of apoptotic percentages were confirmed with flow cytometry analysis. A flow cytometry showed that the treatment of 1.1 mM NaF significantly increased apoptotic cells with DNA hypoploidy compared to the control group (Fig. 16).

6. The alteration of the cell cycle-related proteins in G361 cells treated with NaF

To investigate the alteration of cell cycle-related proteins, Western blot assay was conducted. Western blotting data showed that expression level of Cyclin D1, Cdk2 and Cdk4

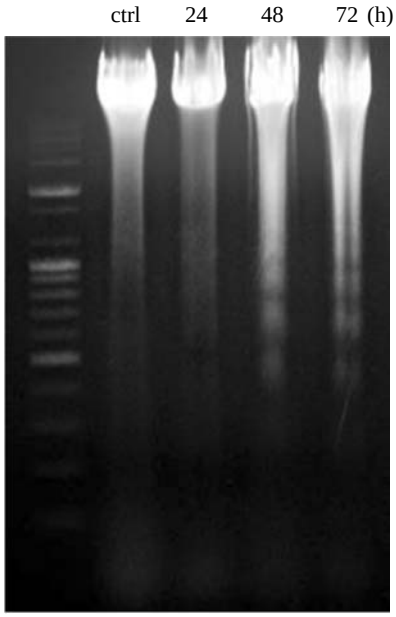

Fig. 7. DNA fragmentation was demonstrated by DNA electrophoresis. Cells treated with 1.1 mM NaF for 24 h showed no DNA ladder, but 48 h and 72 h groups showed DNA degradation characteristic of apoptosis with a ladder pattern of DNA fragments.

regulating G0/G1 phase decreased in a time-dependent manner. The expression level of p53 was remarkably up-regulated in a time-dependent manner (Fig. 17).

Discussion

Human beings and animals are constantly exposed to fluoride compounds. In addition, the increased use of fluorides in industry, medicine and dentistry exposes human beings to even greater levels of fluoride compounds. NaF, one of the most widely used fluoride compounds, was revealed no significant cytotoxicity at lower doses. Song et al. (2002) reported that apoptosis was induced in these cells by a decrease in Bcl-2 immunoreactivity levels and a concomitant activation of caspase-3. In addition, fluoride induces cell death in HL-60 cells by causing the activation of caspase-3 which in turn cleaves PARP leading to DNA damage and ultimately cell death. It is demonstrated that NaF induces apoptosis and that it is mediated by lipid peroxidation. Thus, lipid peroxidation can result in the loss of mitochondrial membrane potential which then leads to the release of cytochrome c from mitochondria, which further initiates the activation of the caspases and leads to

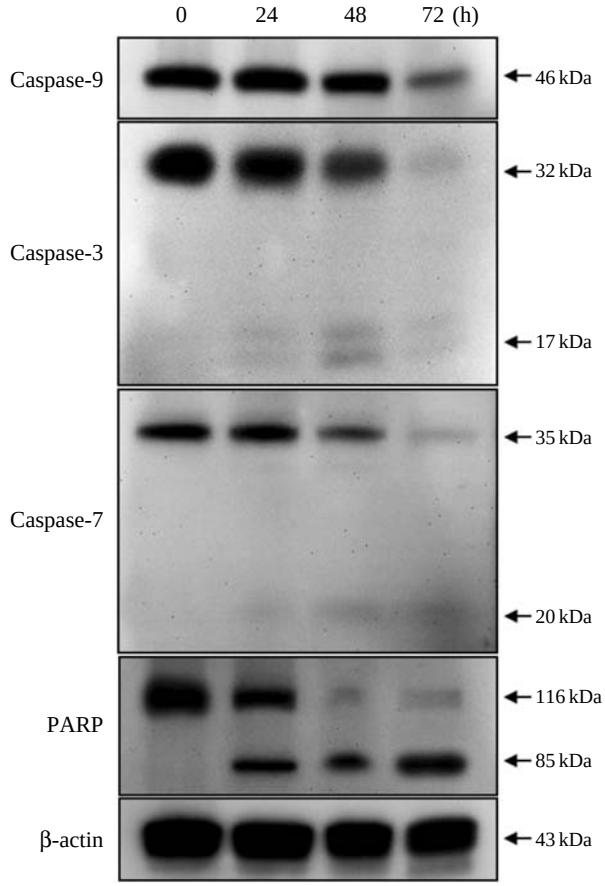

Fig. 8. Western blot analyses of caspase-9, caspase-3, caspase-7 and PARP in G361 treated cells. NaF treatment induced caspase-9, caspase-3, caspase-7 and PARP degradation, and produced the processed caspase-3 17 kDa, caspase-7 20 kDa and PARP 85 kDa cleaved products. The levels of β-actin were used as an internal standard for quantifying caspase-9, caspase-3, caspase-7 and PARP expression.

apoptotic cell death. Further, the downregulation of Bcl-2 may also lead to the loss of Bcl-2-mediated resistance to apoptosis (Anuradha et al. 2000, Anuradha et al. 2001).

The present study investigating the effects of NaF on cell viability in G361 human melanoma cells revealed that NaF produced a dose- and time-dependent reduction on viability of G361 cells in MTT assay. In addition, the clonogenic assay (colony forming assay) confirmed that NaF at 0.025 to 0.5 mM remarkably inhibited the growth of G361 cells. These data indicate that NaF exerts a specific cytotoxic effect on G361 cells.

Apoptosis and necrosis are conceptually distinct forms of cell death and can be distinguished by their specific morphological changes. During apoptosis, apoptotic cells

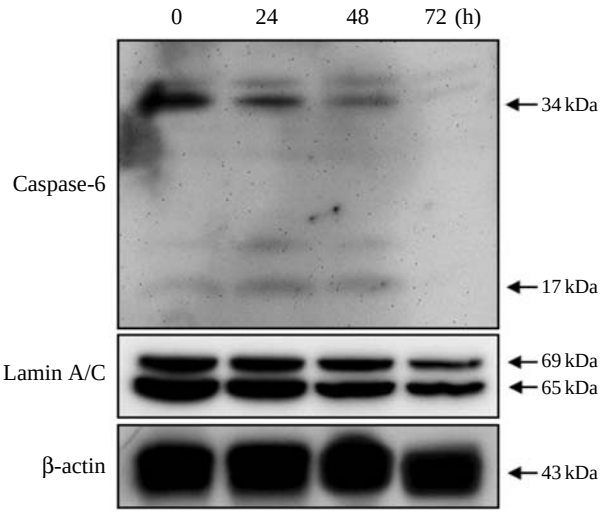

Fig. 9. Western blot analyses of caspase-6 and Lamin A/C in G361 treated cells. NaF treatment induced caspase-6 and Lamin A/C degradation, and produced the processed caspase-6 17 kDa cleaved products. The levels of β-actin were used as an internal standard for quantifying caspase-6 and Lamin A/C expression.

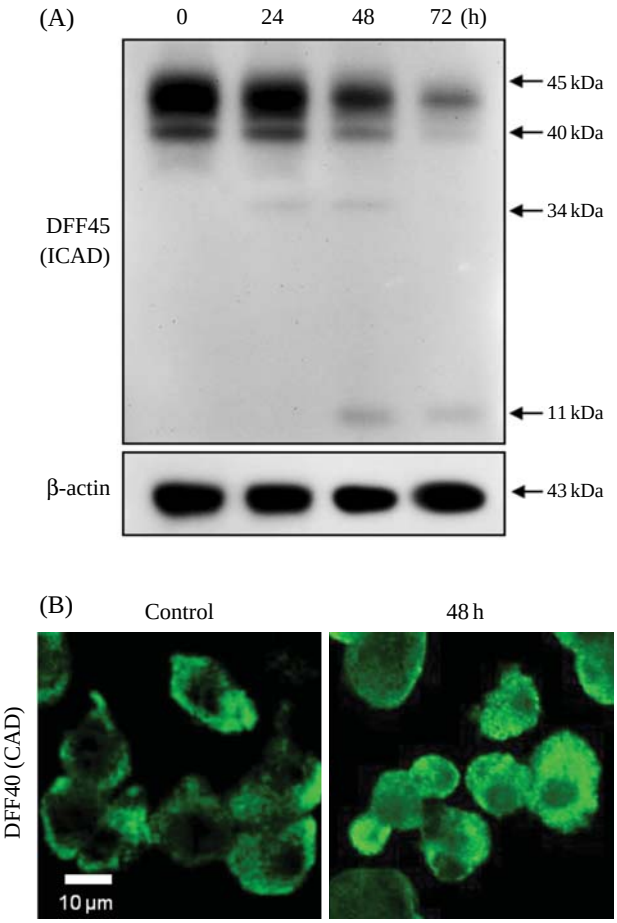

Fig. 10. (A) Western blot analysis showed the activation of DFF45 (ICAD) after treatment of 1.1 mM NaF. DFF45 (ICAD) induced cleaved products (34 kDa and 11 kDa). The levels of β-actin were used as an internal standard for quantifying DFF45 (ICAD) expression. (B) Confocal microscopy showing that DFF40 (CAD) were translocated from cytosol into nuclei. Scale bar, 10 μm.

undergo specific morphological changes such as the cell blebbing, reduction of cell size, cell shrinkage, chromatin condensation and DNA fragmentation (Wyllie et al. 1980, Williams 1991). In the results of Hoechst stain, DNA electrophoresis and TUNEL assay, G361 cells treated with NaF showed apoptotic hallmarks such as the formation of apoptotic bodies, DNA ladder pattern and the increase of TUNEL-positive cells. These results indicate that NaF induced G361 cell death via activating the apoptosis.

Apoptotic stimuli may induce apoptosis by inhibiting the proteasome activity of the target cells (Meng et al. 1999). However, other studies reported that a proteasome inhibitor itself can induce apoptosis in certain cells (Drexler et al. 2000). Generally, the proteasome-mediated step(s) in apoptosis are located upstream of mitochondrial changes and caspase activation, and can involve different systems, including various cyclins, p53, NF-κB, Bax and Bcl-2 (Grimm et al. 1996, Orlowski 1999, Li and Dou 2000). Thus, there is the possibility that NaF may have affected proteasome activity in G361 cells and induced the mitochondrial pathway of apoptosis. In this study, the proteasome activity showed the reduction in G361 cells treated with NaF, time-dependently. This data suggests that NaF induced apoptosis via proteasome pathway.

Because mitochondria plays a crucial role in apoptosis, the induction of the mitochondrial permeability transition plays a key part in the regulation of apoptosis (Kroemer et al. 1997, Green and Reed 1998, Susin et al. 1999, Orrenius 2004). The mitochondrial pathway can also be triggered by various intracellular and extracellular stress signals, which result in activation of pro-apoptotic proteins, including Bax and Bak, or inactivation of anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-xL (Orrenius 2004). As a result of activation/inactivation of Bcl-2 family proteins, changes in 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 (Hengartner 2000, Barczyk et al. 2005). The present study showed

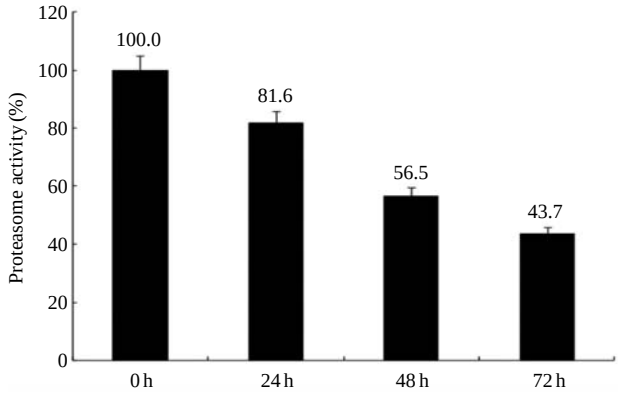

Fig. 11. The proteasome activity which was occurred in the cytoplasmic extract un-treated or treated with 1.1 mM NaF on G361 cells was measured by fluoro-count. Proteasome activity decreased in a time-dependent manner. Three independent assays were performed (24 ~ 72 h, $p < 0.05$).

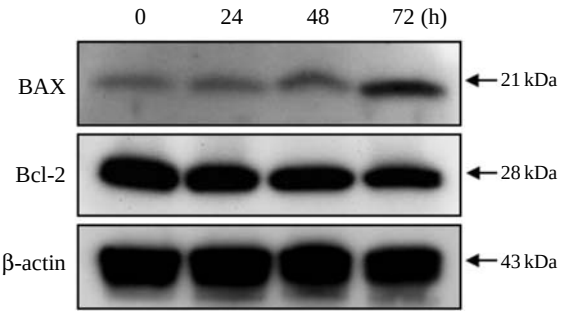

Fig. 12. Bax and Bcl-2 expression level in G361 cells treated with 1.1 mM NaF was detected by Western blot analysis. Pro-apoptotic factor, Bax was significantly up-regulated in a time-dependent manner whereas anti-apoptotic factor, Bcl-2 was down-regulated. The levels of β-actin were used as an internal standard for quantifying Bax and Bcl-2 expression.

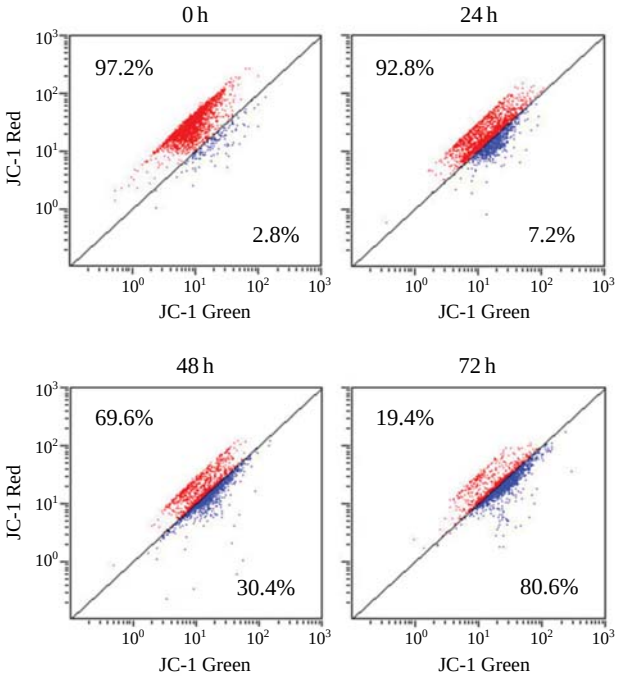

Fig. 13. Mitochondrial membrane potential ($\Delta\Psi m$) at various time points after 1.1 mM NaF treatment. $\Delta\Psi m$ was reduced in G361 cells in a time-dependent manner (48 h, $p < 0.05$; 72 h, $p < 0.01$). $\Delta\Psi m$ was measured by flow cytometry. Three independent assays were performed.

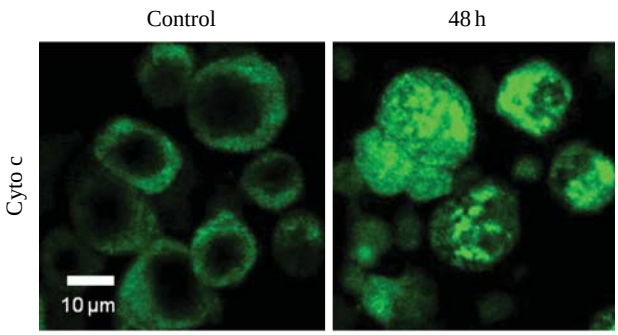

Fig. 14. Confocal microscopy showed that cytochrome c was released from mitochondria into the cytosol of G361 cells treated with 1.1 mM NaF. Scale bar, 10 μm.

a significant shift of the ratio of Bax to Bcl-2 in G361 cells treated with NaF. This result indicates that a shift of the ratio of Bax to Bcl-2 may be the molecular mechanisms by which NaF induces apoptosis of G361 cells. It has been reported that the pro-apoptotic Bcl-2 family in isolated mitochondria induces cytochrome c release, the loss of mitochondrial membrane potential and results in AIF release (Hunter and Parslow 1996, Narita et al. 1998). Cytochrome c release and disruption of mitochondrial membrane potential (MMP) are known to contribute to apoptosis triggered by proteasome inhibition (Wagenknecht et al. 2000, Marshansky et al. 2001). Generally, cytochrome c is released into the cytosol during apoptosis, where it binds to Apaf-1. This 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 (Li et al. 1997, Zou et al. 1999). Released AIF through pro-apoptotic Bcl-2 family activation induces its translocation to the nucleus, resulting in chromatin condensation and large-scale DNA fragmentation (Douglas et al. 2000). In the present study, NaF treatment also induced translocation of AIF from mitochondria

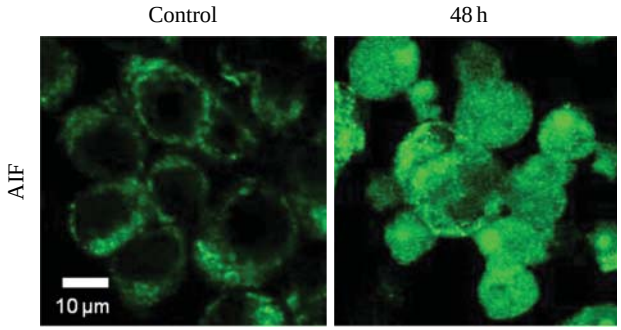

Fig. 15. Confocal microscopy showed that AIF was released from mitochondria into the cytosol, and that translocation onto nuclei was evident in G361 cells treated with 1.1 mM NaF. Scale bar, 10 μm.

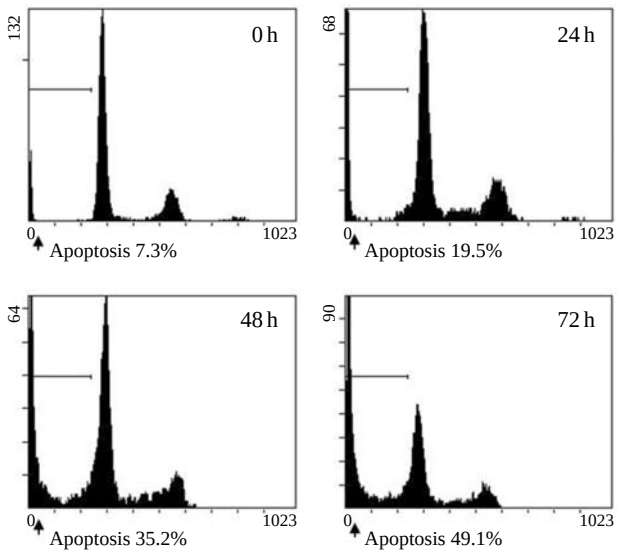

Fig. 16. The kinetic analysis of NaF treatment effect on G361 cell cycle progression and induction of apoptosis using flow cytometry. Representative DNA histograms are shown. NaF treatment significantly showed the increase of apoptotic cells with DNA hypoploidy in a time-dependent manner (24 ~ 72 h, $p < 0.05$). Data shown are representative of three independent experiments.

into nuclei, cytochrome c release from mitochondria into the cytosol, a significant loss of MMP and the production of caspase-9 cleavage. These data have clearly demonstrated that the NaF-induced apoptosis in G361 cells was involved with mitochondrial events as mentioned above.

A common final event of apoptosis is nuclear condensation, which is controlled by caspases, DFF (DNA fragmentation factor), and PARP. Caspases, the aspartate-specific intracellular cysteine protease, play an essential role during apoptotic death (Acehan et al. 2002). 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, leading ultimately 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) (Gross et al. 1999, Porter 1999). 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 (Cheng et al. 2007). Furthermore, in apoptotic cells, the degradation of the lamin A/C, a substrate of caspase-6 was sometimes occurred. In this study, cleavage of caspase-3 and DFF45, and degradation of PARP, caspase-6 and Lamin A/C were shown in NaF-treated G361 cells. Furthermore, confocal microscopy showed that NaF led to the translocation of DFF40 (CAD) from cytosol onto nuclei in G361 cells. Therefore, these data have demonstrated that NaF-induced apoptosis in G361 cells is associated with caspase-3 activation, and activated caspase-3 leads to the activation of PARP and DFF45, and the translocation of DFF40 from cytosol into nucleus which degrades the chromosomes into fragments.

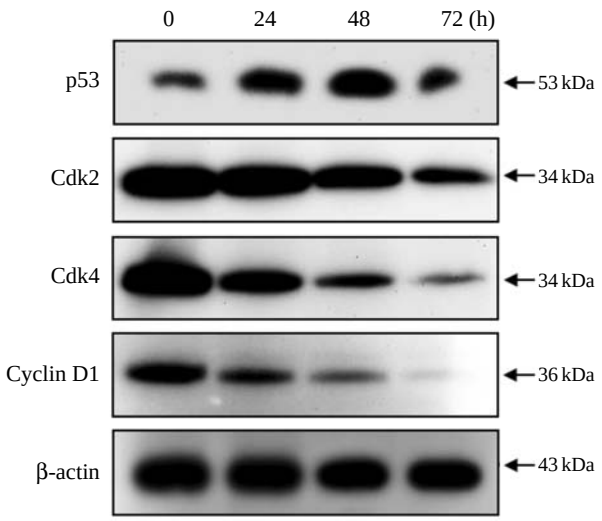

Fig. 17. Western blot analysis of cell cycle-related proteins. Cdk2, Cdk4 and Cyclin D1 were down-regulated in a time dependent manner. p53 was remarkably up-regulated in a time-dependent manner. The levels of β-actin were used as an internal standard for quantifying p53, Cdk2, Cdk4 and Cyclin D1 expression.

Various studies on the molecular analyses of cancers

have revealed that cell cycle regulators are frequently mutated in most common malignancies. Therefore, the 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 (Pavletich 1999). Cdk inhibitors inhibit the active Cdk-Cyclin complex (El-Deiry et al. 1994). $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ have been demonstrated 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 (Polyak et al. 1994, Coats et al. 1996). It is known that the cell cycle G1 arrest may be related to activation of the p53 tumor suppressor protein, which acts as a transcription factor and regulates expression of several components, implicated in pathways that regulate cell cycle progression and apoptosis induction (Teyssier et al. 1999, Colman et al. 2000). In this study, Cdk2, Cdk4, Cyclin D1 and Cyclin D3 and were remarkably down-regulated whereas, p53 were remarkably up-regulated. These data have demonstrated that the NaF-induced apoptosis in G361 cells was involved with the expression alterations of the G1 cell cycle-related proteins. Moreover, we suggest that p53 may play a key role in NaF-induced G361 cell death.

Taken collectively, this study demonstrate that NaF strongly inhibits cell proliferation via the expression modulations of the G1 cell cycle-related proteins and induces apoptosis via proteasome, mitochondria and caspase cascades in G361 cells. Therefore, our data provide the possibility that NaF could be considered as a novel therapeutic strategy for human melanoma.

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사람흑색종세포에서 불화나트륨에 의해 유도된 세포자멸사와 세포주기정지의 기전

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간추림 : 불화나트륨(sodium fluoride, NaF)은 치아우식증을 예방하기 위한 치과용 물질로 널리 사용되어 왔다. 치아우식증 예방을 위한 불소의 장기간 섭취가 인체에 미치는 영향에 대해 알려진 것이 거의 없고, 과복용 시 심각하고 치명적인 독성을 일으킬 수 있다는 보고가 있으나 불소의 안전성은 여전히 논쟁요소로 남아 있다. 그러나 불소는 구강건강을 위한 주요 물질로서 인정받고 있는 것이 사실이다.

NaF은 몇 가지의 정상세포들에서 세포자멸사를 유도하고, 얼마간의 암세포에서 세포주기 정지와 세포자멸사를 유도한다고 알려져 있다. 이 연구의 목표는 사람구강편평상피세포암종세포(G361 세포)에 NaF 적용 후, 세포자멸사의 유도와 세포자멸사에 대한 분자생물학적 기전을 밝히는 것이다.

사람흑색종세포의 생존율은 MTT법으로, G361 세포의 성장억제능의 측정은 clonogenic assay를 사용하였다. NaF 적용시, G361 세포에서 세포자멸사가 유도되는 것을 확인하기 위해서 Hemacolor 염색법, Hoechst 염색법, DNA 전기영동법 및 TUNEL법을 사용하였다. 그리고 G361 세포에 NaF을 적용한 후, Western blot 분석, 세포면역화학염색, 공초점레이져주사현미경 검경, FACScan flow cytometry, 사립체막 전위변화, proteasome 활성도 측정 등을 시행하였다.

NaF로 처리된 G361 세포는 시간 및 용량의존적인 세포생존율 감소, 용량의존적인 성장억제 및 세포자멸사에 의한 세포죽음을 보였다. 그리고 NaF가 적용된 G361 세포에서 핵농축, DNA 조각남, 사립체막전위와 proteasome 활성도의 감소, DNA양의 감소, cytochrome c의 사립체에서의 세포질로의 유리, AIF와 DFF40 (CAD)의 핵으로의 이동, Bax와 Bcl-2의 분율 변화, 그리고 caspase-9, caspase-7, caspase-6, caspase-3, PARP, Lamin A/C, DFF45 (ICAD)의 활성화와 같은 다양한 세포자멸사 증거를 보였다. 더 나아가서 NaF에 처리된 G361 세포에서 G1 세포주기에 관계하는 Cyclin D1, Cyclin D3, Cdk2 그리고 Cdk4의 발현의 감소와 p53의 탁월한 발현의 증가를 보였다.

이 연구를 통하여 NaF가 G1 세포주기정지 단백질의 변형 그리고 proteasome, 사립체 및 caspase 경로의 연속반응을 통해 G361 세포의 세포자멸사를 유도함을 명확하게 증명하였다.

찾아보기 낱말 : 불화나트륨, 세포자멸사, 세포주기정지, 사람흑색종세포

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