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SHORT COMMUNICATION

Differential influence of fluoride concentration on the synthesis of bone matrix glycoproteins within mineralizing bone cells *in vitro*

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Abstract

Objective. This study investigated the influence of fluoride levels on the temporal synthesis of bone-associated glycoproteins, which have been assigned prominent roles in regulating crystal growth, size and shape during the mineralization process. ***Materials and methods.*** Bone marrow stromal cells were isolated from male Wistar rats and cultured under mineralizing conditions, supplemented with 0 M, 10^{-7} M or 10^{-5} M sodium fluoride. The presence of bone-associated glycoproteins was examined 2–13 days post-reseeding by immunocytochemical localization. ***Results.*** All bone-associated glycoproteins increased in 10^{-7} M fluoride, compared to untreated controls, particularly at days 6 and 13 in culture. Conversely, higher 10^{-5} M fluoride concentrations decreased glycoprotein levels, compared to controls. ***Conclusions.*** Results highlight a differential effect of fluoride concentration on glycoprotein synthesis by osteoblasts.

Key Words: *bone, bone-associated glycoproteins, fluoride, mineralization, osteoblasts*

Introduction

Fluoride has long been recognized for its value in the prevention of dental caries. However, other clinical applications for fluoride are increasingly being introduced, including fluoride-modified titanium implant surfaces for improved osseointegration [1–3] and prevention of root resorption during orthodontic tooth movement [4–6]. These clinical applications are supported by observations that fluoride can increase bone mass [7,8]. However, fluoride has also been suggested to cause increased hip fracture incidence in water fluoridated areas [9], implying a decrease in bone mass. Therefore, in order to better assess the healthcare benefits of fluoride, understanding its effects on mineralized tissue formation is of considerable importance.

Bone remodelling and repair involves the recruitment of bone marrow-derived, mesenchymal progenitor cells which, under appropriate signals, proliferate and differentiate into bone-synthesizing osteoblasts. This latter stage is characterized by the synthesis of non-collagenous glycoproteins, such as osteopontin (OPN), osteonectin (ON), bone sialoprotein (BSP) and osteocalcin (OCN), which regulate mineralized crystal deposition [10–12]. Therefore, alterations in glycoprotein expression and overall matrix composition could have major effects on the quality of the mineralized tissue formed [12]. Although reports are currently limited, *in vivo* studies have shown that high fluoride serum levels ($\geq 10^{-5}$ M) impair bone collagen synthesis [13]. *In vitro* studies have also indicted that fluoride influences bone proteoglycans and matrix metalloproteinase (MMP) activities within mineralized tissues, implicated in the formation of a hypomineralized matrix [14,15]. Studies investigating glycoprotein expression by mineralizing bone cells have suggested that lower fluoride concentrations (5–300 μM) exerts no effect on OCN or ON expression, but increases OPN expression. Conversely, higher fluoride levels (500 μM) significantly increase OCN expression [16]. Against this limited evidence, this study reports on the effect of contrasting fluoride concentrations on bone glycoprotein synthesis by mineralizing bone cells *in vitro*, with the aim to further understand how fluoride alters matrix composition

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during bone formation and results in clinically manifested alterations in mineralization.

Materials and methods

Bone marrow stromal cells (BMSCs) were obtained from the bone marrow cavities of 3-week old male Wistar rat femurs, according to Cardiff University approved animal protocols [14,15]. BMSCs were maintained in αMEM, containing 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 mg/ml amphotericin B, 10% foetal calf serum (Invitrogen, Paisley, UK), in addition to the mineralizing supplements (10 nM dexamethasone, 10 mM β-glycerophosphate and 50 µg/ml ascorbic acid, all Sigma, Poole, UK), at 37°C in 5% CO_2/95% air. At day 5 post-isolation, BMSCs were trypsinized (0.05% trypsin-EDTA, Invitrogen), reseeded into 6 well plates at 4×10^4 cells/cm^2; and cultured in the above media supplemented with 0 M, 10^{-7} M or 10^{-5} M sodium fluoride (Sigma); 10^{-7} M fluoride levels reflect *in vivo* serum levels observed during treatment for osteoporosis, whilst higher levels (10^{-5} M) reflect serum levels which can lead to dental and skeletal fluorosis [14,15].

At 2, 6 and 13 days, reflecting periods of cell proliferation, matrix maturation and mineralization, respectively [14], glycoprotein levels were examined by immunocytochemistry. Cells were fixed in 2% formaldehyde (Sigma) for 30 min. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide (5 min) and blocked with 3% horse serum (Vectastain Kit, Vector Laboratories, Peterborough, UK). Cells were incubated for 1 h with the appropriate primary antibody, diluted in 0.1225 M sodium chloride, 0.01 M sodium dihydrogen phosphate and 0.025 M EDTA, with 0.1% Tween 20 and 0.1% bovine serum albumin, pH 7.4. Primary antibodies included goat anti-rat OCN polyclonal (CamBio Ltd., Dry Drayton, UK; 1:10 dilution); and rabbit polyclonal antibodies to OPN (LF123; 1:500), BSP (LF100; 1:500) and ON (LF23; 1:500) [17]. Immunoreactivity was visualized using a universal secondary biotinylated antibody, avidin/biotinylated horseradish peroxidase complex formation and DAB peroxidase (Vectastain Kit). Primary antibody omission served as negative controls. Cultures were viewed by light microscopy and images obtained (×200 magnification). Immunostaining intensity was arbitrarily graded and averaged for each fluoride concentration and time-point by one blinded observer, on a 5-point scale for three images from triplicate wells.

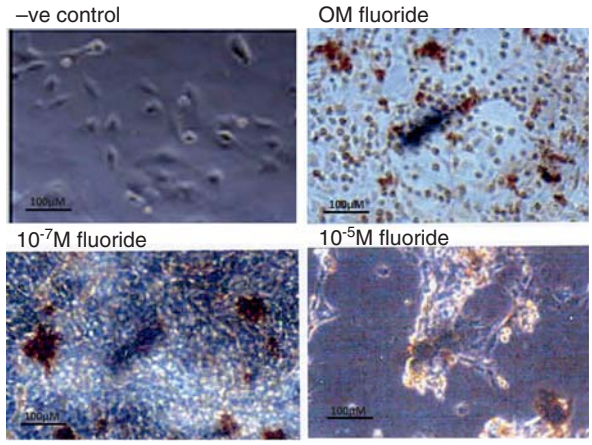

Figure 1. Immunocytochemical localization of bone sialoprotein synthesized by BMSCs cultured for 13 days under mineralizing conditions in the presence and absence of fluoride. Levels of staining were arbitrary graded on a 5-point scale. For this example, bone sialoprotein staining intensity increased in cells cultured in 10^{-7} M fluoride, compared to untreated controls, while a decreased staining intensity was recorded for cells cultured in 10^{-5} M fluoride.

Results

Immunolocalization demonstrated the temporal appearance of BSP, ON, OPN and OCN, which increased with culture duration. As an example, BSP immunolocalization at day 13 is shown in Figure 1. Cells were observed to aggregate into nodules where glycoprotein staining was particularly prominent. The Figure provides examples for 10^{-7} M and 10^{-5} M fluoride concentrations, where staining intensity was graded 5. All graded staining intensities for each glycoprotein are presented in Table I. At day

Table I. Levels of glycoproteins synthesized by BMSCs under mineralizing conditions, in the presence of 0 M, 10^{-7} M or 10^{-5} M sodium fluoride. Images were obtained at ×200 magnification, which were used to grade staining intensity following immunocytochemical localization.

Day	Fluoride concentration	BSP	OPN	ON	OCN
2	0 M	*	**	**	Not detected
	10^{-7} M	*	**	**	Not detected
	10^{-5} M	*	**	**	Not detected
6	0 M	***	***	***	**
	10^{-7} M	****	****	****	***
	10^{-5} M	***	***	***	*
13	0 M	****	****	*****	****
	10^{-7} M	*****	*****	*****	*****
	10^{-5} M	****	***	***	***

Indicates * low through to ***** very high levels of glycoproteins detected.

2, BSP, OPN, OCN and ON levels were low, with no discernible differences between fluoride concentrations and controls. At day 6, however, cells cultured in 10^{-7} M fluoride demonstrated an observable increase in the presence of all glycoproteins. In contrast, glycoprotein levels appeared similar to control cultures in 10^{-5} M fluoride, although a slight decrease in OCN was noted. Similar changes in glycoprotein levels were observed at day 13, although 10^{-5} M fluoride levels decreased OPN, ON and OCN levels, suggesting that glycoprotein synthesis is enhanced by 10^{-7} M fluoride, but not by 10^{-5} concentrations, which conversely decrease bone glycoprotein levels.

Discussion

The data presented indicates that lower fluoride concentrations (10^{-7} M) increase the synthesis of bone glycoproteins, BSP, OPN, ON and OCN, whilst higher fluoride levels (10^{-5} M) decrease OPN, ON and OCN synthesis. These results extend our previous work utilizing this BMSC culture system through distinct periods of proliferation, matrix deposition and mineralization [14,15]. As our previous findings have shown that 10^{-7} M and 10^{-5} M fluoride concentrations have negligible effect on BMSC proliferation or viability, the observed alterations in bone glycoprotein levels appear to be a consequence of altered synthetic activity by cells.

Higher BSP, ON, OPN and OCN levels were identified at days 6 and 13, corresponding to culture periods associated with osteoid deposition and early mineralization. BSP, ON, OPN and OCN are all proposed to regulate bone mineralization, by controlling hydroxyapatite crystal nucleation, growth rate, morphology and size [10,18–21]. Thus, even small changes in glycoprotein levels may impact on crystal growth and mineralization, dictating the extent of deposition and influencing bone quality, as evident with knock-outs for these glycoproteins [10,18,22,23]. Although 10^{-7} M and 10^{-5} M fluoride concentrations were shown to differentially affect glycoprotein levels, previous studies have indicated increased mineral deposition in BMSC cultures in 10^{-7} M or 10^{-5} M fluoride [14]. This may be a consequence of the higher glycoprotein levels at 10^{-7} M fluoride concentrations promoting mineralization and osteogenesis, whilst 10^{-5} M fluoride concentrations reduce glycoprotein levels, but enhance fluorapatite mineral formation [24]. Such events may be compounded by fluoride effects on other bone matrix components, such as proteoglycans, by reducing glycosaminoglycan chain length, sulphation and chondroitin sulphate levels [14]. These fluoride concentrations may also alter MMP activities, influencing matrix remodelling and mineralization [15]. This viewpoint concurs with clinical observations, where 10^{-8} M serum fluoride levels are beneficial as an osteoporosis treatment by increasing bone mass [7,8], whereas higher or more prolonged fluoride levels cause deleterious effects on bone, including increased fracture rates [9].

Overall, the presented findings are significant, as they provide new evidence that fluoride can differentially influence bone glycoprotein metabolism and subsequent mineralization, depending on concentration. The mechanisms by which this occurs remain to be elucidated, although scope for further work in this area is quite apparent. Of clinical significance, if fluoride is used therapeutically, such as for orthodontic tooth movement or osseointegration, fluoride levels and their duration in serum are critical considerations in the maintenance of bone quality.

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