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Involvement of Bcl-2 Family and Caspases Cascade in Sodium Fluoride-Induced Apoptosis of Human Gingival Fibroblasts

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Sodium fluoride (NaF) has been shown to be cytotoxic and elicit inflammatory response in human. However, the cellular mechanisms underlying NaF-induced cytotoxicity in periodontal tissues have not yet been elucidated. This study is aimed to investigate the mechanisms of NaF-induced apoptosis in human gingival fibroblast (HGF). NaF decreased the cell viability of HGF in a dose- and time-dependent manner. NaF gave rise to apoptotic morphological changes including cell shrinkage, chromatin condensation, and DNA fragmentation. However, NaF did not affect the production of ROS. In addition, NaF augumented cytochrome *c* release from mitochondria into the cytosol, and enhanced caspase -9 and -3 activities., cleavage (85 kDa fragments) of poly (ADP-ribose) polymerase (PARP) and upregulation of voltage-dependent anion channel (VDAC) 1. These results demonstrated that NaF-induced apoptosis in HGF may be mediated with mitochondria. Furthermore, NaF elevated caspase-8 activity and upregulated Fas-ligand (Fas-L), suggesting involvement of death receptor mediated pathway in NaF-induced apoptosis. Expression of Bcl-2, an anti-apoptotic Bcl-2 family, was downregulated, whereas expression of Bax, a pro-apoptotic Bcl-2 family, was not affected in NaF-treated HGF. These results suggest that NaF induces apoptosis in HGF through both mitochondria- and death receptor-mediated pathway mediated by Bcl-2 family.

Key Words: NaF, HGF cells, Apoptosis, Bcl-2 family, Caspase, Fas-L

INTRODUCTION

Fluoride has been used widely in dentistry to prevent dental caries, while the safey of fluoride has been a controversial issue. Although the re are rare adverse effects reported on long-term low dose fluoride ingestion, the risk of an overdose can result in serious acute toxicity (Li, 1993; Stephen, 1994; FDI Communication, 2002). Fluoride at high concentrations has been found to inhibit protein synthesis and cell cycle progression (Holland, 1979; Aardema et al, 1989), and epithelial lung cells and alveolar macrophages have been reported to undergo apoptosis after fluoride exposure (Hirano et al, 1996; Refsnes et al, 1999). However, sodium fluoride (NaF)-induced cytotoxicity and its underlying mechanisms in periodontal tissues have still not been elucidated.

Cells undergoing apoptosis show distinct morphological and biochemical changes such as cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation (Kerr et al, 1972; Wyllie et al, 1980). Apoptosis is triggered by a variety of stimuli, mediating the mitochondrial response, endoplasmic reticulum stress, and cell surface receptors such as Fas. Caspases are cysteine proteases that cleave a critical set of cellular proteins to initiate the apoptotic signal including several representatives involved in apoptosis (Roth et al, 2000; Tsujimoto & Shimizu, 2000). Among 14 known mammalian caspases at least, those involved in apoptosis can further be subdivided into the initiators (-2, -8, -9, and -10) and effector caspases (-3, -6, and -7) (Adams & Cory, 1998; Crompton, 2000). According to the pathways for the activating caspases, the main mechanism for apoptosis can be divided into the death receptor-mediated mechanism and the mitochondria-mediated mechanism. The mitochondrial pathway is initiated by the release of cytochrome c from the mitochondria into the cytosol, resulting in the activation of caspase-9 that in turn activates caspase-3 (Green & Reed, 1998). The death receptor pathway is stimulated by cell surface death receptors such as the tumor necrosis factor (TNF) receptor and Fas. The receptors activated by ligands lead to caspase-8 activation, with subsequent activation of caspase-3 (Beer et al, 2000). The mitochondria- and death receptor-mediated pathways share the activation of caspase-3, which cleaves one of its substrates, poly (ADP-ribose) polymerase

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ABBREVIATIONS: NaF, sodium fluoride; ROS, reactive oxide species; VDAC, voltage-dependent anion channel; HGF, human gingival fibroblasts; PARP, poly (ADP-ribose) polymerase; Fas-L, Fas-ligand.

(PARP), during apoptosis in many eukaryotic cells, resulting in apoptotic DNA fragmentation (Ogata et al, 1998). In addition, the Bcl-2 protein family plays an essential role in apoptosis (Tsujimoto & Shimizu, 2000). The Bcl-2 family controls the release of mitochondrial cytochrome c by regulating the mitochondrial permeability transition (PT) pore, which is consisted of the voltage-dependent anion channel (VDAC) in the outer membrane, the adenosine nucleotide translocated (ANT) in the inner membrane, and cyclophilin-D (Cyp-D) in the matrix assemblies (Ankarcrona et al, 1995; Krajewski et al, 1999).

The present study is aimed to investigate the involvements either mitochondria- or death receptor-mediated apoptotic pathways or both and determine the roles of Bcl-2 family in NaF-induced apoptosis of the human gingival fibroblasts (HGF).

METHODS

Cell culture and cell viability assay

HGF cells were obtained from healthy gingival tissue of patient in Chonnam National University Hospital. HGF cells were maintained in DMEM media supplemented with 10% fetal bovine serum (Gibco, USA) under 5% CO_2 at 37 °C. NaF (Sigma, USA) was dissolved in distilled DMEM and sterilized through 0.2 μm filter. Cell viability was determined using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra zolium bromide] assay (Sigma, USA).

Nuclear staining with Diff-Quick

Morphological changes of apoptotic cells were investigated by Diff-Quick stain. Cells were plated in 8-well chamber slide at a density of 1×10^5 and incubated for 18 h, followed subsequently by treatment of 20 mM NaF for 6 h. The cells were then washed with 1×PBS and fixed with acetone and methanol (1 : 1). After incubating for 20 min at −20°C, cells were stained with 10 μg/ml Diff-Quick in PBS and observed under microscope (Olympus, USA).

Detection of ROS production and caspase activity

ROS production was monitored by fluorescence spectrometer (Hitachi F-4500, Japan) using 2', 7'-dichlorofluorbescin diacetate (DCF-DA). Cells were plated on 96-well plate and treated for 1 h with 5 mM N-acetyl-cysteine (NAC, Sigma, USA) and 5~40 mM final concentration of NaF for 6 h. DCF-DA (25 μM) was further added into the media for 10 min at 37°C. Excitation was measured at 480 nm and emission was measured at 530 nm. The caspases activities were assessed using an ELISA reader using the caspase-3, -9 activity assay kit (Calbiochem, CA) and caspase-8 activity assay kit (Santa Cruz, USA), according to the manufacturer's instructions.

Isolation of total RNA and reverse transcription polymerase chain reaction (RT-PCR)

For extraction of total RNA, cells were homogenized with a polytron homogenizer in Trizol reagent (Gibco-BRL, USA). RNA samples were spectrophotometrically quantified at 260 nm. For synthesis of cDNA, 2 μg of total RNA and 2 μl of oligo-dT (10 pmoles) were mixed with 50 μl of RNase-free water, and then incubated at 42°C for 1 h and 94°C for 5 min. PCR products were generated in PCR buffer containing 10 pmoles each of primer using PCR-premix kit (Bioneer, Korea). After the first denaturation step (5 min at 95°C), samples were subjected to 30 cycles consisting of 40 sec at 95°C, 40 sec at 55°C, and 1 min 30 sec at 72°C, with a final extention step of 10 min, on a GeneAmp PCR system (Perkin-Elmer 2400). The following primer pairs were used: for Fas-L, 5'-CAGCCCCTGAATTACCCATATC-3' (sense primer), 5'-CACTCCAGAGATCAAAGCAGTTC-3' (antisense primer). The amplified products were analyzed on 1.5% agarose gels containing ethidium bromide and visualized by UVP Transilluminator/Polaroid camera system (UVP Laboratories, CA). RT-PCR was performed with primers for the housekeeping gene, GAPDH, as a control. The following primer pairs for GAPDH were used: 5'-TGCATCCTGCACCACCAACT-3' (sense primer) and 5'-CGCCTGCTTCACCAC TTC-3' (antisense primer). The intensities of the bands obtained were determined using the NIH Scion Image Software.

Western blotting analysis

Cells were washed twice with PBS, and proteins were solubilized in the lysis buffer (1% NP-40, 500 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM Benzamid, 1 μg/ml trypsin inhibitor) containing a cocktail of protease inhibitor (Complete, Germany). The amount of cytochrome *c* released from the mitochondria into the cytosol was determined using cytosolic fractions, according to the method described (Boulares et al, 2002). To determine cytosolic cytochrome c, pellet was resuspended in extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-NaOH (pH 7.4), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, and 1 mM DTT. Lysates were incubated for 30 min at 4°C, centrifuged at 11,000×g for 20 min and protein concentrations were determined by BCA protein assay (Pierce, USA). Protein extracts (100~300 μg) were boiled for 5 min with SDS-sample buffer and then subjected to 12% polyacrylamide gel electrophoresis. Proteins were electroblotted onto nitrocellulose membrane (Amersham Pharmacia Biotech, UK) and blocked with 5% skim milk (Becton Dickinson, USA) in Tris-buffered saline-0.1%Tween 20 (TBS-T). Primary antibodies used included a rat monoclonal anti-cytochrome *c* (Pharmingen, USA), PARP (Cell Signaling, USA), Bax, Bcl-2, and VDAC (Santa Cruz, USA). Blots were subsequently washed three times in TBS-T for 5 min and incubated with specific peroxidase-coupled secondary antibodies (Sigma, USA). Bound antibodies were visualized using an enhanced chemiluminescent detection system (Amersham Pharmacia Biotech, UK).

RESULTS

NaF induced apoptotic cell death but not ROS production in HGF

To determine the involvement of ROS in NaF-induced cell death of the HGF, ROS production was measured using DCF-DA. Fig. 1 shows that NaF did not affect ROS level in HGF. Pretreatment of the cells with 5 mM NAC, a ROS scavenger, inhibited the production of ROS, as a negative control. The cell viability in NaF-treated HGF was deter-

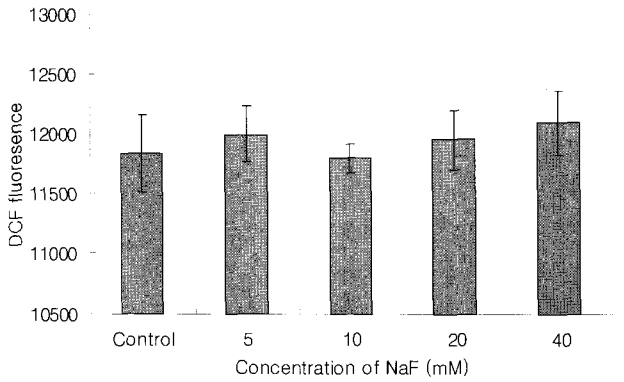

Fig. 1. ROS levels were unchanged in NaF-treated HGF. HGF loaded with DCF were incubated for 6 h with NaF alone or co-incubated with 5 mM N-acetyl-L-cysteine (NAC) for 1 h. The intracellular levels of ROS were determined by measuring the DCF-DA fluorescence. Production of ROS was not changed in NaF-treated HGF. Data are mean±SD obtained from 5 independent experiments.

mined by MTT assay. As seen in Fig. 2, cell viability was gradually reduced in a dose- and time-dependent manner when HGF were exposed to NaF. The cell survival was less than 50% when the cells were treated with 20 mM NaF for 24 h (Fig. 2A), and the percent of cell viability was about 60 % after 6 h of exposure to 20 mM NaF (Fig. 2B). In the presence of 20 mM NaF for 6 h, Diff-Quick staining revealed apoptotic morphological changes, including chromatin condensation and apoptotic body (Fig. 2C).

Caspase-3 was involved in the NaF-induced apoptosis of HGF

Since it is important to identify the intracellular apoptotic pathways in HGF induced by NaF, caspase-3 activity was measured, since active caspase-3 consequently cleaves their substrate at a specific site: DEVD-pNA (200 μM) was used as a substrate for caspase-3. When the cells were treated with different concentrations of NaF, caspase-3 activity was enhanced in a dose dependent manner (Fig. 3A). Also, caspase-3 activity was elevated after treatment of the cells with 20 mM NaF, showing the peak value at 6 h (Fig. 3B). In order to further ascertain the involvement of active caspase-3 in NaF-induced apoptosis of HGF, the cleavage of PARP was investigated by Western blot anaylsis, and PARP fragment (85 kDa) was distinctly detected in the HGF treated for 6 h with NaF (Fig. 3C).

NaF-induced apoptosis in HGF was mediated by mitochondria

To evaluate whether mitochondria are involved in NaF-induced apoptosis of HGF, cytosolic level of cytochrome c was determined, because cytochrome c is released from the mitochondria into the cytosol. Thus, HGF cells were incubated with varying concentrations (5~40 mM) of NaF for 6 h, and subjected to Western blot analysis. As shown in Fig. 4A, cytosolic cytochrome *c* levels were increased in a dose-dependent manner in response to NaF exposure. Furthermore, VDAC-1, a cytochrome *c* releasing channel in mitochondria, was upregulated in a dose-dependent man

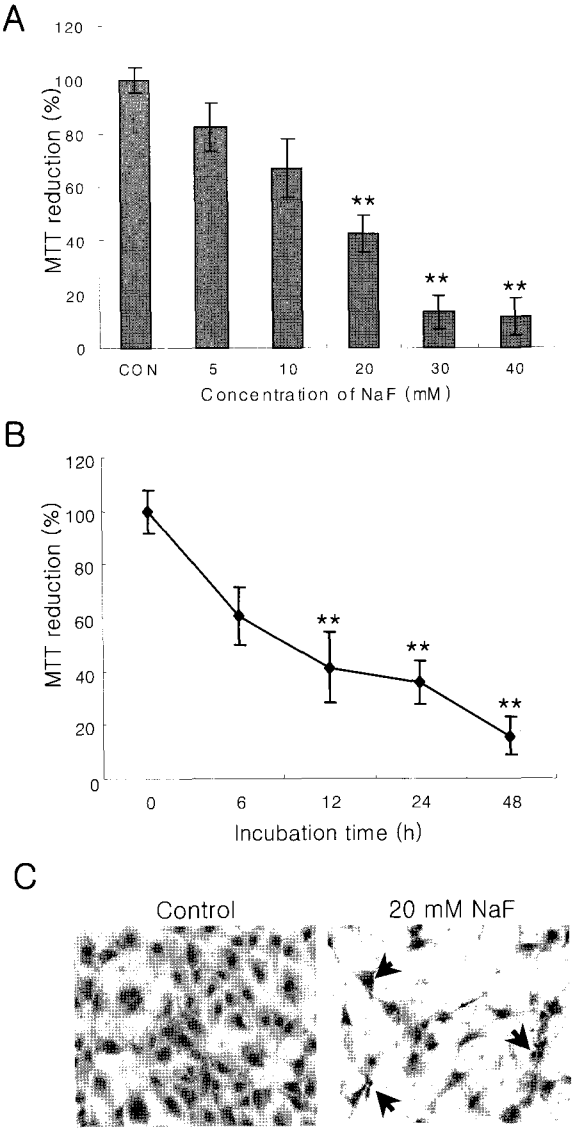

Fig. 2. NaF induced cell death in HGF. Cell viability was determined by MTT assay as described in methods. HGF cells were incubated with different concentrations of NaF (A) and 20 mM NaF for indicated times (B). Data are mean±SD obtained from 5 independent experiments. $p<0.001$, compared with the untreated group (CON). HGF cells were treated with 20 mM NaF for 6 h and were stained using Diff-Quick method. Arrows indicate apoptotic cell (C).

ner (Fig. 4B). These results demonstrate that NaF induces the upregulation of VDAC, resulting in the release of cytochrome *c* from the mitochondria into the cytosol during NaF-induced apoptosis in HGF. Since the activation of caspase-9 by the released cytochrome *c* is important in the mitochondrial pathway, caspase-9 activity in NaF-treated HGF was also examined using the specific substrate for caspase-9, LEHD-pNA (200 μM). When the cells were treated with 20 mM NaF for indicated times, caspase-9 activity was enhanced, showing a peak value at 6 h (Fig. 4C).

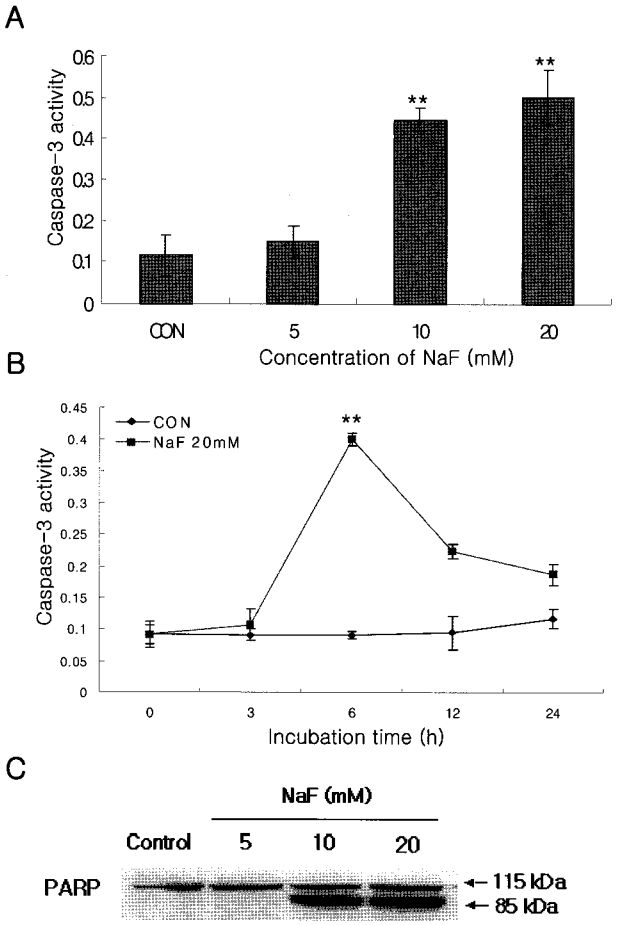

Fig. 3. Caspase-3 activity was enhanced in NaF-treated HGF. After HGF cells were incubated with different concentrations of NaF (A) and 20 mM NaF for indicated times (B), absorbance for caspase-3 activity was measured in the wells at 405 nm by ELISA reader after incubation with DEVD-*p*NA substrate (200 μM) for 24 h at 37°C. Data are mean±SD obtained from 5 independent experiments. $p<0.001$, compared with the untreated group (CON). Cells were treated with 20 mM NaF for 6 h, and cleavage of PARP was measured using Western blot analysis. The lower band (85 kDa) indicates cleaved fragment of PARP (C).

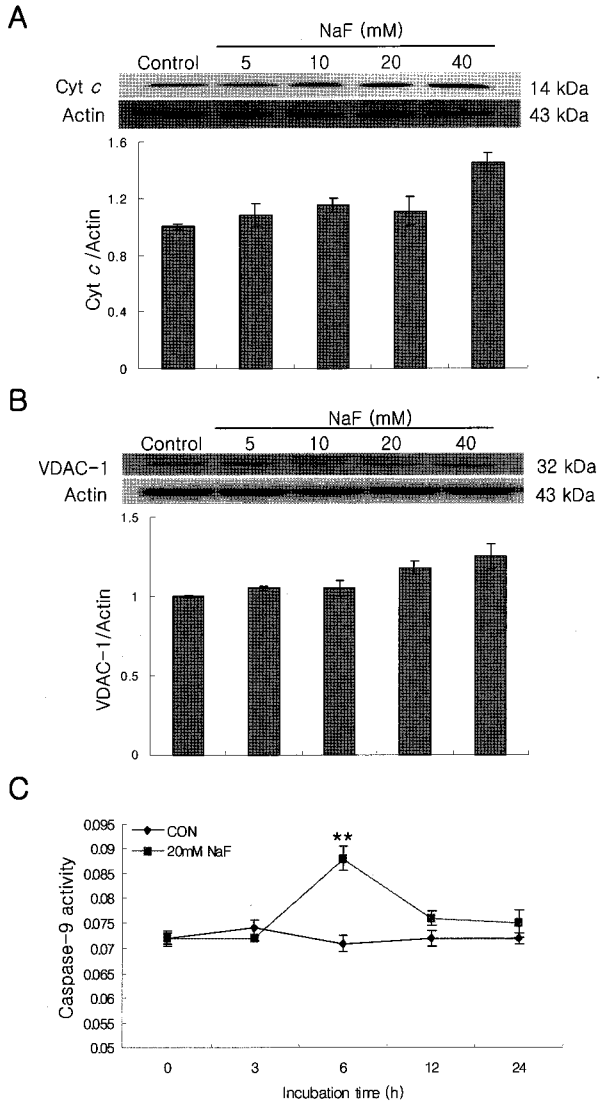

Fig. 4. Enhancement of cytochrome *c* released from the mitochondria into the cytosol, expression of VDAC-1 and the activity of caspase-9 in NaF-treated HGF. Cytosolic cytochrome *c* (A) and VDAC-1 (B) were analyzed by Western blot in HGF treated with different concentrations of NaF for 6 h. Extracts from HGF cells treated with 20 mM NaF for indicated time were assayed for caspase-9 activity using colorimetric peptide substrate (LEHD-*p*NA). Data are mean±SD obtained from 5 independent experiments. $p<0.001$, compared with the untreated group (CON) (C).

NaF-induced apoptosis was mediated with death receptor in HGF

To know whether death receptor-mediated apoptosis pathway is activated in HGF, the mRNA levels of Fas-L, ligand of a death receptor, were determined using RT-PCR analysis. Fas-L in NaF-treated HGF was upregulated in a dose dependent manner (Fig. 5A). Death receptor pathway is stimulated by cell surface death receptor such as Fas, and the receptor activated by ligand leads to caspase-8 activation, with subsequent activation of caspase-3. Therefore, caspase-8 activity in 20 mM NaF-treated HGF was examined using the specific substrate for caspase-8, IETD-pNA (200 μM). When the HGF cells were treated with 20 mM NaF for indicated times, caspase-8 activity was enhanced, showing a peak value at 12 h (Fig. 5B). These results suggest that death receptor mediated pathway may play a crucial role in NaF-induced apoptosis of HGF.

Bcl-2 was downregulated, whereas Bax was unchanged in NaF-induced apoptosis of HGF

Generally, the expression ratio of Bax to Bcl-2 has proven to be significant for apoptosis determination, since a high ratio denotes a low apoptotic threshold. After the treatment of HGF with 20 mM NaF for 6 h, the changes Bax and Bcl-2 protein levels of in HGF were determined using Western blot analysis. Fig. 6A shows that treatment of the cells with 10~40 mM NaF downregulated Bcl-2 level, while

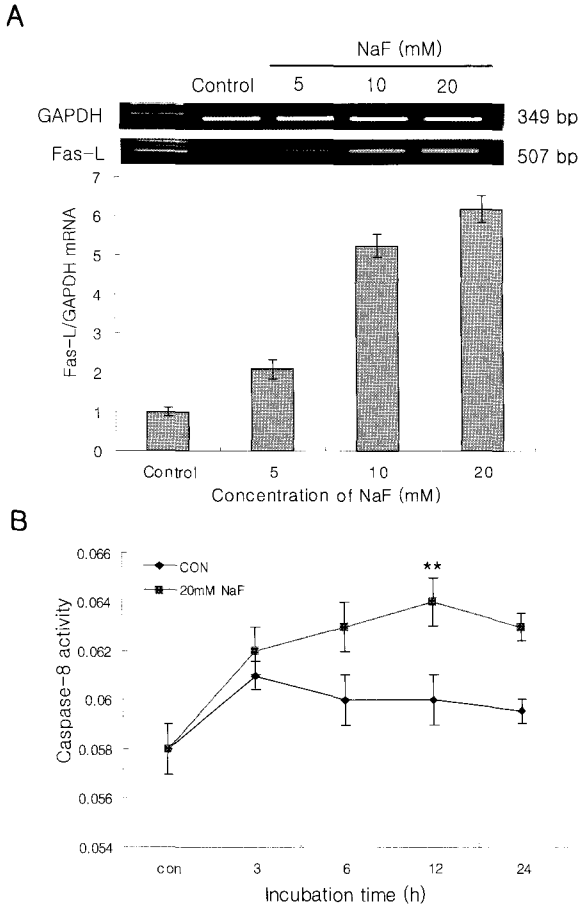

Fig. 5. Enhanced Fas-L level and caspase-8 activity in NaF-treated HGF. The mRNA levels of Fas-L, one component of death receptor assemblies, were determined by RT-PCR. Extracts of HGF cells treated with 20 mM NaF for indicated time were assayed for caspase-8 activity using colorimetric peptide substrate (IETD-*p*NA) (B). Data are mean±SD obtained from 5 independent experiments.

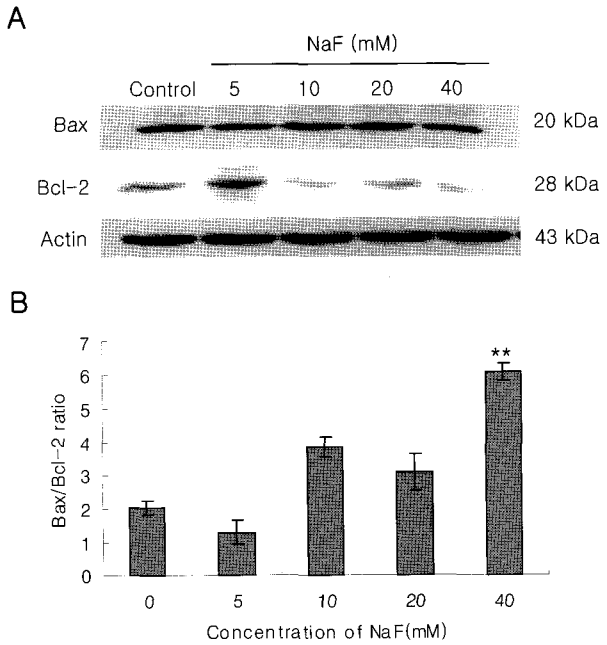

Fig. 6. Expression ratio of Bax and Bcl-2 was enhanced in NaF-treated HGF. After incubation of HGF cells with NaF for 6 h, the Bax and Bcl-2 expressions were analyzed by Western blot (A). The ratio of Bax and Bcl-2 determined by densitometer was calculated and then graphed (B). Data are mean±SD obtained from 5 independent experiments.

it did not change Bax level. As shown in Fig. 6B, the ratio of Bax to Bcl-2 was enhanced when the HGF cells were treated with higher than 10 mM NaF concentration.

DISCUSSION

Fluorides have been known to exert a variety of effects in different cell types. In bone cells, fluorides elicite potentially beneficial effects by stimulating growth of bone cells (Caverzasio et al, 1998). However, in other cells, NaF exhibited the cytotoxic effects including inhibition of protein synthesis (Aardema et al, 1989), alterations in cellular metabolism (Curnutte et al, 1979), induction of inflammatory cytokines (Refsnes et al, 1999), and apoptosis (Hirano & Ando, 1996). Even if there have been several epidemiological, pathogenetic, clinical and cytogenetic studies conducted on fluoride toxicity (Li 1993; Kleinsasser et al, 2001; Refsnes et al, 2003), the mechanism of cytotoxicity by fluoride on periodontal tissue has not yet been investigated.

In the present study, NaF was found to decrease the cell viability of HGF in a dose- and time-dependent manner, and gave rise to apoptotic morphological changes including chromatin condensation, DNA fragmentation, and cell shrinkage in HGF, demonstrating that fluoride induces apoptosis in HGF.

Caspases, which are cysteine proteases, play an important role in apoptosis. In general, caspase-3 is a key and common protease in both mitochondria- and death receptor-dependent pathways (Earnshaw et al, 1999; Bal-Price & Brown, 2000). Previous studies have shown that fluoride induces apoptosis through activating caspase-3 in human leukemia HL-60 cells (Anuradha et al, 2000; Anuradha et al, 2001). The present study showed that caspase-3 activity was enhanced in NaF-treated HGF, in concordance with previous reports in other tissues and cells. The present result implied that caspase-3 plays a pivotal role in NaF-induced apoptosis of HGF. Another polypeptide that has been known to be cleared during apoptosis is PARP, an abundant nuclear enzyme that mediates the conversion of dinucleotide NAD^+ to nicotinamide and protein-linked chains of ADP-ribose (Kaufmann et al, 1993). PARP is a target of caspase protease activity associated with apoptosis: PARP is cleaved from its 116 kDa intact form into 85 kDa and 25 kDa fragments by activated caspase-3 during apoptosis. It has been also reported that it is cleaved before or concomitant with degradation of nuclear DNA into nucleosomal fragments (Kanfmann et al, 1993), and PARP inhibitors delay or prevent apoptosis (Kaufmann et al, 1993). The present study clearly showed that NaF induced the cleavage of PARP into a clear 85 kDa fragment, thus indicating that NaF induces the activation of caspase-3 and then the cleavage of PARP, thereby causing apoptosis in

HGF.

Several studies have revealed the involvement of mitochondria in apoptosis of various cellular systems (Bossy-Wetzel et al, 1998; Mignotte et al, 1998). Many apoptotic stimuli induce the release of cytochrome *c* from the mitochondria into the cytosol, inducing proteolytic activation of caspase-9 through binding to the CED-4 homolog Aparf-1, followed subsequently by activation of caspase-3. The present study showed that NaF resulted in an increment of cytochrome *c* release from the mitochondria into the cytosol in a dose-dependent manner and enhanced the activity of caspase-9. Taken together, mitochondria-dependent apoptotic pathway has been definitely proven to be involved in NaF-induced apoptosis of the HGF, since the cytochrome *c* released into the cytosol and caspase-9 are the major molecules associated with mitochondria- dependent pathway.

The pathway of death receptor-mediated apoptosis is derived from the activation of caspase-8. Once death receptors are activated by their ligands, death receptors recruit the adaptor molecule, Fas associated death domain (FADD), followed by the activation of caspase-8. Recent studies showed that sodium fluoride regulates expression of Fas and Fas-L in the process of osteoclast-like cell apoptosis (Sun et al, 2002). These previous studies suggest a possibility that death receptor-mediated apoptosis pathway may be involved in caspase-3 activated in NaF-induced apoptosis. However, there has so far been no study to examin the death receptor-mediated apoptosis pathway in NaF-induced apoptosis of HGF. The present study showed that Fas-L levels were upregulated and caspase-8 activity was elevated in NaF-induced apoptosis of HGF. Therefore, the present study demonstrated for the first time that death receptor-mediated pathway is involved in NaF-induced apoptosis of HGF. These findings further suggest that NaF-induced apoptosis may be mediated by both mitochondria- and death receptor-dependent apoptosis pathways with involvement of caspases cascade.

The Bcl-2 family is known to be well-characterized regulators of cytochrome c release from the mitochondria into the cytosol by regulating the mitochondrial PT pore which is composed of VDAC, ANT and Cyp-D. The Bcl-2 family is classified into anti-apoptotic proteins such as Bcl-2 and Bcl-X_L, which reduce cytochrome *c* release (Gottlieb et al, 2000; Howard et al, 2002) and pro-apoptotic proteins such as Bax and Bak, which induce the release of cytochrome *c* and a loss of mitochondrial membrane potential (Starkov et al, 2002). Thus, ratio of pro-apoptotic and anti-apoptotic Bcl-2 family may be a pivotal cue to the release of cytochrome *c* from the mitochondria into the cytosol. Therefore, in the present study, the expression of Bcl-2 family was examined to elucidate the involvement of Bcl-2 family in NaF-induced apoptosis, Bcl-2 was shown to be downregulated, whereas Bax was not affected in NaF-treated HGF, thus suggesting that Bcl-2 family may play an important role in NaF-induced apoptosis of HGF. One of the notable interesting results in the present study is that the protein level of VDAC-1, a major component of mitochondrial PT pore, was increased, consequently resulting in the enhancement of cytochrome c release from the mitochondria into the cytosol. To our heat knowledge, this is the first evidence to show that the expression of VDAC, particularly VDAC-1, was altered in NaF-induced apoptosis of HGF. From these results, it is assumed that the altered expression of Bcl-2 family and overexpression of VDAC-1 appear to result in the increase of cytochrome *c* in NaF-induced apoptosis. Nevertheless, further researches are required to elucidate mechanisms underlying of VDAC-1 expression in NaF- induced apoptosis.

In summary, the present results suggest that NaF induces apoptosis in HGF cells through the mitochondria- and death receptor-dependent pathways mediated by Bcl-2 family and caspases cascade such as caspase-3, -8, and -9.

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