The effects of simulated orthodontic forces such as centrifugal force or water pressure on sodium fluoride (NaF)-induced cytotoxicity against mouse osteoblast-like cells MC3T3-E1 were investigated. Loading with centrifugal force (44.5 g/cm²) or water pressure (5 g/cm²) slightly reduced the cell proliferation and additively enhanced the cytotoxic activity of millimolar concentrations of NaF. NaF induced the appearance of phosphatidylserine at outer cell membrane (detected by Annexin staining) but failed to induce caspase-3 activation even under the water pressure. On the other hand, NaF induced autophagic phenotype characterized by the formation of acidic organelles (detected by acridine orange staining). NaF did not increase, but rather dose-dependently reduced the alkaline phosphatase activity, with or without the loading of water pressure. The present study demonstrates that centrifugal force and water pressure partially enhanced the caspase-independent cytotoxicity of NaF against osteoblasts. These simulated orthodontic forces may be a new factor that affects the physiological activity of NaF.

The effects of orthodontic force on the living body have been investigated mainly by histochemical approaches using experimental animals. The loading of orthodontic force in the mice induced the vascular endothelial growth factor receptor (VEGFR) around the blood vessels (1). Experimental tooth movement in the mice induced the expression of connexin-43 (2) and matrix metalloproteinase (MMP-8, MMP-13) (3) in the periodontal ligament cell, and alkaline phosphatase (ALP) in osteoclasts (4). On the other hand, external pressure induced the apoptosis in cultured human lymphoblasts (5). Loading of centrifugal force induced the expression of Runt-related transcription factor 2 (RUNX2) gene in the mouse osteoblasts (6). Centrifugal force also induced the expression of tropoelastin (7) and enhanced the expression of MMP-1 gene (8) in human periodontal ligament fibroblasts. High pressure induced caspase activation in murine erythroleukemia cells (9). However, the effect of external pressures (that can be considered as “simulated orthodontic forces”) on the drug sensitivity of oral cells has not been investigated thus far.

Patients who receive orthodontic treatment belong to the high-risk of caries group. Orthodontists therefore have to pay special attention so as to prevent caries. The prevention of caries is usually performed by cleaning of the tooth and the topical treatment of fluorides. Higher concentrations of fluoride play an important role in the prevention and control of caries in the high-risk group. Residual fluoride after application has been estimated to be at most 21-71 mM, a concentration much below the acute toxicity (10). Recently, NaF has been reported to induce the apoptosis in human promyelocytic leukemia cells HL-60 (11), and that loading of centrifugal force or water pressure increased the sensitivity of HL-60 cells and human periodontal ligament fibroblasts, respectively, to NaF (12, 13). Millimolar concentrations of NaF reduced the protein synthesis and the ATP level in human gingival fibroblast (14) and the growth of mouse rat calvaria cells (15). On the other hand, the effects of higher concentrations of NaF on the periodontal tissues and especially osteoblastic cells under the orthodontic force have been studies to a much lesser extent. Considering this, the effects of simulated orthodontic forces (such as centrifugal force and water pressure) on the NaF-induced cytotoxicity against mouse osteoblastic cells MC3T3-E1 (16) were investigated.

Key Words: Osteoblast, MT3C3-E1, fluoride, orthodontic force, water pressure, centrifugal force, cytotoxicity.
Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: alpha-minimum essential medium (αMEM; Gibco, Grand Island, NY, USA), fetal bovine serum (FBS) (JRH, Bioscience, Lenexa, KS, USA), dimethyl sulfoxide (DMSO), 3-(4,5-dimethylthiazol-2-yl)-2,2-diphenyltetrazolium bromide (MTT) (Sigma Chem Co., Louis, MO, USA); RNase A, proteinase K (Boehringer Ingelheim GmbH, Ingelheim, Germany); acridine orange (Wako Pure Chem., Ltd., Osaka, Japan); MEBCYTO-Apoptosis kit (Medical Biology Lab, Nagano, Japan).

Cell culture. Osteoblastic cells MC3T3-E1 derived from newborn mouse calvaria (16) (kindly provided by Dr. Kikuchi, Meikai University) were cultured in αMEM supplemented with 10% heat-inactivated FBS and incubated at 37˚C in a 5% CO2 incubator. Adherent cells were washed with phosphate-buffered saline without Ca and Mg [PBS(-)], detached with 0.25% trypsin-0.025% EDTA [in PBS(-)] and then subcultured.

Exposure to centrifugal force. MC3T3-E1 cells were inoculated onto a 96-microwell plate (1.5×10^4 cells/well), a 24-well plate (1×10^5 cells/well) or a 6 well plate (3×10^5 cells/well) (Becton, Dickinson, Labware, NJ, USA) and incubated for 24 hours at 37˚C to allow the complete attachment to the plates. The cells were then loaded with the centrifugal force (147 xg) (Appratus: KOKUBO KR/702, Kokubo Shoji, Tokyo) for 30 minutes, or stood at room temperature (26˚C) (control) for the same period (Figure 1A). According to the formula by Redlich (7), the centrifugal force was calculated to be approximately 44.5 g/cm², below the orthodontic force used in the experimental tooth movement (max: 80 g/cm²) (17).

Exposure to water pressure. The loading of water-pressure to the cells was performed through a modification of previous reports (18, 19). MC3T3-E1 cells (5x10⁵ cells) were inoculated onto the 3.5-cm culture dish (Becton, Dickinson, Labware, NJ, USA) and incubated for 48 hours at 37˚C. The medium was then removed and the dish was placed into a beaker. Medium was added up to the 5 cm mark in order to load the cells with a water pressure of 5 g/cm².

The medium was added up to the 0.2 cm mark for the control group cells (water pressure of 0.2 g/cm²) (Figure 1B).

Assay for viable cell number. The cells were replaced with fresh medium containing various concentrations of NaF (0~16 mM) and exposed to centrifugal force (30 min at 145 x g, 44.5 g/cm²) (Figure 1A) or water pressure (0.2 [control] or 5 g/cm²) (Figure 1B). After incubation for 24 hours, the number of viable cells excluding the trypan blue was then determined with hemocytometer or by the MTT method as follows. The cells in 96-microwell plates were incubated for a further 4 hours with 0.2 mg/mL MTT in culture medium. After removal of the medium, the formazan formed was dissolved with 0.1 mL DMSO and the absorbance at 540 nm was determined by microplate reader (Biochromatic Labsystem, Helsinki, Finland).

Assay for caspase activation. MC3T3-E1 cells (1x10⁶ cells) were inoculated on a 35 mm plastic dish and incubated for 24 hours. The cells were replaced with fresh culture medium containing various concentrations (0~8 mM) of NaF and incubated for 24 hours under the simulated orthodontic forces. The cells were then washed twice with PBS(-) and cells were harvested by scraping with a rubber policeman with 0.2 mL of lysis solution (MBL, Nagoya, Japan). The lysate was stood for 10 minutes on ice. The supernatant obtained after centrifugation for 3 minutes at 11,000 xg was collected and 50 μL of it (equivalent to 200 μg protein) was mixed with 50 μL 2x reaction buffer (MBL) containing substrate for caspase-3 (DEVD-pNA (p-nitroanilide)). The enzymic cleavage of the substrate was done by incubation at 37˚C for 4 hours and the absorbance at 405 nm of the liberated chromophore pNA was measured by a plate reader (20).

Annexin V staining. MC3T3-E1 cells (3x10⁴ cells) were inoculated onto a glass bottom dish (Matsunami Glass, Tokyo) and incubated for 24 hours. The cells were replaced with fresh culture medium containing 4 mM NaF and incubated for 4 hours. Cells were washed twice with PBS(-) and suspended in 85 μL of binding buffer (Medical Biology Laboratory, Nagano). The cells were then stained for 15 minutes with 10 μL of Annexin V-FITC and 5 μL of propidium iodide (PI), and observed under laser scanning microscope (LSM 510, Carl Zeiss Microscopy) (21).
Acridine orange staining. MC3T3-E1 cells (3×10⁴ cells) were inoculated onto a glass bottom dish (Matsunami Glass, Tokyo) and incubated for 24 hours. The cells were replaced with fresh medium containing 0 or 4 mM NaF and further incubated for 24 hours. After washing twice with PBS(-), the cells were stained for 20 minutes with 10 μg/mL acridine orange and observed with a confocal laser scanning microscope (excitation: 448 nm, emission: green 505-530 nm, red <650 nm) (LSM 510, Carl Zeiss Microscopy, Berlin, Germany) (20).

Effect of NaF on ALP activity in MC3T3-E1 cells. MC3T3-E1 cells (1×10⁶ cells) were inoculated on 35 mm plastic dish and incubated for 24 hours. The cells were replaced with fresh culture medium containing various concentrations (0~8 mM) of NaF and incubated for 24 hours under the simulated orthodontic forces. Cells were washed twice with PBS(-) and harvested by scraping with a rubber policeman with 0.2 mL of lysis solution (0.2 % NP-40 in PBS [-]). The lysate was stood for 10 minutes on ice. The supernatant obtained after centrifugation for 5 minutes at 11,000 × g was collected and 20 μL of it was mixed with 100 μL of substrate solution (p-nitrophenylphosphate disodium). After incubation at 37˚C for 15 minutes, the reaction was stopped by 80 μL of 0.2 M NaOH, and the absorbance at 405 nm was measured by microplate reader (16).

Statistical treatment. Significance of the difference between the two groups were evaluated by Student’s t-test with p<0.05.

Results

Cytotoxic activity. NaF below 2 mM did not significantly affect the growth of mouse osteoblast-like cells MC3T3-E1 whereas NaF above 4 mM significantly (p<0.05) reduced the number of viable cells that excluded the trypan blue (Figure 2A). Loading of centrifugal force alone reduced the viable cell number slightly (approximately by 10%), and additively enhanced the cytotoxicity of NaF, as demonstrated by either MTT method (Figure 2B) or cell counting method (Figure 3B). Loading of water pressure alone reduced the viable cell number approximately by 12% and additively enhanced the cytotoxicity of NaF (Figure 3B).

Failure of apoptosis induction. It has been reported that caspase-3 is activated during the apoptotic cell death via either extrinsic (22) or mitochondria-mediated intrinsic (23) pathways. The possibility that NaF may have induced the activation of caspase-3 in MC3T3-E1 cells was next investigated. Although actinomycin D time-dependently activated caspase-3 (Figure 4A), NaF (0-8 mM) did not do so, regardless of the loading of water pressure (Figure 4A and 4B). Loading of water pressure did not further enhance the caspase-3 activation induced by actinomycin D (Figure 4B).

Whether NaF induced the appearance of phosphatidylserine at the outer cell membrane (known as early apoptosis marker) was next investigated using Annexin V staining technique (24). NaF treatment induced the production of Annexin-positive MC3T3-E1 cells (Figure 5). However, nuclei were stained with PI, suggesting the possible occurrence of membrane injury by NaF treatment.

Induction of acidic organelles. The possibility that NaF induced autophagy was next investigated. NaF induced the formation of acidic organelles (Figure 6B), detected by acridine orange which has a high affinity to autophagolysosomes (25). Water pressure alone weakly induced the formation of acid organelles (Figure 6C), and the combination of NaF with water pressure induced the formation of acid organelles to a similar extent as that attained by NaF alone (Figure 6D).

Inhibition of alkaline phosphatase. NaF dose-dependently inhibited the ALP activity in MC3T3-E1 cells (Figure 7).
NaF (4-8 mM) and actinomycin D (10 μg/mL) inhibited the ALP activity approximately by 80-90%. Loading of water pressure slightly enhanced the inhibitory activity of lower concentration (1-2 mM) of NaF (Figure 7).

**Discussion**

It has been previously reported that pressure induces G2 arrest of cell cycle and apoptosis in mouse erythroleukemia cells via the activation of caspase (9). The magnitude and exposure time of a centrifugal force positively regulates the cytotoxicity of NaF (12). These reports suggest that the external pressure is one of the causative factors of NaF cytotoxicity. The apoptosis induced by vitamin C in HL-60 cells was modified by various factors such as temperature (26), pH (27), medium components (i.e., amino acids) (28) and oxygen tension (29). NaF induced apoptosis in HL-60 cells (11), however, the study of NaF on the cells derived from normal periodontal tissues has been limited. This has urged the investigation of the combination effect of NaF and external forces on NaF sensitivity in osteoblastic cells.

It has also been reported that centrifugal force (40.3 g/cm²) induced the transient expression of Runt-related transcription factor 2 (RUNX2) in human osteoblasts (6). The responses of osteoblasts against simulated orthodontic forces ex vivo have been reported (30-33). The effect of external pressures (centrifugal force and water pressure) on the responses of mouse osteoblastic cells MC3T3-E1 to NaF cytotoxicity was investigated. Higher concentrations of NaF (>4 mM) were found to induce cytotoxicity in a short time (24 hours) even without external pressure suggesting a local cytotoxic action of NaF against osteoblasts. However, external forces such as centrifugation or water pressure produced a weak additive cytotoxicity to NaF.
Figure 5. Induction of early apoptotic marker by NaF. MC3T3-E1 cells were treated for 4 hours without (G) (control) or with 10 \( \mu g/mL \) actinomycin D (A-C) or with 4 mM NaF (D-F) on the glass bottom dish. The cells were then washed and stained with Annexin V-FITC and propidium iodide (PI). PI staining (A, D), Annexin V-FITC staining (B, E). Merge (C, F).

Figure 6. Induction of the formation of acidic organelles by NaF. MC3T3-E1 cells were treated for 24 hours without (control) (A), with NaF (4 mM), water pressure (C) or NaF + water pressure (D) on the glass bottom dish. The cells were then washed with PBS(-), stained with acridine orange staining for 20 minutes.
The present study demonstrated the induction of early apoptosis marker (such as production of Annexin V-positive cells) by NaF, without the induction of a late apoptosis marker (caspase-3 activation). On the other hand, NaF induced autophagy, characterized by the formation of acidic organelles (secondary lysosomes). This suggests that NaF induced the apoptosis at earlier stage, followed by a caspase-independent cell death (34), possibly autophagy. The type of cell death (either apoptosis, autophagy or necrosis) induced by NaF may depend on the cells used (35).

ALP is distributed into various tissues such as liver, bone and small intestine and has been used as an osteogenic marker (36). It has been reported that ALP activity in the osteoclasts is elevated during tooth movement (4). The present study demonstrated that NaF treatment induced the decline of ALP activity in MC3T3-E1 cells, with or without water pressure. This may be the secondary result of cytotoxicity induced by NaF.

In conclusion, the present study demonstrates that simulated orthodontic force, such as centrifugal force and water pressure, partially enhanced the caspase-independent cytotoxicity of NaF against mouse osteoblastic cells. External forces such as centrifugal force and water pressure may be a new factor that affects the physiological activity of NaF.

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