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Induction of Apoptosis by Sodium Fluorosilicate Treatment in Human Osteogenic Sarcoma (HOS) Cells

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Abstract. *Fluorine compounds are widely used for the prevention of caries, and recently sodium fluorosilicate has been used in water fluorination. The cytotoxic effects of sodium fluorosilicate in several osteosarcoma and oral cancer cells were evaluated in this study by measurement of inhibition of cell proliferation. Human osteogenic sarcoma (HOS) cells were the most sensitive to sodium fluorosilicate treatment. Induction of apoptosis, such as nucleosomal DNA fragmentation and the appearance of apoptotic bodies, were observed in HOS cells by agarose gel electrophoresis and by flow cytometric analysis, respectively. The molecular mechanism of apoptosis induction in HOS was investigated by Western blot analysis. The level of Bcl-2 was decreased and consequent release of cytochrome c was increased. Caspase-3 was activated and the cleavage of poly (ADP-ribosyl) polymerase was increased. In conclusion, sodium fluorosilicate induces apoptosis in HOS cells through decrease in Bcl-2, the release of cytochrome c to the cytosol and activation of caspase-3.*

Fluorine compounds are naturally present in soil, water and food. These compounds have also been used for many years as additives in toothpaste, mouthwash and drinking water in order to reduce the incidence of caries. Although fluorides are normally used in low concentrations, the possible cytotoxic effects of these compounds must be considered.

Fluoride is beneficial for reducing the incidence of caries (1-3) and for treatment of osteoporosis and prevention of fracture (4-6), probably due to an uptake of fluoride by hard tissue and the subsequent formation of fluoroapatite, and to stimulation of bone-forming cells (7-9). However, it has been reported that fluoride causes acute adverse effects including fetal poisoning. Many studies have shown the ability of sodium fluoride to cause genotoxic effects to cultured mammalian cells (10, 11). The reported genotoxicity and/or cytotoxicity were chromosomal aberration, mutation, morphological change, neoplastic transformation, sister-chromatid exchange, unscheduled DNA synthesis and formation of micronuclei (10-16). Also, cytotoxicity of sodium fluoride on human oral mucosal cells and human pulp cells was reported (17, 18). Recently, it has been reported that sodium fluoride mediates apoptosis (19, 20) and the involvement of caspase-3 activation in human leukemia cells (21, 22) and mitogen-activated protein kinase p38 in epithelial cells (23).

Recently, sodium fluorosilicate has been used in water fluoridation. In order to contribute to a better understanding of the mechanism of sodium fluorosilicate cytotoxicity, we studied and report the mechanism of sodium fluorosilicate-induced apoptosis in osteosarcoma (HOS) cells as a model system.

Materials and Methods

Fluorine compounds and cell culture. The fluorine compounds used in this study were sodium fluorosilicate (F_6Na_2Si, Fluka, Switzerland) and sodium fluoride (NaF, Sigma, USA). Human osteogenic sarcoma cells (HOS, ATCC CRL-1543) were cultured in Dulbeccos' modified Eagles medium (DMEM, Gibco BRL, USA) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 unit/ml of penicillin, and 100 mg/ml of streptomycin in a humidified environment containing 5% CO_2 at 37˚C. A253 (human submaxillary gland epidermoid carcinoma, ATCC HTB-41), KB (human oral epidermoid carcinoma, ATCC CCL 17) and MG-63 (human osteosarcoma, ATCC CRL-1427) cells were cultured in appropriate media as recommended by the supplier.

Cytotoxicity analysis. Each cell line was seeded in a 96-well plate at an adequate concentration. After 6 h, the cells were treated with the indicated concentrations of sodium fluorosilicate, sodium fluoride or vehicle, as described in the figure legends. After 48 h, the general viability of cultured cells was determined by assaying the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan (24). All experiments were performed in triplicate.

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Key Words: Sodium fluorosilicate, cytotoxicity, apoptosis, osteosarcoma (HOS) cells.

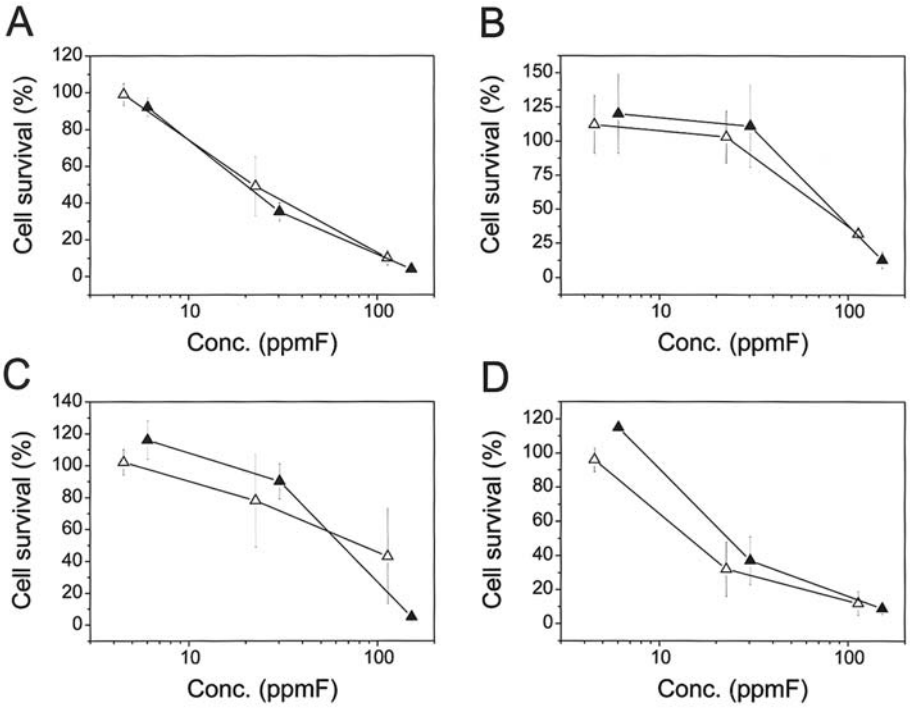

Figure 1. *Inhibition of proliferation by sodium fluorosilicate treatment. Osteo-sarcoma [HOS (A) and MG-63 (B)] and oral carcinoma [KB (C) and A253 (D)] cells were treated with 0, 10, 50, or 250 ppm of sodium fluorosilicate (F_6Na_2Si, ▲) or sodium fluoride (NaF, △) and cell survival was measured after 48 h of incubation with fluorine compounds. The relative cell survival of each cell line upon treatment of fluorine compounds was plotted against the fluorine content of the fluorine compounds.*

DNA fragmentation assay. DNA was purified as described previously (25). Human osteogenic sarcoma (HOS) cells were exposed to sodium fluorosilicate for different time periods and concentrations as described in the figure legends.

Flow cytometry analysis of apoptosis. The effects of sodium fluorosilicate on cell proliferation were evaluated by measuring the distribution of the cells in different phases of the cell cycle by flow cytometry, as described elsewhere (26). HOS cells were treated with 100 ppmF of sodium fluorosilicate for the times indicated in the figure legends.

Preparation of cytosolic extracts for cytochrome c analysis. HOS cells were treated with 100 ppmF of sodium fluorosilicate for the times indicated in the figure legends. HOS cells were harvested by centrifugation at 750 x g for 5 min, washed with ice-cold PBS and resuspended in ice-cold lysis buffer (20 mM HEPES-KOH, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 250 mM sucrose, pH 7.0). After incubation on ice for 20 min, the cells were homogenized with 10-15 strokes of a homogenizer (Bel-Art Pro., USA), and the homogenates were centrifuged at 1,000 x g for 10 min at 4˚C and at 10,000 x g for 15 min at 4˚C. The supernatants were subjected to the next centrifugation at 100,000 x g for 1 h at 4˚C and stored at -70˚C for the analysis of cytochrome c.

Western blot analysis. Western blot analysis was performed as described elsewhere (26), except that the blots were probed with an additional antibody, anti-cyochrome c (Pharmingen, USA).

Results

Cytotoxicity of sodium fluorosilicate. To determine a concentration which leads to 50% growth inhibition (IC_{50} values), we performed cytotoxicity analysis by MTT assay. HOS, MG-63, KB and A-253 cells were treated with 0-250 ppm of sodium fluorosilicate or sodium fluoride. The cells were treated with the fluorine compound for 48 h, and relative cell survivals were observed. The cell survivals decreased with increasing dose of fluorine compounds (Figure 1). The relative cell survival of each cell line was plotted against the fluorine content of sodium fluorosilicate (60.6%) and sodium fluoride (45.3%). IC_{50} values of sodium fluorosilicate were 20 ppmF for HOS, 22 ppmF for A253, 64 ppmF for KB and 82 ppmF for MG63. Similar IC_{50} values were obtained with sodium fluoride; 21 ppmF, 14 ppmF, 73 ppmF and 80 ppmF for HOS, A253, KB and MG63, respectively. MG63 cells were most resistant to both sodium fluorosilicate and sodium fluoride treatment. A253 cells were a little more sensitive to sodium fluoride. Among the cell lines used in this study, HOS cells were the most sensitive to sodium fluorosilicate treatment. Therefore, HOS cells were used to examine the effect of sodium fluorosilicate in the following experiments.

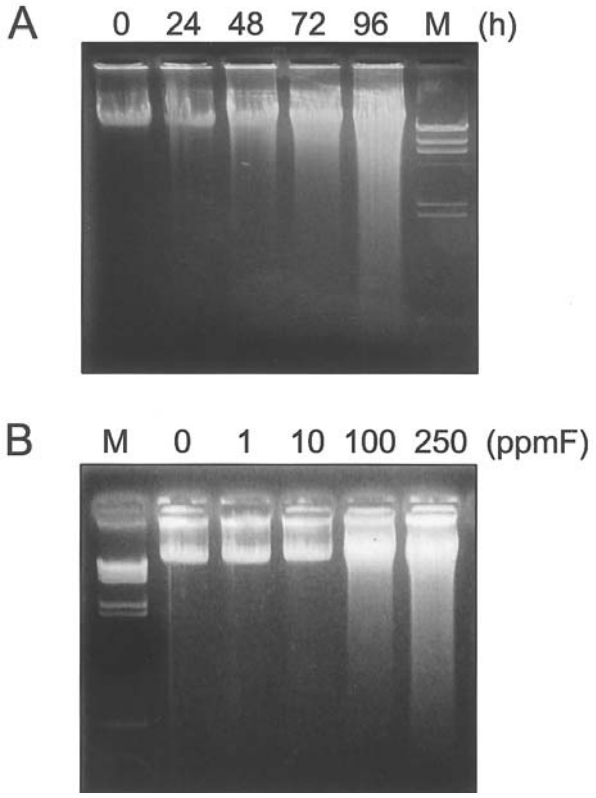

Figure 2. *Nucleosomal DNA fragmentation of sodium fluorosilicate-treated HOS cells. HOS cells were treated with 100 ppmF of sodium fluorosilicate for 0, 24, 48, 72, or 96 h (A) or HOS cells were treated with 0, 1, 10, 100 or 250 ppmF of sodium fluorosilicate for 72 h (B) and chromosomal DNA was isolated and analyzed by agarose gel electrophoresis.*

Sodium fluorosilicate induces DNA fragmentation and apoptotic bodies. In order to determine whether the cytotoxic effect of sodium fluorosilicate was associated with the induction of apoptosis, nucleosomal DNA fragmentation patterns by sodium fluorosilicate treatment in HOS cells were observed in time- and dose-dependent manners. The nucleosomal DNA fragmentation is the most well-known event that occurs in apoptotic cells. As shown in Figure 2, analysis of DNA extracted from sodium fluorosilicate-treated cells revealed a progressive time-dependent increase in non-random fragmentation of DNA (Figure 2A). Similarly, DNA fragmentation was increased in a dose-dependent manner. At lower concentrations of sodium fluorosilicate, 1 and 10 ppm, little fragmentation of DNA was observed, but significant DNA fragmentation was observed at higher concentrations of 100 and 250 ppm (Figure 2B).

To further analyze the effect of sodium fluorosilicate on the induction of apoptotic bodies in HOS cells, cell cycle analysis was performed using flow cytometry. HOS cells were incubated with 100 ppmF of sodium fluorosilicate for 0, 24, 48, 72 or 96 h and the cell cycle distribution was analyzed. As shown in Figure 3, there was a progressive increase in apoptotic bodies in sodium fluorosilicate-treated cells as the incubation time increased; 13.0%, 25.0% 45.5% and 57.5% of the cells were apoptotic cells after 24, 48, 72 and 96 h of sodium fluorosilicate treatment, respectively. There were no noticeable changes in the cell cycle distribution.

Western blot analysis. To begin to address the mechanism by which sodium fluorosilicate causes apoptosis, we first examined whether caspase-3 protease is involved in the cell death response in HOS cells. HOS cells were cultured in 100 ppmF of sodium fluorosilicate-containing media for 0, 1, 7, 24, 48, 72, or 96 h. Total protein was isolated at the indicated time points and caspase-3 protein expression was estimated (Figure 4). A decline in the 32 kDa pro-caspase-3 level was observed, which implied an increase of the caspase-3 level. To be certain of the increase of pro-caspase-3 cleavage and enzymatic activation, PARP cleavage was measured, which results from caspase-3 protease activation. Increase of 85 kDa fragment formation and decrease of 116 kDa pro-PARP were observed in a time-dependent manner, which means increase of PARP cleavage. It is reported that apoptosis involves a disruption of mitochondrial membrane integrity that is decisive for the cell death process. We next evaluated the effect of sodium fluorosilicate on the release of mitochondrial cytochrome c into the cytosol. Further, changes in the level of Bcl-2, an anti-apoptotic molecule known to inhibit mitochondrial megachannel opening, was monitored. As shown in Figure 4, treatment with sodium fluorosilicate induced the release of mitochondrial cytochrome c into the cytosol. The level of Bcl-2 slightly decreased upon treatment with sodium fluorosilicate. Taken together, these results suggest that the mitochondria play an important role in the activation of caspase-3, proteolytic cleavage of PARP and the induction of apoptosis on treatment with sodium fluorosilicate in HOS cells.

Discussion

Our study demonstrated that sodium fluorosilicate, a fluorine compound used for caries prevention, inhibits proliferation of several cancer cells including osteosarcoma (HOS and MG63) cells, submaxillary gland carcinoma (A253) cells and oral carcinoma (KB) cells at higher concentrations. The present study also demonstrated that sodium fluorosilicate induces apoptosis in HOS cells *via* caspase-3 activation and subsequent cleavage by activated DNase.

Apoptosis is an active process that ultimately leads to the activation of endonuclease and cleavage of DNA into fragments of about 180-200 base pairs (27, 28). The observed breakage of the cellular DNA of HOS cells by sodium fluorosilicate, detected by agarose gel electrophoresis, showed the characteristics of apoptotic cell death. Besides the biochemical indicators of DNA

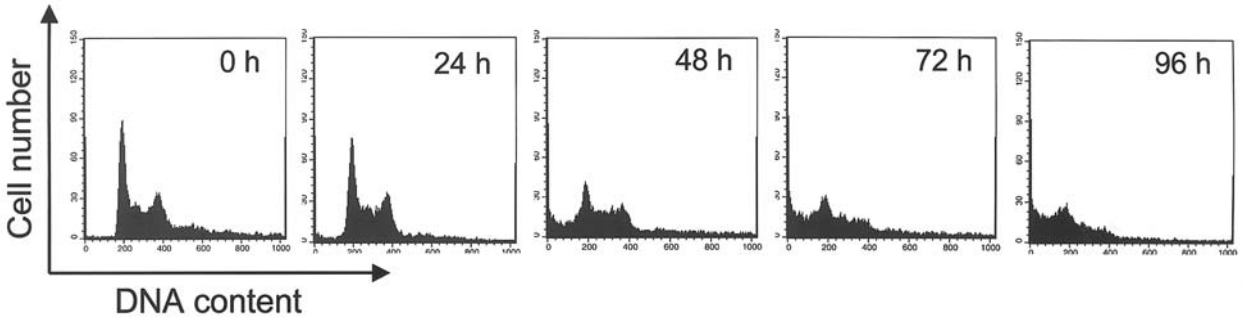

Figure 3. *Flow cytometric analysis of apoptosis induced by sodium fluorosilicate treatment. HOS cells were treated with 100 ppmF of sodium fluorosilicate for the indicated periods of time, fixed and stained with propidium iodide. Cells were analyzed by flow cytometry as described in Materials and Methods.*

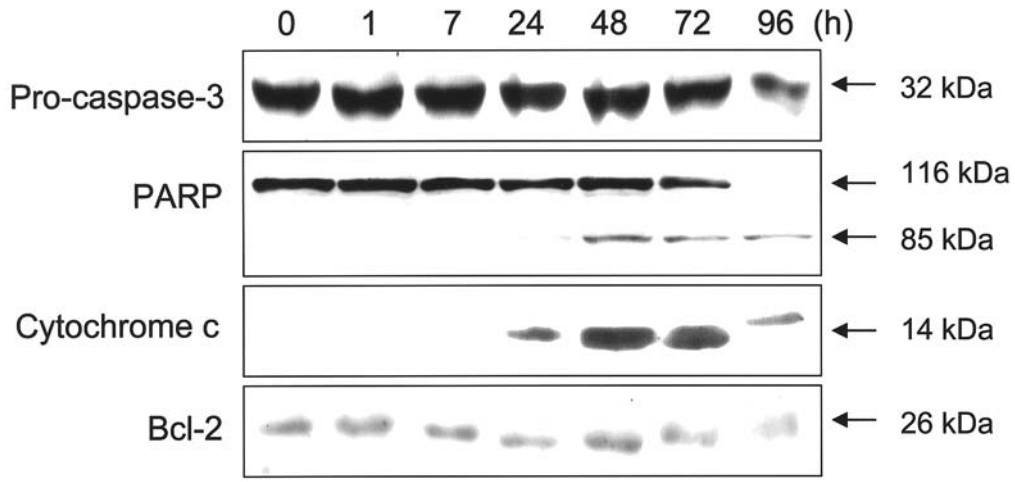

Figure 4. *Regulation of caspase-3, PARP, cytochrome c and Bcl-2 immunoreactivity levels by sodium fluorosilicate treatment. HOS cells were treated with 100 ppmF of sodium fluorosilicate for the indicated periods of time. Cells were lysed and the resulting cell lysates were subjected to immunoblotting with anti-caspase-3, anti-PARP, anti-cytochrome c and anti-Bcl-2 antibodies as described in Materials and Methods.*

fragmentation, cells with sub-G1 DNA content were detected by flow cytometry. These cytofluorimetric characteristics showed very similar time dependence to the biochemical assays of DNA fragmentation. In this study, it was shown that sodium fluorosilicate treatment induced the release of cytochrome c into the cytosol. Cytochrome c normally resides in the mitochondrial intermembrane space, where it serves as a transducer of electrons in the respiratory channel. Mitochondrial release and cytosolic accumulation of cytochrome c and activation of caspase-3 has been shown during apoptosis (29, 30). Apaf-1, cytochrome c and caspase-9 participate in the activation of caspase-3 (31). It seems that the activation of caspase-3 results from the decrease in Bcl-2 in sodium fluorosilicate-treated cells. The proto-oncogene Bcl-2 has been shown to be an intracellular suppresor of apoptosis and thus serve a cyto-protective function (32, 33). Our results suggest that sodium fluorosilicate treatment slightly decreases Bcl-2 immunoreactivity levels in HOS cells. The induction of apoptosis by sodium fluorosilicate may be caused by a decrease in Bcl-2 level and the release of cytochrome c into the cytosol.

In summary, sodium fluorosilicate inhibited the cell proliferation of several oral cancer and/or osteosarcoma cells. Treatment with sodium fluorosilicate induced apoptosis in HOS cells by a decrease in Bcl-2 levels, a concomitant release of cytochrome c to the cytosol and subsequent activation of capsase-3. Taken together, these findings provide a new insight into the molecular mechanism by which sodium fluorosilicate induces apoptosis.

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