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Changes in Fluoride Sensitivity During *In Vitro* Senescence of Normal Human Oral Cells

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Abstract. *We have previously reported that sodium fluoride (NaF) showed slightly higher cytotoxicity against human oral tumor cell lines than normal human oral cells. Possible changes in the NaF sensitivity of three normal human oral cell types (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF) during in vitro ageing were investigated in the present study. When these cells were subcultured at 1:4 split ratio every week, their saturation density declined with increasing population doubling level (PDL), and they ceased to divide when they reached 20 PDL. Mitochondrial function, evaluated by MTT stainability per cell basis, was elevated at the terminal phase. NaF dose-dependently reduced the viable cell number, but did not show any beneficial (growth promoting) effect (so-called "hormesis") at lower concentrations. NaF produced large DNA fragments, without induction of internucleosomal DNA fragmentation, possibly due to weak activation of caspases -3, -8 and -9. Higher concentrations of NaF were required to reduce the number of viable senescent cells than younger cells, indicating that cells become resistant to cytotoxicity of NaF with in vitro ageing.*

Fluoride oral treatment has shown both beneficial and adverse effects (1). Fluoride prevents the development of dental caries at relatively low concentrations, but produces fluorosis at higher concentrations. Fluoride shows cytotoxic activity against various cultured cells, to extents varing from cell to cell (2-4). We have recently reported that sodium fluoride (NaF) showed slightly higher cytotoxicity against human tumor cell lines (oral squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG, promyelocytic leukemia HL-60) than normal human cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF), with an approximate tumor specificity index of 1.8 (5). The cytotoxic activity of NaF against tumor cells has been reported to be modified by antioxidants, oxidants, metals, saliva (6), antitumor agents (7) and endodontic agents (8). There is a possiblity that NaF sensitivity may also fluctuate during the ageing process of these human normal cells, with limited *in vitro* lifespan. NaF has been used in several studies with ageing animal models (9-17). NaF was shown to induce mutation by its genotoxic effect (9) and to increase the crystallinity of the bioapatite (10), in similar manners to those observed during the ageing process. During ageing, the responses to β-adrenergic receptor agonists (11-13), dopamine (14) and thymulin (15, 16) have been reported to decline, due to the decrease in either adenylate cyclase activation (11, 13) or receptor density (13, 14), both causing the defect of receptor-G protein coupling. During development, an ATP-dependent process seems to be important for the formation and/or compaction of the myelin sheath, and an ATP-independent mechanism is likely to play a role in myelin maintenance (17). NaF has been used for these studies as a stimulator of adenylate cyclase (11-16) and as a glycolysis inhibitor (17). The extent of adenylate cyclase stimulation by NaF has been contradictory, with reports of both decreased activity (11) and no change (13). There have been no detailed data regarding the change in NaF sensitivity

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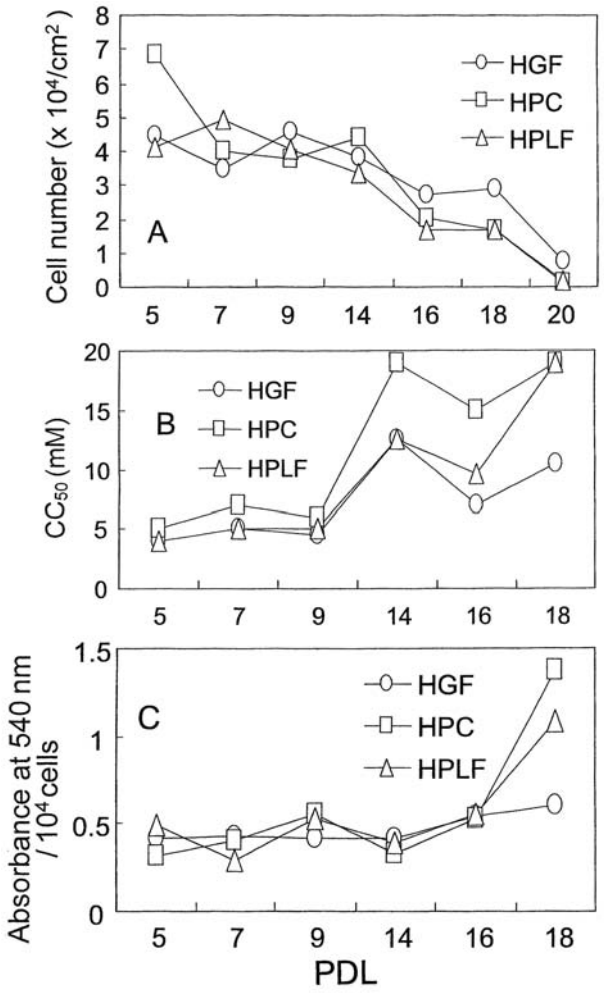

Figure 1. *Changes in saturation density (A), NaF sensitivity (B) and mitochondrial function (C) during ageing of HGF (○), HPC (□) and HPLF (△). (A) The saturation cell density of these cells at the indicated PDL. (B) NaF sensitivity was expressed as CC_{50}, which was determined from the data of Figure 2. (C) Mitochondrial function was determined by incubating the cells with MTT reagent for 4 hours and expressed as absorbance at 540 nm/10^4 cells.*

during ageing at the cellular level, prompting our investigation of how the sensitivities of three normal cell types (HGF, HPC, HPLF) change during the ageing process. Many toxic agents, endocrine disruptors, as well as radiation affect growth in a biphasic manner (so-called "hormesis"), with growth stimulation (beneficial) effects at lower concentration and growth inhibitory (adverse) effects at higher concentration (18). Whether NaF shows such biphasic effects on the growth of the three normal human cell types harvested at different population doubling levels (PDL) was also investigated.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the indicated companies: Dulbecco's modified Eagle medium (DMEM) (Gibco BRL, Grand Island, NY, USA); 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) (Sigma Chem. Ind., St. Louis, MO, USA); fetal bovine serum (FBS) (Gemini Bio-Products, Woodland, CA, USA) ; NaF, dimethyl sulfoxide (DMSO) (Wako Pure Chem Ind, Osaka, Japan).

Cell culture. HGF, HPC and HPLF cells were cultured at 37˚C in DMEM, supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere. These cells were isolated from the gingival biopsies from a 10-year-old girl, according to the Guideline of Meikai University Ethics Committee, after obtaining informed consent from the patient. The cells were subcultivated at the split ratio of 1:4 once a week, and the medium was changed once between the subcultures.

Cytotoxic activity assay. HGF, HPC and HPLF cells were inoculated at 12 x 10^3 cells/well in 96-microwell (Becton Dickinson Labware, NJ, USA), unless otherwise stated. After 24 hours, the medium was removed by suction with an aspirator, and replaced with 0.1 mL of fresh medium containing different NaF concentrations. Cells were incubated for another 24 hours, and the relative viable cell number was then determined by the MTT method. In brief, cells were replaced with fresh culture medium containing 0.2 mg/mL MTT and incubated for another 4 hours. The cells were lysed with 0.1 mL DMSO, and the absorbance at 540 nm (A_{540}) of the cell lysate was determined, using a microplate reader (Labsystem, Helsinki, Finland). The A_{540} of the control cells was usually in the range of 0.40 to 0.90. The 50% cytotoxic concentration (CC_{50}) was determined from the dose-response curve.

DNA fragmentation assay. Cells were lysed with 50 μL lysate buffer [50 mM Tris-HCl (pH 7.8), 10 mM EDTA, 0.5% (w/v) sodium *N*-lauroyl-sarcosinate]. The solution was incubated with 0.4 mg/mL RNase A and 0.8 mg/mL proteinase K for 1-2 hours at 50˚C. After incubation, the lysate was mixed with 50 μL NaI solution [7.6 M NaI, 20 mM EDTA-2Na, 40 mM Tris-HCl, pH 8.0], and then with 100 μL of ethanol. After centrifugation for 20 minutes at 20,000 xg, the precipitate was washed with 1 mL of 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). Samples (10-20 μL) were applied to 2% agarose gel electrophoresis in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA). DNA from apoptotic HL-60 cells, induced by UV irradiation (19) and DNA molecular marker (Takara), were used for calibration. The DNA fragmentation pattern was examined in photographs taken under UV illumination.

Caspase activation assay. Cells were washed with PBS and lysed in lysis solution (MBL, Nagoya, Japan). After standing for 10 minutes on ice followed by centrifugation for 5 minutes at 10,000 xg, the supernatant was collected. The lysate (50 μL, equivalent to 200 μg protein) was mixed with 50 μL 2x reaction buffer (MBL) containing substrates for caspase-3 (DEVD-*p*NA(*p*-nitroanilide)), caspase-8 (IETD-*p*NA) or caspase-9 (LEHD-*p*NA). After incubation for 2 hours at 37˚C, the absorbance at 405 nm of the liberated chromophore (*p*NA) was determined according to the manufacturer's instructions (MBL).

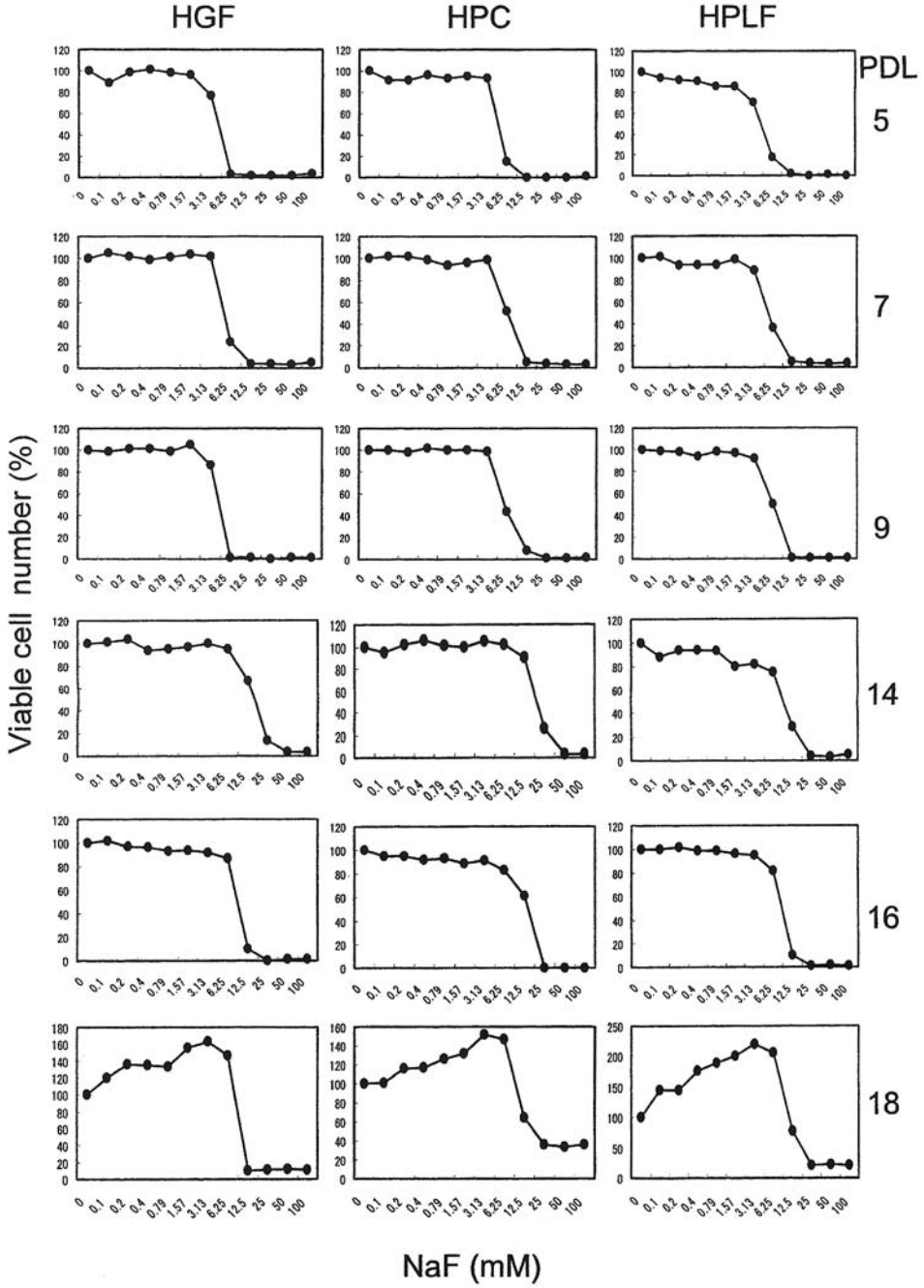

Figure 2. *Cytotoxic activity of NaF against three normal oral cells at different PDLs. Near confluent cells at the indicated PDL were treated for 24 hours without (control) or with the indicated concentrations of NaF, and the viable cell number was determined by MTT method and expressed as % of control. Reproducible results were obtained in another independent experiment.*

Results

When normal human cells (HGF, HPC, HPLF) were subcultured at 1:4 split ratio every week, their saturation density declined gradually with increasing PDL (Figure 1A). They ceased to grow when they reached 20 PDL. The effect of ageing on NaF sensitivity was investigated and the data are provided in Figure 2. NaF dose-dependently reduced the viable cell number, but did not induce any beneficial (growth promoting) effect (so-called "hormesis") in any of the three cell types, except for those cells at the terminal phase (Figure 2). From the dose-response curve in Figure 2,

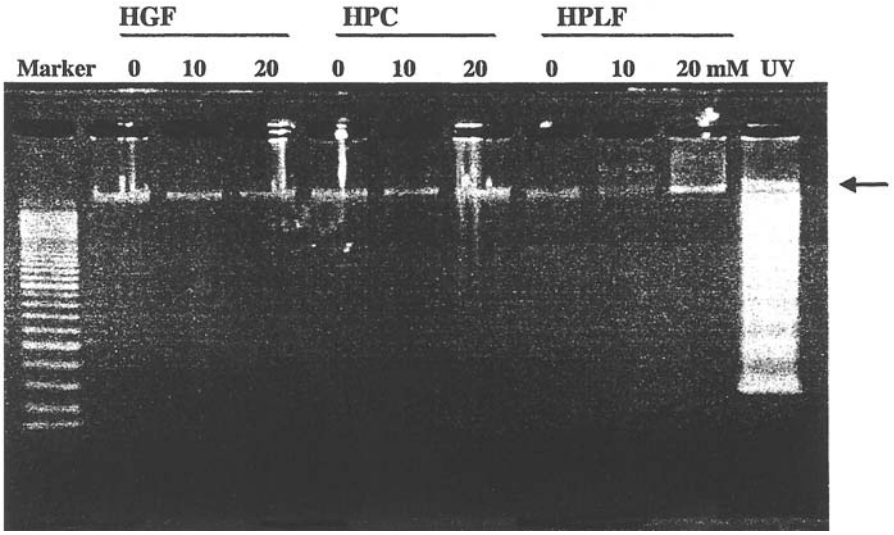

Figure 3. *Induction of the production of large DNA fragment by NaF. Near confluent cells (7 PDL) were incubated for 6 hours with the indicated concentrations of NaF, and DNA was extracted and applied to agarose gel electrophoresis. Marker, DNA molecular marker. UV, DNA from apoptotic HL-60 cells induced by UV irradiation (ref. 19).*

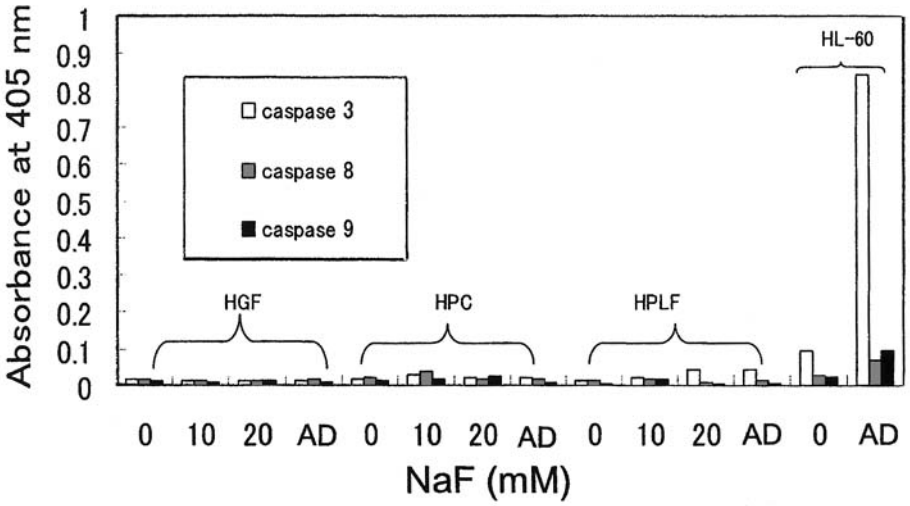

Figure 4. *Activation of capases -3, -8 and -9 by NaF. Near confluent cells (8 PDL) were incubated for 4 hours with the indicated concentrations of NaF or 1 μg/mL actinomycin D (AD, used as a positive control), and caspase activity was determined by substrate cleavage assay.*

we calculated the CC_{50} of NaF as an index of sensitivity to NaF cytotoxicity (Figure 1B). We found that the CC_{50} of NaF slightly increased after 14 PDL, and higher CC_{50} values were maintained thereafter (Figure 1B), indicating that cells became resistant to NaF with ageing. Mitochondrial function, evaluated by MTT stainability per cell basis, did not decline, but instead slightly increased at the terminal phase of ageing (18 PDL) (Figure 1C).

NaF failed to induce internucleosomal DNA fragmentation in the normal cell lines, in contrast to its effect on apoptotic HL-60 cells (induced by UV irradiation) (Figure 3). Higher NaF concentrations (20 mM) induced the production of large DNA fragments in HPC and HPLF cells (indicated by arrow in Figure 3). NaF activated caspases -3, -8 and -9, to much lesser extents than that attained by actinomycin D-treated HL-60 cells (Figure 4).

Discussion

The present study demonstrates, for the first time to our knowledge, that three normal human oral cell lines became resistant to NaF during *in vitro* ageing. This might

be due to the age-related increase of cell (20) and nuclear (21) volume and cellular protein content (22, 23), which may dilute NaF near the target site. However, no NaF target molecule has yet been identified in cultured cells, although enolase in the glycolytic pathway (17, 24), adenylate cyclase (11-16) and others have been hypothesized as candidates. It also remains unclear whether NaF is actually incorporated into the cells, especially in the presence of calcium and, if so, whether the incorporation and excretion (*via* some transporter proteins) change during the ageing process.

The results reported herein also demonstrate that NaF did not induce a biphasic growth modulation effect. Thus, hormesis is not a phenomenon universal to all toxic agents. Codeinone, a morphine analogue with apoptosis-inducing activity (25), has also been shown not to induce hormesis in human leukemia HL-60 cells (Takeuchi *et al.*, submitted). More extensive studies with a variety of toxic compounds are required to confirm the universality of hormesis in different biological systems.

NaF did not induce apoptotic characteristics, such as internucleosomal DNA fragmentation or caspase activation in three normal cells. The lack of induction of DNA fragmenation might be due to the limited extent of caspase -3, -8 and -9 activation. Further investigations should examine the possibility that NaF may induce non-apoptotic cell death such as necrosis (characterized by cell swelling) or autophagy (characterized by vacuolization and expression of ATG 7 and beclin 1) in normal cells (26-28).

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