Fluoride-Treated Bio-Resorbable Synthetic Hydroxyapatite Promotes Proliferation and Differentiation of Human Osteoblastic MG-63 Cells

Motofumi Ohno, DDS[1]
Kazunari Kimoto, DDS, PhD[1]*
Toshihisa Toyoda, DDS, PhD[2]
Kazushige Kawata, DDS[1]
Hirohisa Arakawa, DDS, PhD[1]

When resorbable hydroxyapatite (HA) granules, which are used as a bone supplement material, were treated in neutral 4% sodium fluoride (NaF) solution, formation of a reactant resembling calcium fluoride was observed on the surface of the granules. Immediate and slow release of fluoride from fluoridated HA (HA+F) granules was observed after immersion in culture fluid, and the concentration increased over time to 1.25 $\pm$ 0.05 ppm F at 0.5 hours, 1.57 $\pm$ 0.12 ppm F at 24 hours, and 1.73 $\pm$ 0.15 ppm F at 48 hours. On invasion assay, migration of human osteoblast-like MG-63 cells exposed to the released fluoride was confirmed in comparison to the cells incubated with a nonfluoridated control sample ($P < .01$). In addition, fluoride added to the medium increased MG-63 cell proliferation in a manner dependent on fluoride concentrations up to 2.0 ppm ($P < .05$). At 5.0 ppm, however, fluoride significantly inhibited cell proliferation ($P < .005$). Activity of the osteogenic differentiation marker, alkaline phosphatase (ALP), also increased with fluoride after exposure for 1 week, increasing significantly at 1.0 ppm ($P < .05$). The promotion of MG-63 cell migration and proliferation, as well as increased ALP activity, suggested that fluoride released from the surface of resorbable HA granules, which were fluoridated by prior treatment with neutral 4% NaF solution, can provide a superb method to supply fluoride and promote osteogenic cell differentiation.

Key Words: fluoride slow release, bio-resorbable hydroxyapatite, cell migration, proliferation, ALP activity

INTRODUCTION

Implants cannot be placed in deficient bone and pretreatments such as guided bone regeneration are necessary in such cases. Materials currently used for guided bone regeneration include autograft bone, allograft bone, xenogeneic bone, and artificial materials such as hydroxyapatite (HA) and tricalcium phosphate granules.[1,2]

In a previous study, fluorapatite was prepared from resorbable synthetic HA granules by treating them with 4% sodium fluoride (NaF) at neutral pH for 2 minutes. Rinsed powder was then subjected to analyses using electron microscopy, 1-dimensional and 2-dimensional X-ray diffraction, energy dispersive X-ray (EDX) analysis, and EDX mapping.[3] We showed that the reaction of fluoride ions with the HA granules produced crystalline fluorapatite having characteristic and sharp X-ray diffraction patterns on the surface of granules. EDX analysis revealed a fluoride peak at 0.70 KeV that was not seen on the surface of nonfluoridated control granules. Furthermore, EDX mapping showed an evenly distributed needle crystalline particulate

[1] Division of Oral Health, Department of Health Science, Kanagawa Dental College, Yokosuka, Japan.
[2] Tokyo Plastic Surgery Dental Society, Tokyo, Japan.
* *Corresponding author,* e-mail: kimotokz@kdcnet.ac.jp
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pattern over the entire surface of the fluorapatite granules. It was concluded that the reaction of synthetic resorbable HA with 4% NaF solution produced fluorapatite-coated HA.[3] This suggests that fluoridated HA granules can act as a source of fluoride and that fluoridated HA, when it is used as a biomaterial, merits further investigation.

Implant products have been developed in recent years with a variety of modified surfaces that exert biologic effects on protein attachment, cell attachment, and cell proliferation on the surface. These include HA or calcium-phosphate coating of titanium surfaces, blasting or acid processing, or both in combination.[4] In particular, implant products with a surface texture created by sandblasting or acid processing with hydrogen fluoride have been developed and marketed by Swedish medical equipment manufacturers. It has been reported that osteoblast proliferation and differentiation judged by alkaline phosphatase (ALP) activity are facilitated through minute amounts of fluoride retained on the surface.[5–9] Fluoride studies, such as that of Farley et al,[10] have been carried out for many years and have found that fluoride clearly activates osteoblasts, improving the rate of mineral apposition in early osteogenesis.[11] However, the concentrations of fluoride used in these studies vary considerably, depending on the research and evaluation methods. This wide range has not been narrowed down to an optimal fluoride concentration.

There have been numerous studies on fluoridated HA, which was used in the present study, carried out from the perspective of preventing caries. A concept to emerge from the report by Caslavska et al[12] is the classification of fluoride on either HA or enamel as either "firmly" bound fluoride (fluoride bound within the HA or enamel) or "loosely" bound fluoride (fluoride generated on the surface). These are alkali-insoluble and alkali-soluble fluoride, respectively, with respect to solubility with potassium hydroxide. Successive studies focused on the mobility of fluoride after application to HA or enamel.[13–19] We used neutral 4% NaF solution in the previous and present studies, which is close to the maximum solubility of NaF in distilled water at 20°C. This alkaline-soluble fluoride forms a loosely bound fluoride from the reactants, which is maximally generated on the surface of the HA granules.

Release of low-level fluoride can be expected to facilitate cell adhesion, cell proliferation, and cell differentiation—the series of biologic reactions leading to osteogenesis. One purpose of this study was to use the MTS assay to determine whether the effect of fluoride is required for the proliferation of osteoblast cells and whether differences in low-level fluoride concentration could contribute to the differential responsiveness of osteoblast cells to the migration and differentiation (invasion assay and ALP activity). The present study aimed to investigate the biologic effects of released fluoride from the surface of fluoridated resorbable HA granules on MG-63 human osteoblastic cells.

Materials and Methods

Reagents and cell culture

Fluoridated HA granules were prepared using 3.0 g of OsteoGen 300–400 μm synthetic resorbable HA granules (Lot no. U1201B, Impladent, NY, NY), which were immersed in 50 mL of neutral 4% NaF solution for 3 minutes and then immediately washed twice in 200 mL of saline (Otsuka Normal Saline, Otsuka Pharmaceutical, Tokyo, Japan) and allowed to dry naturally. The control sample was nonfluoridated HA granules immersed for 3 minutes in distilled water and examined in the same way for comparison.

Elution medium was prepared using 0.55 g of HA+F granules immersed in 45 mL of Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, St Louis, Mo) containing no serum for 24 hours at 37°C. After immersion, the HA+F granules were precipitated out by centrifugation at 1500 rpm for 5 minutes, and the supernatant was collected. The supernatant was then filter-sterilized through a 0.2-μm pore size filter (Corning, Corning, NY) to yield HA+F elution medium. HA elution medium was produced in the same manner using a nonfluoridated control sample of HA granules. Fluoride concentrations in each culture medium were measured using the ion-selective electrode method (Model 96-09 Orion Fluoride Electrode, Thermo Scientific). An equal amount of TISAB II (Orion) was used as total ionic strength adjustment buffer.

Human osteoblast-like cells, MG-63 cells, were obtained from the American Type Culture Collection (Rockville, Md) and cultured in DMEM containing 10% fetal bovine serum (Wako Pure Chemical Industries,

Japan), with 5% CO_2 at 37°C. All assays were performed in triplicate and repeated least 3 times.

Measurement of released fluoride from HA+F granules over time

The concentration of fluoride released from HA+F granules was measured by immersing 10 mg of HA+F granules in 1.25 mL of DMEM (Sigma-Aldrich) at 37°C, and fluoride concentrations were determined at 0.5, 1, 2, 4, 8, 12, 24, and 48 hours after immersion.

Invasion assay

A BD BioCoat Matrigel Invasion Chamber (Becton, Dickinson and Company, Franklin Lakes, NY) was used for invasion assay. HA+F granules (10 mg) were added to 0.75 mL of DMEM in each well of a BD Falcon Companion Plate for cell culture, and Falcon Cell Culture Inserts fitted with an 8-μm PET membrane were placed inside the wells. MG-63 cell culture (0.5 mL) with cells suspended at a density of 2.5×10^4 cells/well was seeded inside the cell culture inserts, followed by culture for 24 hours at 37°C under 5% CO_2. The culture was then fixed with 100% methanol and stained with hematoxylin-eosin. The number of migrating cells was counted by photographing 9 fields per well (field of view, 2.34 mm^2) at ×100 magnification. The concentration of fluoride released into the culture medium was also measured in the outer chamber using the ion selective electrode method. As a control, invasion assay was performed using nonfluoridated HA granules.

Cell proliferation assay (MTS assay)

Cells were seeded at a density of 4×10^3 cells/well in a 96-well plate using DMEM-containing serum, and cultured for 24 hours. After 24-hour incubation without serum, medium was replaced with HA+F elution medium diluted to final concentrations of 0.5, 1.0, 2.0, and 5.0 ppm fluoride. Cell proliferation was assessed following an additional 24-hour period using MTS assay with CellTiter 96 AQueous One Solution Reagent (Promega, Southampton, UK). HA elution medium prepared in the same manner was used for control cultures.

Measurement of ALP activity

In a 3.5-cm dish, 1×10^5 cells were seeded and when the cells reached confluence, the medium was replaced with HA+F elution medium diluted to 0.5, 1.0, and 2.0 ppm F. Medium-containing eluted fluoride at each dilution was replaced every 3 days. After 1 week, cells were washed with ice-cold phosphate-buffered saline, scraped off, and collected with a rubber policeman in RIPA Lysis Buffer (Santa Cruz Biotechnology, Santa Cruz, Calif) on ice. MG-63 cells were disrupted by homogenization with ultrasound for 10 seconds and kept on a rotary shaker for 30 minutes at 80 rpm under ice-cold conditions. After centrifugation for 15 minutes at 15,000 rpm, the resulting supernatant was collected for measurement. Proteins in each measurement sample were quantified using the Quick-Start Protein Assay (Bio-Rad Laboratories, Hercules, Calif), which is a modification of the Bradford method, according to the manufacturer's protocols.

ALP activity was calculated from the product concentration (nmol *p*-nitrophosphate/μg protein/hour) using LabAssay ALP (Wako Pure Chemical Industries) in a microplate reader at 405 nm. Control activity was measured with HA elution medium prepared in the same manner.

Statistical analysis

JMP 7.0 J (SAS Inc) was used for statistical analysis. Student *t* test was used to compare means between 2 groups. The vertical bars represent ± one standard error of the mean.

Results

Measurement of fluoride released from HA+F granules over time

The concentration of fluoride released from HA+F granules fluoridated in neutral 4% NaF solution is shown in Figure 1. The concentration of fluoride released from HA+F granules was increased markedly (1.25 ± 0.05 ppm) at 0.5 hours after immersion of the granules. The concentration was increased gradually (1.57 ± 0.12 ppm at 24 hours) and peaked at 48 hours (1.73 ± 0.15 ppm), thus confirming the immediate release as the first phase and the slow release as the second phase from HA+F granules.

Invasion assay

We tested whether the concentration of fluoride would affect the cell migration. Migrating MG-63

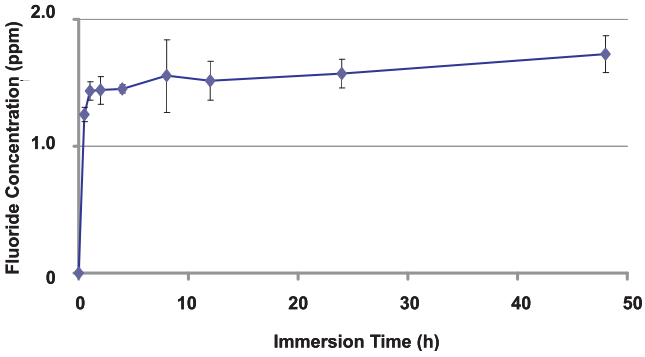


Figure 1. Fluoride released into culture medium from fluoridated hydroxyapatite granules. Release of fluoride from fluoridated hydroxyapatite granules was measured with a fluoride ion-selective electrode as described in Materials and Methods. Filled diamonds represent mean values with standard error (vertical bars).

cells were stained with hematoxylin-eosin following culture for 24 hours (Figure 2). Fluoride concentration was approximately 1.5 ppm in the chamber with HA+F granules. The number of MG-63 cells counted per view (2.34 mm^2) was 12.8 $\pm$ 2.5 cells for HA+F granules and 4.6 $\pm$ 1.6 cells for control HA granule samples. The number of MG-63 cells was significantly greater for HA+F granules ($P < .01$) (Figure 3), thus confirming migration of MG-63 cells toward the released fluoride. As seen in Figure 2b, MG-63 cells showed good spreading and motility. The effect on MG-63 cell migration of fluoride released from HA+F granules was thus clear.

Cell proliferation assay (MTS assay)

To analyze the effect of fluoride on cell proliferation, MTS assay was determined in MG-63 cells. Cell proliferation after 24 hours was the highest at fluoride concentrations of 1.0 and 2.0 ppm ($P < .05$), and the lowest at a fluoride concentration of 5.0 ppm ($P < .005$) (Figure 4). Fluoride at concentrations in the range of 1.0–2.0 ppm thus promoted MG-63 cell proliferation.

Measurement of ALP activity

Consistent with the cell proliferation and in vitro cell migration, ALP activity, a marker for early osteoblast differentiation, was promoted in the HA+F elution medium in comparison to the control. Although it was significantly greater at 1.0 ppm F ($P < .05$), the increase was not significant at 2.0 ppm F (Figure 5). Low concentrations of fluoride released from HA+F granules contributed to the differentiation of MG-63 cells.

Discussion

The results of experiments with MG-63 showed that 80% of the fluoride released by fluoridated granules over a 24-hour period was released within 30 minutes of immersion, and 90% was released within 1 hour. Afterwards, the amount of fluoride released continued to increase until 48 hours. The released fluoride clearly influenced subsequent MG-63 cell

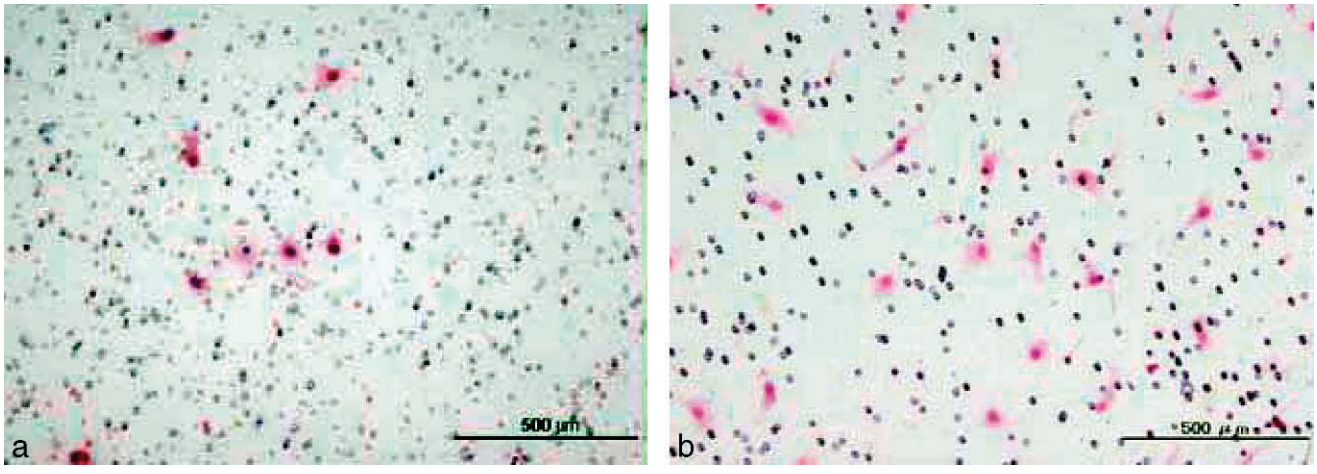
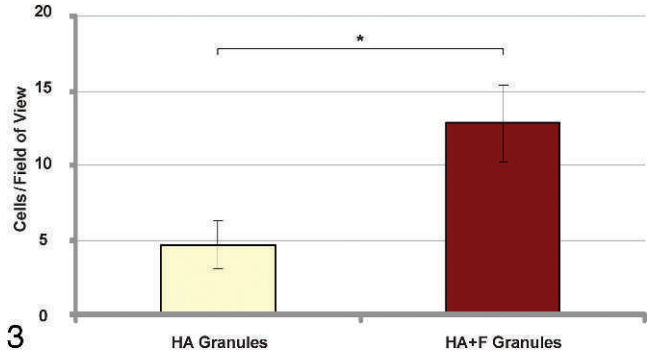
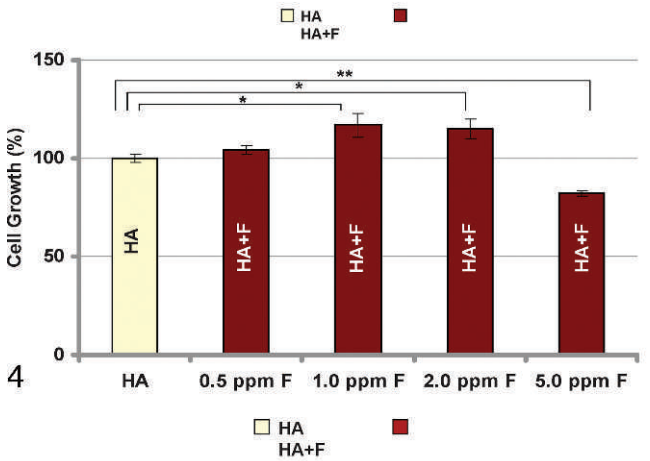
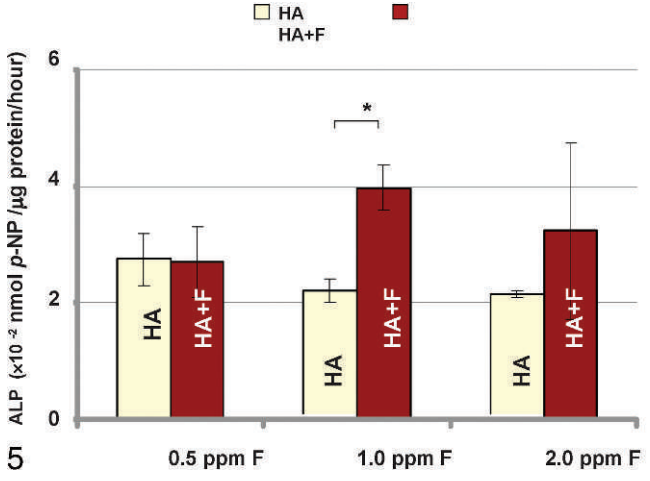


Figure 2. MG-63 cell migration due to fluoride released from fluoridated hydroxyapatite granules. MG-63 cells were stained with hematoxylin-eosin after 24-hour culture in the presence of nonfluoridated hydroxyapatite granules (a) and fluoridated hydroxyapatite granules (b). Fluoride concentrations were less than 0.01 ppm F (a) and 1.51 $\pm$ 0.10 ppm F (b), respectively, after 24-hour incubation.







Figures 3–5. Figure 3. Cell migration in the presence of fluoridated or vehicle-treated fluoridated hydroxyapatite granules. Cell migration was assessed by invasion assay as described in Materials and Methods. Number of MG-63 cells, which migrated to the outer layer, was counted after the 24-hour incubation period with either fluoridated or mock-treated hydroxyapatite granules as in Figure 2. Vertical scale represents number in the field of view, 2.34 mm^2. Fluoride concentrations were the same as in Figure 2. *$P < .01$. **Figure 4.** Effects of released fluoride on MG-63 osteoblastic cell proliferation. Cell proliferation was assessed by the MTS assay as described in Materials and Methods. Differences in MG-63 cells before and after 24-hour incubation with control hydroxyapatite medium were set at 100%. In culture medium with diluted, released fluoride, cell proliferation was significantly higher than in controls at 1.0 and 2.0 ppm F, and significantly lower than in controls at 5.0 ppm F. *$P <$ .05. **$P < .005$. **Figure 5.** Effects of released fluoride on alkaline phosphatase (ALP) activity in MG-63 cells. ALP activity was measured after 1-week culture in the medium with diluted, low concentration of fluoride, 0.5, 1.0, and 2.0 ppm F. ALP activity was significantly higher with medium containing 1.0 ppm F than with control medium. *$P < .05$.

migration. Moreover, cell proliferation in the medium with adjusted fluoride concentrations clearly indicated a specific range of fluoride concentrations levels (1.0–2.0 ppm F) optimal for MG-63 cells. We also showed that the osteoblastic cell differentiation was promoted by low-level fluoride concentrations, in the range of 1.0–2.0 ppm F.

Wang et al[20] recently investigated the response of MG-63 cells to fluoridated HA coating on a titanium alloy (Ti6Al4V) substrate by cell morphology, proliferation, ALP activity, and osteocalcin levels. For differentiation of MG-63, they recommended hydroxyfluoroapatite with 0.8 to 1.1 M fluoride substitution.[20] In other reports, mouse stem cells and osteoblast-like ROS cells responded to similar, or slightly lower, ranges of fluoride substitution and showed good proliferation.[21,22] Arakawa et al,[23] on the other hand, focused on the presence of minute quantities of fluoride from 5 to 50 µM (0.095–0.95 ppm F) in the culture medium, which promoted cell proliferation of human gingival epithelial cells (HGEC) and human epidermal keratinocytes (HaCaT) after 48-hour exposure. A significant difference was reported for proliferation of both cell types. The presence of minute quantities of fluoride led to the production of fibronectin and laminin-5, which play a major role in cell adhesion and proliferation.[23] These studies indicated the need for further research to establish a method for slow release of fluoride during the early stages of cell differentiation.

In our study by using proliferation assay, significant inhibition of MG-63 cell proliferation was observed at the higher fluoride concentration of 5.0 ppm. However, cell proliferation was significantly promoted at 1.0–2.0 ppm F. In cells such as HGEC and HaCaT, which are derived from cells growing in the oral environment, 48-hour exposure to 0.95 ppm F resulted in a significant increase in cell proliferation (HGEC, $P < .01$; HaCaT, $P < .05$).[23] The fact that minute quantities of fluoride facilitated HGEC and HaCaT cell proliferation supports the

notion that fluoride has a beneficial effect on human gingival and epidermal tissue.

In our experiments, ALP activity of MG-63 cells after 1-week exposure was significantly higher for 1.0 ppm F, while the result with 2.0 ppm F was variable. It should be noted that previous reports with different types of cells have used fairly high fluoride concentrations,[24–29] and apoptotic responses have been reported.[29] Further investigation is needed to establish beneficial effects for low fluoride concentrations around 1.0–2.0 ppm. Changes in osteocalcin and bone sialoprotein expression may be suitable for assessing maturation of osteoblastic cells in relation to mineralization.[30]

Another aspect of the biologic effects of fluoride, the inhibition of bacterial enzymes, has been reported with the titanium materials used for implant. Specific antibacterial action on proliferation of *P. gingivalis* or *A. actinomycetemcomitans* suggests that fluoride inhibits bacterial glucose metabolism after bacterial adhesion to the titanium surface.[31,32] At the same time, no inhibition of fibroblastic L929 cell proliferation was observed. Our preliminary animal study, as well as previous studies, suggested that degradative enzymes and inflammatory cytokines are suppressed by fluoride at high concentrations, while inflammatory responses of uninfected tissue and osteolysis are prevented.[33–35]

In clinical applications, fluoride solution is used at a concentration around neutral 4% NaF solution (18 000 ppm F) on the surface of HA granules as a supplement. Based on the present study and the above-mentioned antibacterial effect, we may conclude that this stimulates bone regeneration in a stepwise action; transient antibacterial effects are expressed near the site of application, where fluoride concentrations are highest, and the subsequent slow release of fluoride from the HA granules facilitates osteogenesis. Further studies are necessary to confirm such a 2-phase effect for released fluoride.

Conclusions

The promotion of MG-63 cell migration and proliferation, as well as increased ALP activity, suggests that released fluoride from the surface of resorbable HA granules, which were fluoridated by a prior treatment with neutral 4% NaF solution, can be a beneficial and convenient method of fluoride supply.

Abbreviations

ALP: alkaline phosphatase
EDX: energy dispersive X ray
HA: hydroxyapatite
HaCaT: human epidermal keratinocytes
HGEC: human gingival epithelial cells
NaF: sodium fluoride

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