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Fluoride mediates apoptosis in osteosarcoma UMR 106 and its cytotoxicity depends on the pH

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Abstract Although an excess intake of fluoride has been reported to cause skeletal fluorosis, very little is known about the mechanism of adverse effects of fluoride on bone. In the present study cytotoxic effects of fluoride were studied using the osteosarcoma cell line, UMR 106. The DNA ladder formation upon agarose electrophoresis and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining revealed that UMR 106 underwent apoptosis following exposure to 5 mM fluoride for 8 h. On the other hand exposure to A23187, a calcium ionophore, caused necrosis while co-exposure to fluoride and A23187 inhibited fluoride-mediated apoptosis in UMR 106. The proliferation of UMR 106 cells cultured for 6 days in the presence of 0.5 mM fluoride was significantly decreased compared to the control culture. The cytotoxic effects of fluoride were modulated by both the cell density and the pH of the culture medium. The fluoride-induced viability loss in UMR 106 was enhanced in culture of high cell-density and inversely correlated with pH of the culture medium. Enhancement of fluoride cytotoxicity at acidic pH was also observed in rat alveolar macrophages and RAW 264, a macrophage cell line. The results suggest that fluoride-mediated apoptosis and culture conditions, including pH of the medium, should be taken into consideration to evaluate toxicity of fluoride in vitro.

Key words Fluoride · A23187 · Apoptosis · UMR 106 · pH

Introduction

Fluoridation of drinking water with 1 ppm sodium fluoride (NaF) is believed to prevent dental caries (Whitford 1990). Fluoride is also recognized as a stimulator for bone-forming cells (Farley et al. 1983), and slowly releasing forms of sodium fluoride are considered to have a vast potential for the therapy of osteoporosis (Mundy 1995). Those beneficial actions of fluoride are probably due to uptake of fluoride by hard tissues and the subsequent formation of fluoroapatite (McNeill et al. 1991; Prostak et al. 1991). It has been shown that bones obtained from rats fed a fluoride-containing diet dissolved slower than those fed a normal diet in the buffer solution of various pH (Grynpas and Cheng 1988).

However, fluoride appears to be a two-edged sword for human health, because it has been reported that fluoride causes acute adverse effects including fetal poisoning (Yamaguchi et al. 1986; Whitford 1990, 1992; Kono 1994). Sowers et al. (1991) reported that a relative risk of bone fracture in women in the high-fluoride community (4 mg F/l in the water supply) was 2.1 in their epidemiological study in Iowa. Boulton et al. (1995) reported that intake of drinking water containing 80 μg F/ml for 84 days caused dental lesions in mice and wild small mammals. Willinger et al. (1995) reported that fluoride caused renal function changes in rats. Heindel et al. (1996) reported that the no-observed-adverse-effect level (NOAEL) of NaF in drinking water for maternal toxicity was 150 ppm in rats and 200 ppm in rabbits, while NOAEL for developmental toxicity in rats and rabbits was more than 300 and 400 ppm, respectively.

Fluoride is reported to be nongenotoxic (Tong et al. 1988), but affects cellular functions and enzyme activities. The fluoride-induced biochemical changes are activation of Ca^{2+}-independent phospholipase D (English et al. 1991) and inhibition of azurophilic degranulation in neutrophils (Niessen et al. 1994); decreases in mRNA for osteocalcin and α1-type collagen in rat calvaria cells (Li and DenBesten 1993); disturbance of vesicular transport in secretory ameloblasts (Matsuo et al. 1996); increase in amylase secretion in rats (Shahed et al. 1985); stimulation of O_2^- production in polymorphonuclear leucocytes (PMN; McPhail et al. 1981); and inhibition of phosphatase (Leis and Kaplan 1982) and cholinesterase activities (Dybing and Loe 1956).

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Recently, we reported that rat alveolar macrophages underwent apoptosis following exposure to fluoride in vitro (Hirano and Ando 1996). In the present report we show that UMR 106, a rat osteoblastic cell line, also undergoes apoptosis following exposure to fluoride and the cytotoxicity of fluoride depends on acidity of the culture medium. These results contribute a better understanding of the mechanism of fluoride toxicity.

Materials and methods

Cell preparation

UMR 106 (a rat osteosarcoma cell line) and RAW 264 (a mouse macrophage cell line) were obtained from Dainippon Pharmaceutical Co. (Osaka, Japan) and Riken Cell Bank (Tsukuba, Japan), respectively. UMR 106 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 25 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS). RAW 264 cells were subcultured in RPMI 1640 containing 20 mM HEPES, 4 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and supplemental 10% FBS.

Specific pathogen-free male Sprague-Dawley rats were purchased from Clea Japan (Tokyo, Japan). Rats were maintained in a clean air-conditioned room (temperature 22 °C, relative humidity 50–60%) with a 12:12 h light/dark cycle and allowed free access to commercial chow and distilled water. At 8 weeks old rats were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight) and killed by exsanguination from abdominal aorta. The lungs were exposed and lavaged eight times with endotoxin-free saline (Otsuka Pharmaceutical Co., Naruto, Japan). The washout fluid was centrifuged at 400 *g* for 5 min (4 °C), and the pellet washed with serum-free RPMI 1640 containing 4 mM glutamine, 20 mM HEPES, 100 U/ml penicillin, and 100 µg/ml streptomycin. The viability of the lavaged cells was more than 94% as assayed by the ability to exclude trypan blue. More than 97% of the cells were alveolar macrophages as determined by differential cell counting on cytocentrifuged preparation stained with Diff-Quik (Kokusai Shinyaku Co., Kobe, Japan). Unless otherwise specified, the pH of the culture medium was adjusted to 7.4.

Dose-dependent cytotoxicity of fluoride in UMR 106

UMR 106 cells were suspended in DMEM at 0.03×10^6 or 0.12×10^6 cells/ml. A sample of 100 µl of the cell suspension was aliquoted into a 96-well culture dish (Costar, Cambridge, Mass., USA). After 3 days of culture the conditioned medium was replaced with fresh DMEM containing 0–10 mM NaF and the cells were further cultured for 8 h. The cell monolayer was washed twice with Hanks' balanced salt solution (HBSS) and once with phenol red-free DMEM. The viability of the cells was measured using a modified thiazolyl blue (MTT) assay kit according to the manufacturer's specification (WST-1; Dojindo, Kumamoto, Japan). Briefly, WST-1 solution was added to each well and the dish was incubated at 37 °C for 1 h. At the end of incubation optical density (OD) at 450 nm was measured with a reference of 650 nm using a microtiter plate reader (CS9300; Shimadzu, Kyoto, Japan). The increase in OD at 450 nm correlates linearly with the number of viable cells.

DNA content of UMR 106

UMR 106 cells were suspended in DMEM at 0.03×10^6 cells/ml and a 0.5 ml sample of the cell suspension was aliquoted into a 24-well culture dish (Costar). After 24 h of culture, the conditioned medium was replaced with fresh medium containing 0, 0.1, 0.2, and 0.5 mM NaF. The monolayers were further cultured for the following 6 days in the presence or absence of additional 10 mM sodium phosphate. The medium was changed at days 3, 4, and 5. UMR 106 cells were cultured in the presence or absence of additional phosphate, because this cell line has been reported matrix vesicles to produce in the presence of excess (10 mM) phosphate like osteoblasts in the bone (Stanford et al. 1995). After washing the monolayers twice with saline, the detached and adhesive cells were combined and lysed in 0.5 ml of 20 mM TRIS-HCl buffer (pH 8.0) containing 10 mM EDTA disodium salt and 0.5% sodium dodecyl sulfate (SDS). The DNA content in the lysate was determined by the diphenylamine reaction (Burton 1956) using calf thymus DNA (Sigma, St. Louis, Mo., USA) as standard.

Microscopic examination

UMR 106 cells were grown to subconfluence and exposed to 5 mM NaF or 5 µg/ml A23187 (Calbiochem, La Jolla, Calif., USA) for 8 h. A23187 stock solution (1 mM) was prepared in dimethyl sulfoxide (DMSO). The monolayers were washed twice with calcium- and magnesium-free phosphate buffered saline (PBS) and suspended into single cells by brief trypsinization (in 0.25% trypsin/1 mM EDTA at 37 °C for 1 min). The detached cells in PBS and the trypsinized cell suspension were combined. The cell suspension was cytocentrifuged, fixed in 4% paraformaldehyde for 15 min, and stained both by Diff-Quik and terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) using an in situ apoptosis detection kit (MK500; Takara Biochemicals, Otsu, Japan). For fluorescent microscopy to detect TUNEL-positive cells, SlowFade™ Antifade (Molecular Probes, Eugene, Ore., USA) was used to reduce photobleaching of fluorescein isothiocyanate.

Detection of DNA ladder formation

UMR 106 cells were suspended in DMEM at 0.03×10^6 or 0.12×10^6 cells/ml and a 2 ml sample of the cell suspension was aliquoted into a 6-well culture dish (Costar). After 3 days of culture the conditioned medium was replaced with fresh DMEM containing 5 mM NaF or 5 µg/ml A23187 and the cell monolayers were further cultured for 8 h. The cell monolayer was washed twice in PBS. The detached and adhesive cells were combined and lysed in 0.7 ml of 20 mM TRIS-HCl buffer (pH 8.0) containing 10 mM EDTA disodium salt and 0.5% SDS. Four wells in the culture of low cell-density were combined to equalize the cell number between the cultures of low and high cell-density. Cells were lysed as described, the cell lysate was centrifuged at 20 000 *g* for 10 min at 4 °C, and the supernatant incubated overnight with 0.2 mg/ml protease K (Wako Pure Chemicals, Osaka, Japan) at 50 °C. The sample was extracted twice with the same volume of phenol/chloroform (50:50, v/v). DNA was precipitated overnight at –20 °C with two volumes of ethanol in the presence of 0.3 M sodium acetate. The precipitated sample was digested with ribonuclease (20 µg/ml, Wako) at 37 °C for 1 h, electrophoresed in duplicate on 1.8% agarose gel at 70 V for 4 h, and visualized with ethidium bromide.

Effects of pH on cell viability

UMR 106 cells were suspended in DMEM at 0.03×10^6 cells/ml. A 100 µl sample of the cell suspension was aliquoted into a 96-well dish and the cells were cultured for 3 days. The conditioned medium was replaced with fresh DMEM containing 5 mM NaF or 5 µg/ml A23187 (the pH of the medium was adjusted at 6.6, 7.4, and 8.2). At 8 h of culture each well was washed with HBSS and the cytotoxicity assay was carried out using WST-1 as described above.

Effects of the pH on fluoride-induced cytotoxicity were also examined using rat alveolar macrophages and RAW 264. A 100 µl sample of alveolar macrophage suspension (1.0×10^6 viable cells/ml in serum-free RPMI 1640) was aliquoted into a 96-well culture dish and the cells were allowed to adhere to the dish for 20 min in a CO_2 incubator. RAW 264 cells were trypsinized and the cells were suspended in RPMI 1640 supplemented with 10% FBS at 0.25×10^6 viable cell/ml. A 100 µl sample of RAW 264 cell suspension was aliquoted into a 96-well culture dish and the dish was incubated for the

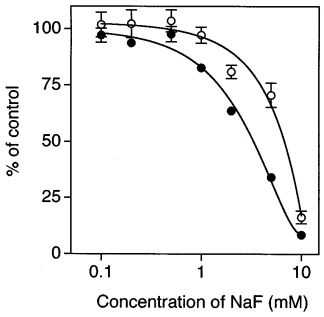

Fig. 1 Dose-dependent cytotoxicity of NaF. UMR 106 cells were suspended in Dulbecco′s modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) at 0.03×10^6 (low density, *open circles*) or 0.12×10^6 viable cells/ml (high density, *closed circles*) and 100 µl aliquots were cultured in a 96-well dish for 3 days. The conditioned medium was replaced with fresh medium containing 0–10 mM NaF, and the monolayers were further cultured for 8 h. The viability of UMR 106 cells was measured using WST-1 (see the Materials and methods)

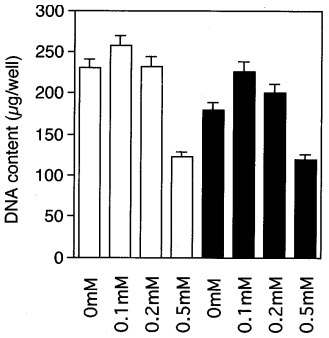

Fig. 2 Dose-dependent inhibition of cell proliferation by NaF. UMR 106 cells were cultured in a 24-well dish for 6 days in the presence or absence of 0–0.5 mM NaF and with (*closed columns*) or without additional 10 mM sodium phosphate (*open columns*). At the end of culture the monolayer was lysed and the DNA content of the lysate was measured

following 18 h. The culture medium of alveolar macrophages and RAW 264 cells was replaced with fresh serum-free RPMI1640 containing 0–5 mM NaF (the pH was adjusted at 6.6, 7.0, 7.4, 7.8, 8.2, and 8.6) and the cells were further cultured for 2 h. The viability of the cells was assayed using WST-1 as described above.

Statistics

Each value represents the mean ± SE of five or six determinations for UMR 106 and RAW 264 cells. For alveolar macrophages each value represents the mean ± SE of four rats with duplicate replications. Statistical analyses were performed using one-way analysis of variance followed by Bonferroni's post hoc comparison for alveolar macrophages. A probability value <0.05 was accepted as indicative of a significant difference.

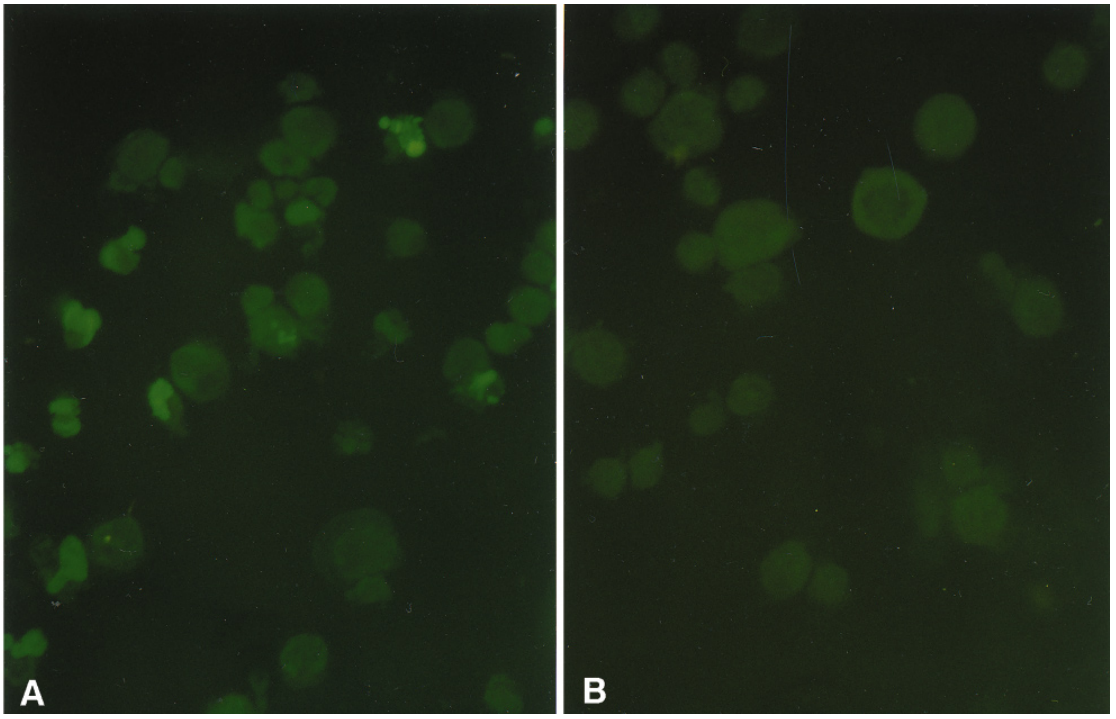

Fig. 3 A, B Fluorescent micrographs of UMR 106 cells stained with fluorescein thiocyanate-tagged TUNEL. UMR 106 cells were exposed to **A** 5 mM NaF or **B** 5 µg/ml A23187 for 8 h. The whole cell suspension was cytocentrifuged and stained by TUNEL. Apoptotic cells were stained brighter than other cells. (*TUNEL* Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-lebeling)

Results

Because a preliminary study suggested that cytotoxicity of fluoride depended on the cell density, two cell densities (0.03×10^6 and 0.12×10^6 viable cells/ml) were chosen when UMR 106 cell suspension was aliquoted into culture dishes. In culture of high cell-density UMR 106 achieved early confluence after 2 days of culture. Figure 1 shows that

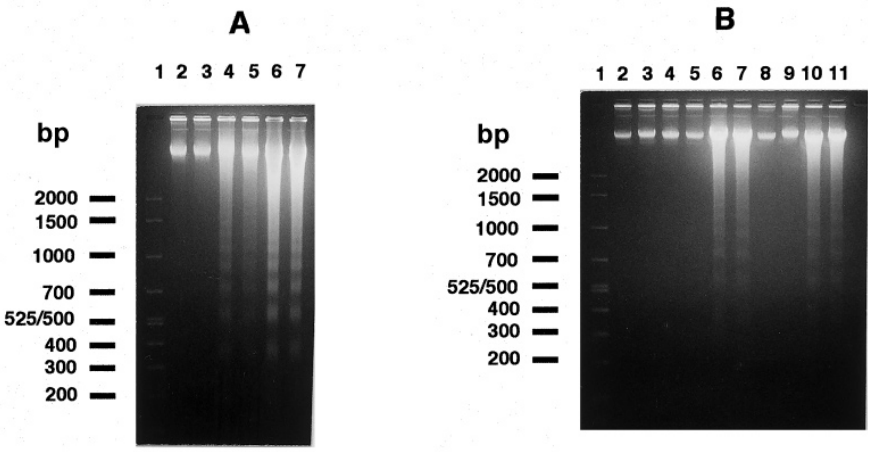

Fig. 4A, B Fluoride-induced DNA fragmentation in UMR 106. **A** UMR 106 cells were suspended in RPMI 1640 containing 10% FBS at 0.03×10^6 (low density) or 0.12×10^6 viable cells/ml (high density) and 2 ml aliquots were cultured in 6-well culture dishes for 3 days. The conditioned medium was replaced with fresh medium and the monolayers were further cultured for 8 h in the presence or absence of 5 mM NaF. DNA was extracted in duplicate as described in the Materials and methods. For the low cell-density culture DNA was extracted from four wells to adjust the cell number between the cultures of low and high cell-density. Lane 1, DNA marker; lanes 2 and 3, control (high density); lanes 4 and 5, 5 mM NaF (low density); lanes 6 and 7, 5 mM NaF (high density). **B** UMR 106 were cultured at high density as described above and the monolayers were exposed to either NaF or A23187, or the combination of them for 8 h. Numbers on *left* indicate size (in bp). Lane 1, DNA marker; lanes 2 and 3, control (0.5% DMSO); lanes 4 and 5, 5 μg/ml A23187; lanes 6 and 7, 5 mM NaF; lanes 8 and 9, 5 μg/ml A23187 + 5 mM NaF; lanes 10 and 11, 5 mM NaF + 0.5% DMSO (*DMSO* Dimethyl sulfoxide)

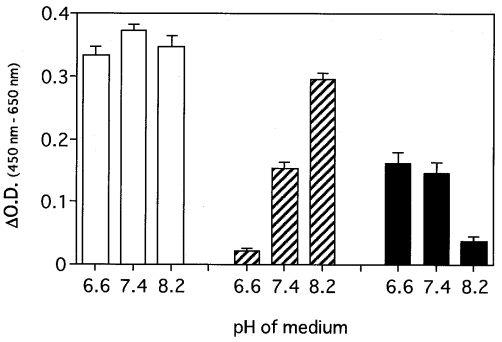

Fig. 5 Cytotoxic effects of NaF and A23187 on UMR 106 cells at the different pH values of medium. A 100 μl sample of 0.03×10^6 UMR 106 cells/ml was aliquoted into a 96-well culture dish and the cells were cultured for 3 days. The conditioned medium was replaced with pH-preadjusted fresh medium (pH of 6.6, 7.4, and 8.2) containing 5 mM NaF or 5 μg/ml A23187 and the monolayers were further cultured for 8 h. *Open columns*, control; *hatched columns*, fluoride; *closed columns*, A23187. The decrease in optical density (OD) difference (ΔOD 450–650 nm) indicates loss of viable cells

fluoride decreased the viability of UMR 106 cells dose-dependently and UMR 106 cells cultured at the high cell-density were more sensitive to fluoride than those cultured at the low cell-density. When a curve of the secondary degree was fitted to the dose-effect relationship, LC_{50} of NaF for the low and high density cultures were calculated to be 7.1 and 2.9 mM, respectively.

Figure 2 shows that the DNA content of UMR 106 monolayer cultured for 6 days in the presence of 0.5 mM NaF was decreased compared to that of the control culture, suggesting that the proliferation of UMR 106 was inhibited in the presence of 0.5 mM NaF. The presence of 0.1 mM NaF appears to have slightly increased the cell proliferation. Similar findings of the DNA content of UMR 106 cells were also observed in the presence of additional 10 mM sodium phosphate (closed columns in Fig. 2).

Although the viability of UMR 106 decreased to ca. 50% of the control value following 8-h exposure to either 5 mM NaF or 5 μg/ml A23187, a calcium ionophore, at the neutral pH (see Fig. 5), the death mode is different between NaF- and A23187-exposed UMR 106 cells. There were many TUNEL-positive cells in NaF-exposed cells, while most A23187-exposed cells were TUNEL-negative (Fig. 3). Photomicroscopic findings with Diff-Quik staining revealed that nuclei of fluoride-exposed UMR 106 cells were fragmented and those of A23187-exposed cells were not fragmented but swollen (data not shown). The DNA ladder formation was observed in fluoride-exposed cells, whereas no DNA ladder was observed in A23187-exposed cells (Fig. 4A, B). The presence of TUNEL-positive cells, fragmentation of nuclei, and DNA ladder formation clearly demonstrated that UMR 106 underwent apoptosis following exposure to fluoride. The absence of DNA fragmentation with a significant viability loss and morphological observation indicated that A23187-exposed UMR 106 cells underwent necrosis. It is of interest to note that the DNA ladder formation was not observed following co-exposure to NaF and A23187, suggesting that A23187 inhibited fluoride-induced apoptosis in UMR 106 (Fig. 4B).

Figure 5 shows that cytotoxic effects of fluoride on UMR 106 were modulated by the pH of the culture medium. Cytotoxicity of fluoride was reduced at the alkaline pH and enhanced at the acidic pH. In the absence of fluoride, the pH did not affect the viability of UMR 106 cells. Contrary to the finding in fluoride-exposed cells, the viability of A23187-exposed UMR 106 cells was not changed at the acidic pH but decreased at the alkaline pH. The pH-dependent cytotoxicity of fluoride was also examined using rat alveolar macrophages and the mouse

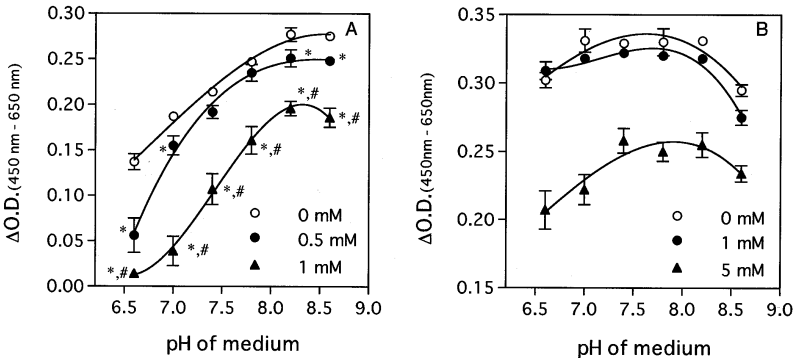

Fig. 6 **A** Cytotoxic effects of NaF on rat alveolar macrophages and **B** RAW 264 cells at different pH values of medium. A 100 µl sample of cell suspension was aliquoted into a 96-well dish and cultured as described in the Materials and methods (see also legend to Fig. 5). Cells were exposed for 2 h to 0–1 mM NaF for alveolar macrophages and 0–5 mM NaF for RAW 264 cells in serum-free RPMI 1640 medium of various pH. *, Significantly different from the control culture (0 mM). #, Significantly different from the culture with 0.5 mM NaF

RAW 264 macrophage cell line (Fig. 6). The cytotoxicity of fluoride was again enhanced at acidic pH in both of the cell lines. Unexpectedly, the viability of control alveolar macrophages was also decreased, when cultured at the acidic pH.

Discussion

Several lines of evidence suggest that a low dose of fluoride is beneficial for bone formation. Farley et al. (1983) reported that treatment with sodium fluoride (1–100 µM) increased thymidine incorporation in embryonic chick cells in vitro with a peak at 10 µM. Chavassieux et al. (1993) reported that thymidine incorporation and alkaline phosphatase activity were significantly increased in osteoblastic cells derived from NaF-treated rats (100 ppm NaF in drinking water for 1 month) than those derived from control rats. Those changes were not found, however, when the cells were exposed to fluoride in vitro (10 µM). The results presented in Fig. 2 suggest that 0.1 mM NaF appeared to slightly increase proliferation of UMR 106 during 6 days of culture and 0.5 mM clearly inhibited the cell proliferation. However, 8-h exposure to 0.5 mM NaF did not decrease the apparent viability of UMR 106 at the neutral pH (Fig. 1).

The DNA ladder formation on the agarose gel and the presence of TUNEL-positive cells are considered to be hallmarks of apoptosis (Gavrieli et al. 1992; Patel et al. 1995). Figures 3 and 4A show that there were many TUNEL-positive cells following exposure to 5 mM fluoride and the DNA extracted from fluoride-exposed UMR 106 cells was fragmented into nucleosomal units. These results indicate that UMR 106 cells underwent apoptosis following exposure to fluoride. The intensity of DNA ladders in the high cell-density culture appeared greater than those in the low cell-density culture, although DNA extraction was performed using the same number of cells (Fig. 4A). This result reinforces the enhancement of fluoride cytotoxicity in the culture at the higher cell-density (Fig. 1). There are a variety of environmental toxicants that cause apoptosis in mammalian cells such as silica-exposed peritoneal macrophages (Sarih et al. 1993), asbestos-exposed alveolar macrophages (Hamilton et al. 1996), tributyltin-exposed thymocytes (Aw et al. 1990), cadmium-exposed renal cells (Ishido et al. 1995), and mercury-exposed brain cells (Kunimoto 1994). The present study and our previous report (Hirano and Ando 1996) demonstrated that fluoride is also an environmental apogen (Corcoran et al. 1994).

It is generally accepted that calcium ionophores elevate cytosolic free calcium concentration resulting in activation of endonucleases and DNA fragmentation, while perturbation of intracelluar free calcium is implicated in necrosis (Fawthrop et al. 1991). Thus, we had expected that A23187 would cause apoptosis in UMR 106 cells as well as NaF. However, UMR 106 cells underwent necrosis following exposure to A23187. Matsubara et al. (1994) reported that myelogenous cell lines (HL60, U937, KG-1) underwent apoptosis, whereas T-lymphoblastic leukemia cell lines (Molt-4, Molt-3, CEM) underwent necrosis following exposure to 1 µM A23187. The same authors also reported that although the morphological pattern of cell death was different between myelogenous and T-lymphoblastic cell lines, initial rises of intracellular free calcium concentrations after treatment with A23187 were the same between those cell lines. In the present study A23187 inhibited fluoride-induced apoptosis in UMR 106 cells (Fig. 4B), suggesting that Ca^{2+}-dependent endonucleases do not play an important role, if any, in fluoride-mediated apoptosis in this cell line.

One of the most important findings in the present study is the dependence of fluoride cytotoxicity on the pH of the culture medium. As shown in Fig. 5, cytotoxic effects of fluoride were markedly reduced with the increase in the pH, while the alkaline pH enhanced A23187-mediated cell death in UMR 106. The modulation of fluoride cytotoxicity by the pH was also observed in rat alveolar macrophages and RAW 264 cells (Fig. 6). It has been reported that hydroxy radical-induced oxidative cytotoxicity at pH 7.4 was significantly reduced when the pH of the medium was lowered to 6.8 in a hepatocyte line (ch/ch cells), and the lethality correlated with the depletion of intracelluar glutathione (Zhu et al. 1996). Thus, a mechanism of fluoride-induced apoptosis in UMR 106 cells is different from that of oxidation-induced cytotoxicity in hepatocytes with respect to the pH-dependency.

One possible explanation for the modulation of fluoride cytotoxicity by the pH is that hydrogen fluoride (HF) is a more effective molecule than its ionic form (F^-). Whitford (1990) reported that unlike most substances, an appreciable amount of fluoride can be absorbed from the stomach by diffusion and the gastric absorption is inversely related to the pH. Because HF is a weak acid (pK_a of 3.2) compared to other acids that are major ingredients of culture media (cf. hydrochloric acid, pK_a of -6.1; sulfuric acid, pK_{a1} of -3.0; phosphoric acid, pK_{a1} of 2.1) (Dean 1973), the greater amount of fluoride should be changed into HF at the acidic pH. The influx of HF into cells by diffusion possibly precedes apoptosis in UMR 106 cells and macrophages.

The present study also shows that cytotoxicity of fluoride depends on the cell density of UMR 106 and the LC_{50} value was lower in the culture at higher cell-density (Fig. 1). In general, the pH of the medium decreases during culture of cells. Thus, it is safe to suppose that the pH of the microenvironment of UMR 106 monolayer cultured at the high cell density was lower than that cultured at the low cell density, and fluoride exerted its cytotoxicity more effectively at higher cell density.

An ancillary but interesting finding is that the viability of control rat alveolar macrophages was significantly decreased at the acidic pH even after a short period of culture (Fig. 6A). Recently, Demaurex et al. (1996) reported that spreading on adhesive substrates caused a rapid cytosolic alkalinization in human neutrophils and the increase of intracellular pH was mediated by Na^+/H^+ exchange. In the present study alveolar macrophages were cultured without FBS to promote rapid adhesion to the plastic dish. Thus, the acidic microenvironment might have affected functional changes of alveolar macrophages following adhesion and spreading. Further study is required to elucidate the early decline in the viability of alveolar macrophages at acidic pH.

In summary we have shown that fluoride-exposed UMR 106 cells underwent Ca^{2+}-independent apoptosis. The cytotoxicity of fluoride was markedly enhanced in UMR 106, RAW 264, and rat alveolar macrophages at the acidic pH. Dai (1988) reported that indoor air as well as drinking water has been polluted by fluoride in rural areas of China, because fluoride-contaminated coal has been used for cooking and heating without effective exhaust facilities. In those cases respiratory effects of airborne fluoride may be underestimated, because acid aerosols generated by coal combustion can increase fluoride toxicity to alveolar macrophages near the deposition sites in the lung.

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