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Sodium Fluoride Induces Changes on Proteoglycans Synthesized by Avian Osteoblasts in Culture

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Abstract The results reported here show that sodium fluoride (NaF) at low concentration (up to 10 μM) increased four times the proliferation rate of avian osteoblasts in culture. Also NaF increases, in a concentration dependent manner, 10 times the alkaline phosphatase activity. However, NaF decreased the incorporation of ^{35}S-sulfate into proteoglycans (PGs) synthesized by osteoblasts (60–65%). Also, we observed that PGs synthesized in the presence of NaF (50 μM) exhibited a higher sensitivity to chondroitinase ABC than PGs synthesized by osteoblasts in the absence of NaF, suggesting an increase in the chondroitin sulfate moieties associated with the core protein of PGs. The modification of glycosaminoglycan (GAG) chains composition was evidenced also by change in the mean charge density of the PGs observed by ion exchange chromatography. Since the ratio of $^{35}SO_4/^3$H-glucosamine incorporated into PGs was similar in the presence and in the absence of NaF (8.2 and 7, respectively), it is not possible to explain differences in mean charge density by changes in the sulfation extent of PGs. No differences were observed in the hydrodynamic size of PG synthesized in the presence of NaF, nor in the hydrodynamic size of the GAG chains. According to these results, we speculate that the stimulatory effect of fluoride on bone mineralization may be mediated, in part, by the changes in the rate of synthesis or in the structural characteristics of bone PGs. The changes produced by fluoride in PGs suggest that these molecules play an inhibitory role in the bone mineralization process. J. Cell. Biochem. 83: 607–616, 2001.

Key words: osteoblasts; proteoglycans; fluoride; osteoporosis

Several studies have reported that sodium fluoride (NaF) stimulates bone formation. However, results from in vitro studies showed that the effects of fluoride on bone cells are variable and dependent on the culture conditions [Tenenbaum et al., 1991; Marie et al., 1992; Modrowski et al., 1992; Chavassieux et al., 1993; Kassem et al., 1994]. Thus, some studies demonstrated that NaF increased osteoblast numbers, indicating that the osteogenic effect of fluoride is mediated through changes in osteoblasts proliferation rate [Takada et al., 1996]. Other studies showed completely different results as fluoride did not appear to modify the activity or proliferation rate of human osteoblastic cells [Chavassieux et al., 1993]. This variability may be explained on the ground that different stages of osteogenic cell differentiation have particular responsiveness to fluoride. It has been shown that NaF increases the proliferation rate of osteoblastic precursors but not that of differentiated osteoblasts. On the other hand, NaF increases alkaline phosphatase activity both in osteoblastic precursors and in mature osteoblasts. These results suggest that osteoblast precursors are more sensitive than mature osteoblasts to NaF action, and that the stimulation of proliferation and differentiation of committed osteoblast precursors in bone marrow may mediate the in vivo effects of NaF on bone formation. [Kassem et al., 1994]. In addition, other studies showed that NaF affects bone mineralization by increasing calcium phosphate deposition or by decreasing the solubility of hydroxyapatite crystals [Grynpass, 1993].

The effects of fluoride on bone mentioned above explain why this agent has been widely used in the treatment of osteoporosis. Primary osteoporosis, in both males and in females,

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results from an imbalance between new bone formation and resorption of trabecular bone, leading to bone loss and eventual mechanical failure [Modrowski et al., 1992; Kassem et al., 1994].

The cause for the relative unresponsiveness of some osteoporotic patients to NaF is unknown, in part because the precise mechanism of action of fluoride at the cellular level remains unclear [Modrowski et al., 1992]. A direct demonstration that fluoride may promote bone formation in patients through stimulation of bone cell proliferation and/or differentiation is not yet available. The divergent findings in humans have fostered numerous efforts to understand the mechanisms of action of fluoride on bone cells [Tenenbaum et al., 1991; Modrowski et al., 1992].

In addition to its cellular components, a functional extracellular matrix is an important requisite for the formation and mineralization of normal bone. The major components of the extracellular matrix, which are synthesized by osteoblasts, include type I collagen and noncollagen proteins such as bone sialoproteins and phosphoproteins, osteonectin, osteocalcin, and proteoglycans (PGs) [Klaushofer and Peterlik, 1996]. Different studies have investigated the effects of NaF on bone matrix formation [Reed et al., 1993; Kassem et al., 1994]. No significant effects of NaF were observed on the synthesis of type I collagen; however, in the presence of 1,25-dihydroxivitamin D_3, NaF enhances the formation of type I collagen by osteoblasts in culture [Kassem et al., 1993].

Fluoride has previously been shown to have effects on the PGs of mineralized tissues [Waddington and Langley, 1998]. The exact role that PGs play in bone is not known. It has been proposed that PGs may be involved in mineralization and bone remodeling [Tian et al., 1986]. This proposal is assessed by studies demonstrating that PG aggregates inhibited the mineralization process and that they must be removed for mineralization to begin [Dean et al., 1994].

Thus, it has been described that, under conditions favorable for mineralization in vitro, matrix vesicles produced by osteoblasts become enriched in metalloproteinases capable of digesting PGs, which are potential inhibitors of matrix calcification.

In this study we evaluated the effect of NaF on the synthesis and/or structural characteristics of PGs produced by avian osteoblasts under nonmineralizing culture conditions, to obtain information that may help to understand the mechanisms of action of fluoride in bone cells.

MATERIALS AND METHODS

Isolation and Culture of Avian Osteoblasts

Avian osteoblasts were isolated from 17-day-old chick embryos (30 embryos) as described previously [Gerstenfeld et al., 1989]. Briefly, calvarial bones were carefully removed from the posterior aspect of the cranium; once all the calvaria were removed, any adherent soft tissue was eliminated, and the bones were transferred into a collagenase solution (0.3%, Worthington, Class II), and incubated for 10 min at 37°C. The collagenase solution was removed and the bones were rinsed with Tyrode's solution (Sigma Chemical Co, St Louis, MO). Cells liberated by this digestion, mainly red blood cells and damaged cells, were discarded. Fresh collagenase solution was added to the bones and these were further incubated for 1 h at 37°C. The collagenase solution was then removed, the bones were rinsed with Tyrode's solution, and combined with the collagenase solution just removed. This cell suspension is called Fraction 2 and is enriched in osteoblasts. Fresh collagenase was then added to the bones, which were incubated for an additional hour at 37°C. This final digestion liberated cells is referred to as Fraction 3.

One milliliter of calf serum was added to fractions 2 and 3 to stop the digestion. Both fractions were centrifuged and the cells were resuspended in BGJ_b medium (Sigma Chemical Co.) supplemented with 10% fetal calf serum (FCS) (Gibco-Rockville, MD). Fractions 2 and 3 were pooled and adjusted to a cell density of 1–2 million per milliliter. Cells were plated on uncoated 16-mm plastic dishes (Nunc, Roskilde, Denmark) at a density of 7×10^4 cells per well, and cultured at 37°C in an atmosphere of 5% CO_2. Cells were incubated the following day with fresh medium.

Cell Proliferation

Cells were cultured to a density of 1.2×10^5 cells/well (Multiwells, 24 wells, Nunc, Naperville, IL) in culture medium supplemented with different concentrations of NaF (up to 50 μM). This was used for alkaline phosphatase activity

measurements, radiolabeling of proteoglycans with ^{35}S-sulfate and ^{3}H-glucosamine. After 4 days, cells were removed by a mild treatment with trypsin (0.25%, 10 min, 37°C) and counted in a hemocytometer.

Alkaline Phosphatase Activity

Avian osteoblasts obtained as already described (1.2×10^5 cells/well) were cultured for 4 days and following this, the medium was replaced by culture medium supplemented with different concentrations of NaF (up to 30 μM). The alkaline phosphatase activity produced by osteoblasts was measured directly on the plastic. For this procedure, osteoblasts attached to the plastic dishes were fixed with 3.7% ethanol/formaldehyde for 30 sec. The enzymatic activity was assayed in 2 ml of 50 mM sodium carbonate/bicarbonate buffer (pH 9.6) containing 1 mM magnesium chloride and 1 mM disodium *p*-nitrophenol-phosphate (SIGMA 104 phosphatase substrate, Sigma Chemical Co.) added as substrate to the culture dishes. After 20 min at 37°C, the reaction mixture was removed and 0.5 ml of 3N sodium hydroxide was added. The color developed during the reaction was measured spectrophotometrically at 405 nm.

Radiolabeling of Proteoglycans With ^{35}S-Sulfate

Avian osteoblasts (1.2×10^5 cells/well) were grown in culture medium supplemented with 20 μCi/ml sodium ^{35}S-sulfate (ICN, 43 Ci/mg S) as glycosaminoglycan (GAG) radioactive precursor at 37°C for 24 h. At the end of the labeling period, the culture medium was removed and the cell layer was washed twice with phosphate-buffered saline (PBS). The washes were combined with the culture medium (culture medium fraction) and 0.4 g/ml of solid guanidine-HCl, 1.25 mg/ml *N*-ethylmaleimide, and 0.18 mg/ml phenylmethylsulfonylfluoride were added [Rodríguez et al., 1995]. Cells were detached by mild treatment with trypsin (0.25%, 10 min, 37°C) and counted in an hemocytometer.

To remove unincorporated ^{35}S-sulfate, guanidine-HCl, and other chemicals, the culture medium fractions were applied onto a Sephadex G-50 column (0.8 cm × 5 cm) equilibrated with a buffer containing 8M urea, 0.05M sodium acetate, 0.15M sodium chloride, and 0.5% Triton X-100, at pH 7.0 (buffer A).

Radiolabeling of Proteoglycans With ^{3}H-Glucosamine

Avian osteoblasts (1.2×10^5 cells/well) were cultured in culture medium supplemented with 5 μCi/ml ^{3}H-glucosamine (ICN, 40 Ci/mmol) as a glycosaminoglycan (GAG) radioactive precursor at 37°C for 24 h. At the end of the labeling period, the culture medium was removed and processed as described in the preceding paragraph.

To remove unincorporated ^{3}H-glucosamine, guanidine-HCl, and other chemicals the culture medium fractions were applied onto a Sephadex G-50 column as already described.

Ion Exchange Chromatography

Fractions containing the excluded material (Vo) from the Sephadex G-50 columns were pooled and loaded onto a DEAE-Sephacel column (0.6 cm × 10 cm), equilibrated, and washed with buffer A. The retained material was eluted with a linear gradient of sodium chloride (0.15–0.8 M) prepared in buffer A. Fractions (0.8 ml) were collected and aliquots measured for radioactivity and sodium chloride content [Rodríguez, 1995].

Molecular Sieve Chromatography

To analyze the hydrodynamic size of intact PGs and their GAG chains, the species eluted from the DEAE-Sephacel column were pooled, dialyzed exhaustively against distilled water, lyophilized to dryness, reconstituted in a solution containing 4 M guanidine-HCl, 0.05M sodium acetate, 0.5% Triton X-100, at pH 6.0 (buffer B), and applied to a Sepharose CL-4B column equilibrated with buffer B. Fractions (0.45 ml) were collected and aliquots were used for measurements of radioactivity [Rodríguez et al., 1995].

Preparation and Characterization of Glycosaminoglycan Chains

To further characterize the PG molecules synthesized by the osteoblasts, GAG chains were released from the core protein and analyzed by molecular sieve chromatography; additionally, their sensitivity to enzymatic treatments was studied.

To release GAG chains from the core protein, PGs were treated with 0.1 M sodium hydroxide in the presence of sodium borohydride (75.5 mg/ml) for 48 h at 45°C [Carney, 1986]. To analyze

the hydrodynamic size of GAG chains, the released chains were applied to a Sepharose CL-6B column equilibrated in buffer B. Fractions (0.45 ml) were collected and aliquots used for radioactivity measurements.

GAG chains were also subjected to chondroitinase ABC [Minguell and Tavassoli, 1989] and heparitinase treatments. The extent of the enzymatic digestion was detected by the appearance of low molecular weight-labeled species after Sephadex G-25 chromatography [Keller et al., 1989].

Statistical Analysis

The statistical significance of difference between control and the different concentrations of NaF were determined by Student's *t*-test.

RESULTS

Cell Proliferation

After 4 days in culture in a medium supplemented with different concentrations of NaF, osteoblasts were removed by a mild treatment with trypsin and counted in a hemocytometer. The results showed that NaF affects the proliferation of avian osteoblasts in a bimodal fashion. Thus, at low NaF concentrations, up to 10 μM, NaF had a stimulatory effect on the proliferation rate, with an increase of four to five times in cell numbers. This effect was observed within a narrow range of concentrations, because at concentrations >10 μM, NaF caused an inhibitory effect on osteoblast proliferation. This inhibitory effect also occurred at a narrow range of concentrations, since it was abolished at NaF concentrations >25 μM, when the stimulatory effect on proliferation became apparent again (Fig. 1).

Alkaline Phosphatase Activity

Alkaline phosphatase activity was measured in situ in osteoblasts cultured in the presence of different concentrations of NaF (up to 50 μM). Osteoblasts expressed alkaline phosphatase activity both in the absence and in the presence of NaF. As shown in Figure 2, NaF induced an increase on the enzymatic activity and this increase was dependent on the concentration of fluoride. Thus, the enzymatic activity reached its maximum value in cultures supplemented with 20–25 μM NaF. At concentrations of NaF >25 μM, alkaline phosphatase activity decreased (data not shown).

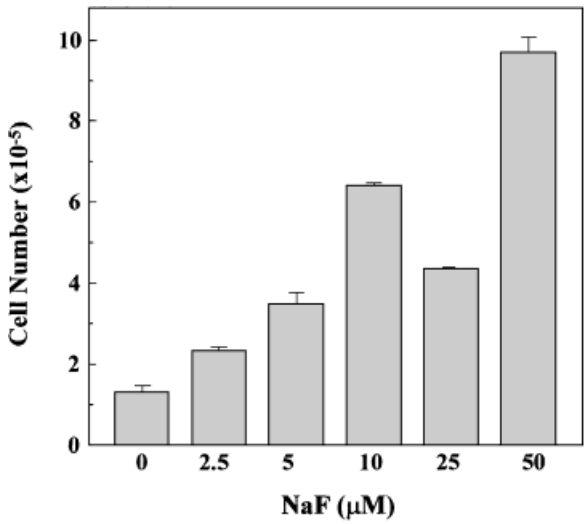

Fig. 1. Proliferation of osteoblasts cultured in the presence of different concentrations of NaF. Cells were cultured to a density of 1.2×10^5 cells/well in a medium supplemented with different concentrations of NaF (up to 50 μM). After 4 days, cells were removed by mild treatment with trypsin (0.25%, 10 min, 37°C) and counted in a hemocytometer. Each experiment was performed in triplicate and the results expressed as mean ± SD.

Proteoglycan Synthesis

Osteoblasts actively incorporated ^{35}S-sulfate into PG molecules in the presence of different concentrations of NaF. As shown in Figure 3, NaF at low concentrations (up to 10 μM) induced a decrease in the incorporation of ^{35}S-sulfate into PGs as compared with its incorporation in the absence of NaF (control). Thus, at 10 μM NaF, sulfate incorporation into PG was around of 35–40% of the PGs produced under control conditions. At concentrations of NaF >10 μM, incorporation of ^{35}S-sulfate increased reaching a value close to 70% of control values at 50 μM NaF.

To evaluate whether NaF affects some structural characteristics of the PG molecules, in addition to their synthesis, PGs were analyzed by ion exchange chromatography, molecular sieve chromatography, and by their sensitivity to specific enzymatic treatments.

DEAE-Sephacel Chromatography

Figure 4 shows the elution profiles of PGs synthesized and released into the culture medium by osteoblasts cultured in the absence (A) or in the presence of 50 μM NaF (B). PGs synthesized by osteoblasts cultured in the absence of NaF eluted from the ion exchange

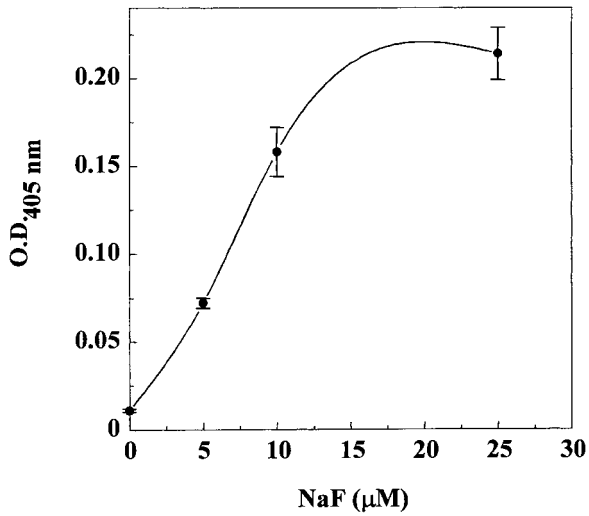

Fig. 2. Alkaline phosphatase activity associated with osteoblasts cultured in the presence of different concentrations of NaF. Cells were cultured to a density of 1.2×10^5 cells/well and after 4 days the medium was replaced by another supplemented with concentrations of NaF up to 25 μM. Alkaline phosphatase activity was measured spectrophotometrically at 405 nm using *p*-nitrophenolphosphate as substrate. Each experiment was performed in triplicate and the results expressed as mean ± SD.

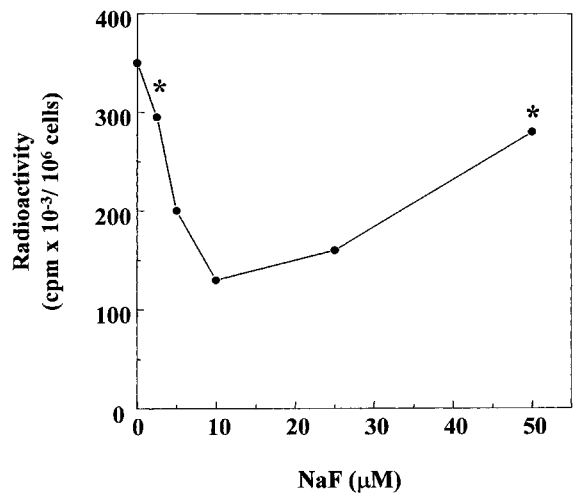

Fig. 3. Incorporation of $^{35}SO_4$ into PG produced by osteoblasts cultured in the presence of different concentrations of NaF. Avian osteoblasts were cultured in medium containing different NaF concentrations (up to 50 μM), and supplemented with 20 μCi/ml sodium ^{35}S-sulfate. Cells were incubated for 24 h at 37°C. For each concentration of NaF, the culture medium from three different wells were pooled and applied onto a Sephadex G-50 column (see Materials and Methods) to remove unincorporated $^{35}SO_4$, guanidine-HCl, and other chemicals. Incorporation of ^{35}S-sulfate into proteoglycans at each concentration of NaF was expressed as cpm/1×10^6 cells. The statistical significance of difference was determined by Student's *t*-test. Asterisk show nonsignificant differences.

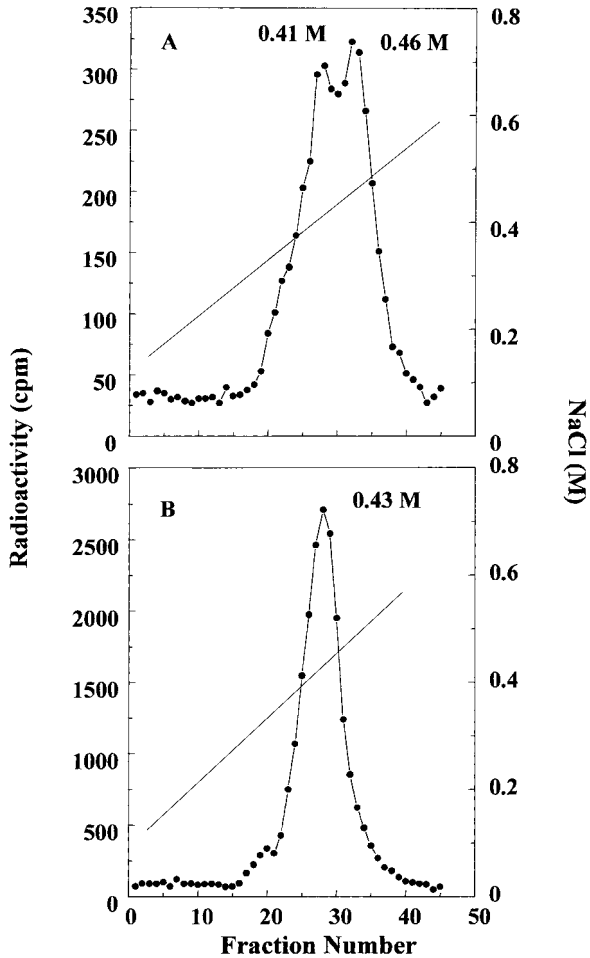

Fig. 4. DEAE-Sephacel chromatography of PG synthesized by osteoblasts in the absence (**A**) and presence (**B**) of 50 μM NaF. Radioactive material (see Materials and Methods) from osteoblasts in culture, was applied to a DEAE-Sephacel column. Retained material was eluted with a linear gradient of sodium chloride (0.15–0.8 M), and aliquots measured for radioactivity and salt concentration.

column as two different peaks with 0.41 and 0.46M NaCl, respectively (Fig. 4A). On the other hand, cells cultured in the presence of 50 μM NaF synthesized PG eluting from the DEAE-Sephacel column as a single, well-defined peak at 0.43 M NaCl (Fig. 4B). This suggests that the addition of NaF to the culture medium induces changes in some of the structural characteristics of the PGs produced by osteoblasts. Thus, the homogenous peak of labeled material eluted from the DEAE-Sephacel column suggests that osteoblasts cultured in the presence of 50 μM NaF synthesized proteoglycan species (one or more) with a similar mean charge density. On the other hand, in the absence of fluoride,

osteoblasts synthesized at least two PG species with different mean charge density (Fig. 4A).

Sepharose CL-4B Chromatography

To evaluate whether the changes observed in mean charge density of PGs synthesized at low and high concentrations of NaF were also related to changes in their hydrodynamic sizes, PGs were analyzed by molecular sieve chromatography on a Sepharose CL-4B column. Analysis of their elution profiles (Fig. 5A and 5B) indicated that the addition of 50 μM NaF to the culture medium did not produce changes in the elution profiles of PGs synthesized by avian osteoblasts. In both cases the radioactive material was recovered from the Sepharose CL-4B column as two different peaks ($K_{av} = 0.47$ and 0.53, respectively). This suggests that osteoblasts cultured in the absence or the presence of NaF (50 μM) produce two PGs differing in their hydrodynamic sizes.

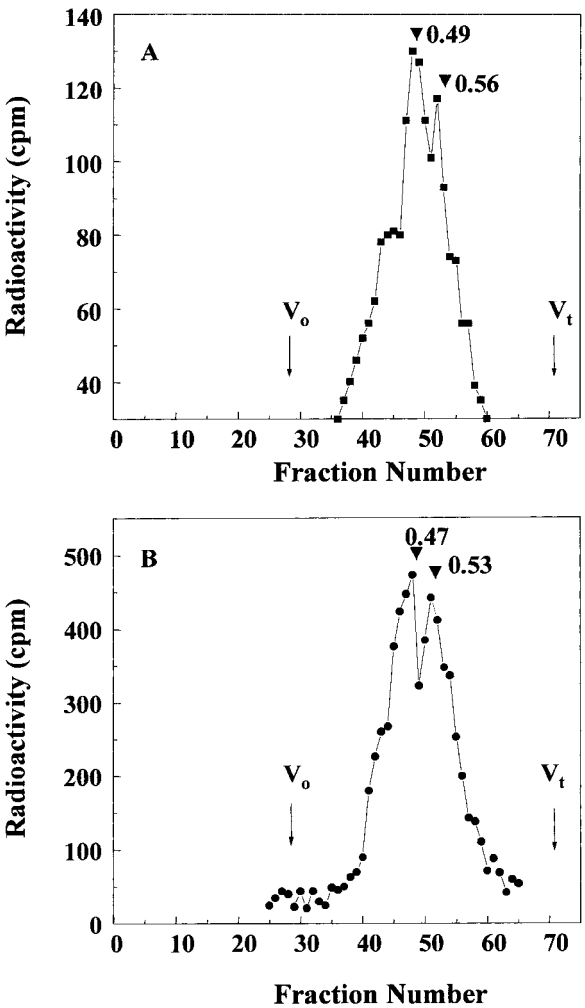

Fig. 5. Sepharose CL-4B chromatography of PG produced osteoblasts in the absence (**A**) and in the presence of 50 μM NaF (**B**). Aliquots of the column fractions were taken for measurement of radioactivity. Arrowheads indicate K_{av} values.

The chromatographic analysis of GAG chains released from PGs synthesized by osteoblasts in the presence or in the absence of NaF in a Sepharose CL-6B column revealed the existence of two species with different hydrodynamic sizes with $K_{av} = 0.38$ and 0.48, respectively (data not shown). This suggests that the fluoride in the culture medium does not affect the length of the GAG chains.

Radiolabeled PGs With ^{3}H-Glucosamine

To elucidate whether changes observed in the ion exchange chromatography are due to changes in the sulfation of GAG chains, PGs were labeled with ^{3}H-glucosamine, and the incorporation of ^{3}H-glucosamine and ^{35}S-sulfate were compared.

The results showed that the patterns of incorporation both of ^{3}H-glucosamine and ^{35}S-sulfate into PGs were similar. At low concentrations of fluoride (up to 10 μM), incorporation of ^{3}H-glucosamine into PGs decreased by about 50% compared with the incorporation in the absence of NaF. At concentrations of NaF >10 μM, ^{3}H-glucosamine incorporation increased, reaching values close to those observed in the controls. (Fig. 6)

The ratio of ^{35}S-sulfate to ^{3}H-glucosamine incorporated into PGs, calculated from the individual isotope incorporation, was similar in the absence and in the presence of different concentrations of NaF (Fig. 7). The largest difference between the ratio values in the presence of 50 μM NaF and the absence of fluoride was of around 10–15%. These results suggest that the effect of fluoride on the structural characteristics of PGs is not due to modifications of the degree of sulfation of GAG chains.

Sensitivity to Chondroitinase ABC and Heparitinase Treatments

To investigate whether the results obtained by ion exchange chromatography corresponded to changes in the nature of the GAG moieties associated to the core protein, PGs produced by osteoblasts at different concentrations of NaF were subjected to enzymatic treatment with chondroitinases ABC and heparitinase.

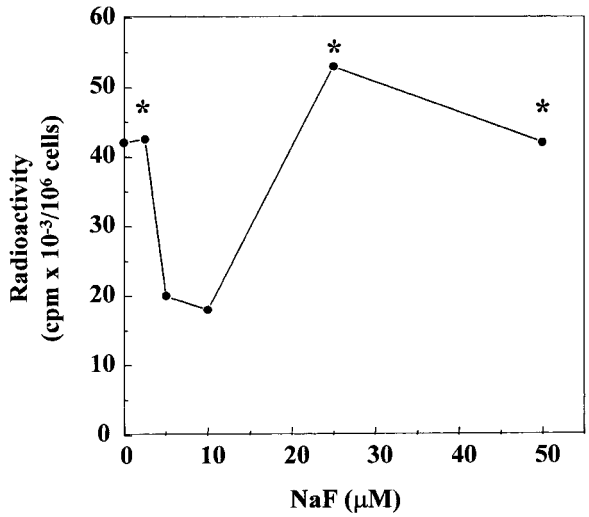

Fig. 6. Incorporation of ^{3}H-glucosamine into PG produced by osteoblasts in the presence of different concentrations of NaF. Osteoblasts were cultured for 24 h at 37°C in a medium supplemented with 5 μCi/ml ^{3}H-glucosamine (ICN, 40 Ci/mmol). For each concentration of NaF, the culture medium from three different wells were pooled and applied onto a Sephadex G-50 column as described in Materials and Methods. The incorporation of ^{3}H-glucosamine into proteoglycans, at each concentration of NaF was expressed as cpm/1 × 10^{6} cells. The statistical significance of difference was determined by Student's *t*-test. Asterisks show nonsignificant differences.

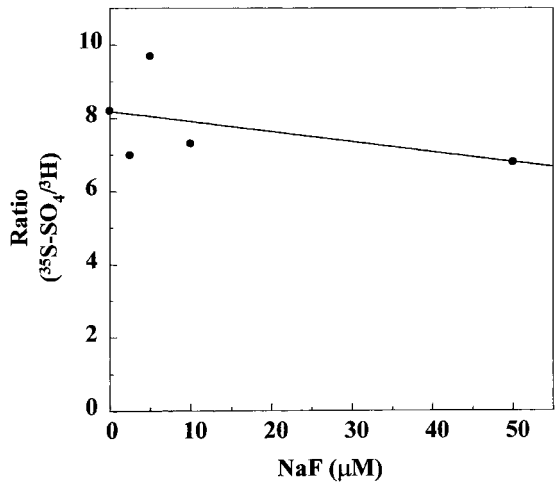

Fig. 7. Ratio of $^{35}SO_4$ and ^{3}H-glucosamine incorporated into PG produced by osteoblasts at different concentrations of NaF. For each concentration of NaF, the ratio of ^{35}S-sulfate and ^{3}H-glucosamine incorporated into PGs was calculated from the individual isotope incorporation. Individual values were fitted to a linear regression.

Results in Figure 8 show that PGs synthesized by osteoblasts cultured at low concentrations of NaF (0–2.5 μM) exhibited different sensitivity to treatment with chondroitinase ABC than PGs produced at higher concentration of NaF (5–50 μM). Thus, 60% of the radioactivity associated with the PGs produced by osteoblasts cultured in the absence or in the presence of 2.5 μM NaF is susceptible to this enzymatic treatment. The sensitivity to chondroitinase ABC increases to above 80%, when cells were cultured in the presence of higher concentrations of NaF (5–50 μM).

Neither PGs produced in the presence nor in the absence of NaF were sensitive to heparitinase treatment (data not shown).

DISCUSSION

Although the exact function of many of the extracellular matrix components in the mineralization process is not well understood, PGs have been implicated as important components in the organization of the extracellular matrix and in mineralization. [Boskey, 1989]

The precise nature of the mechanisms by which PGs intervene in the mineralization process is poorly understood, but there have been suggestions that they bind to the mineral

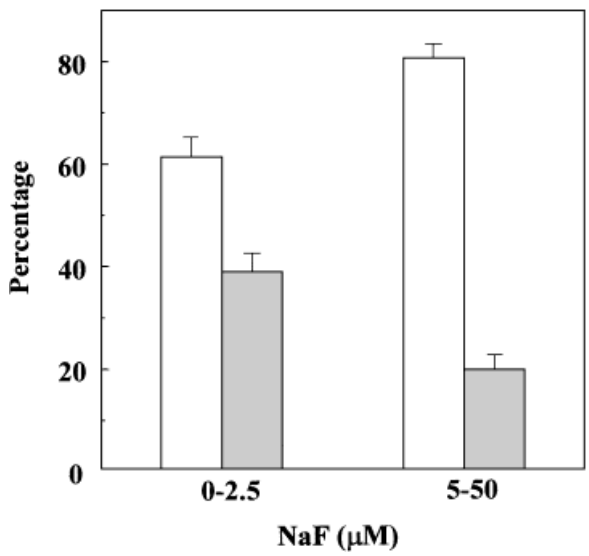

Fig. 8. Sensitivity to chondroitinase ABC of PG synthesized in the absence or presence of NaF. The extent of the enzymatic digestion was evaluated by the appearance of low molecular weight-labeled species after Sephadex G-25 chromatography. Values represent the mean of percentages of ^{35}S-sulfate molecules sensitive (white bars) and resistant (gray bars) to chondroitinase ABC in each range of concentration of NaF (0–2.5 and 5–50 μM). Each experiment was performed in triplicate and values expressed as mean ± SD.

fractions, thus regulating the size and shape of the crystals deposited [Boskey et al., 1997]. Different studies both in vivo and in vitro have been carried out on mineralized tissues [Dziewiatkowski and Majznerski, 1985] to elucidate the role of PGs in the mineralization process. However, their specific role in bone mineralization is still controversial. Some studies concluded that PGs and GAG act as inhibitors of mineralization [Dziewiatkowski and Majznerski, 1985; Takeuchi et al., 1990; Dean et al., 1994], because they showed that partial degradation of PGs at the osteoblastic mineralization front is a requisite for this process. On the other hand, other reports suggest that GAG act as a cation-exchanging calcium reservoir and, when immobilized on a surface, PGs act as mineralization promotors [Lormée et al., 1996]. In vitro crystallization studies have demonstrated that PGs induce changes in the size and morphology of calcium carbonate crystals, a concentration-dependent effect [Wu et al., 1994].

Fluoride has been shown to influence cellular activity and the synthesis and metabolism of the extracellular matrix [Susheela and Mukerjee, 1981; Wergedal et al., 1988; Lau et al., 1989; Waddington et al., 1993; Hall et al., 1996; Lau and Baylink, 1998]. In addition, low concentrations of fluoride are used in the treatment of osteoporosis in an attempt to increase bone mineral density [Farley et al., 1983].

Considering the different effects of NaF in osteoporotic patients, several in vitro studies have been performed to determine which are the target cells and what is the mechanism(s) of NaF action in bone. These studies have reported important differences in osteoblasts responsiveness to NaF depending on their differentiation stage [Tenenbaum et al., 1991; Chavassieux et al., 1993; Kassem et al., 1994]. For instance, the response of the proliferation rate of human precursor osteoblastic cells to fluoride is higher than that of mature cells [Kassem et al., 1994]. Similar results were obtained with respect to alkaline phosphatase activity, and synthesis of procollagen type I and of osteocalcin. However, few studies have focused on the effect of NaF on the rate of synthesis and structural characteristics of the extracellular matrix components produced by bone cells [Kassem et al., 1993].

The results reported here show that NaF increased the proliferation rate of avian osteoblasts in culture as well as the alkaline phosphatase activity. However, the presence of NaF in the culture medium decreased the incorporation of ^{35}S-sulfate into PGs synthesized by osteoblasts. In this study we observed that NaF not only induced changes in the incorporation of sulfate, but that it also induced changes in some characteristics of PGs. Thus, PGs synthesized by osteoblasts in the presence of 50 μM of NaF were more sensitive to chondroitinase ABC than PGs synthesized in its absence. This strongly suggests that NaF induced changes in the GAG chains bound to the core protein of PGs. The increased sensitivity to chondroitinases ABC of PGs synthesized in the presence of NaF indicated an increase in the chondroitin sulfate moieties associated with the core protein, as compared with PGs synthesized in the absence of NaF. Since GAG chains were not completely sensitive to chondroitinase ABC, this suggests the presence of chains that are not chondroitin sulfate, but bound to the core protein. Using enzymatic analysis we were not able to demonstrate heparan sulfate chains associated with these PGs. The predominant GAG identified in the previous study was chondroitin sulfate (CS), with dermatan sulfate (DS) and hyaluronan (HA) also being detected [Waddington and Langley, 1998]. CS has previously been identified as the main constituent of PGs in the extracellular matrix of mineralized tissues. The modification of GAG chain composition was also evidenced by the change in the mean charge density of PGs observed by ion exchange chromatography (Fig. 4). Since the ratio of $^{35}SO_4$/ ^{3}H-glucosamine incorporated into PGs was similar in the presence and in the absence of NaF (8.2 and 7, respectively), it is not possible to explain the differences in mean charge density, by changes in sulfation of GAG chains bound to PGs in the presence of NaF. However, differences in the elution patterns of PGs after ion exchange chromatography may be explained by changes in the composition of GAG associated with the core protein, as demonstrated by their different sensitivities to chondroitinase treatment. Our results agree with those of previous studies [Smalley and Embery, 1980; Waddington et al., 1993; Waddington and Langley, 1998]. However, studies analyzing PGs extracted from fully mineralized extracellular matrix found some discrepancy in the effect of fluoride on the

composition of GAG chains, as the percentage of dermatan sulfate increased [Waddington et al., 1993].

Several factors induce changes in the rate of synthesis or in the structural characteristics of PGs. We have previously observed that the sulfation of PGs is determined by the state of growth of the cell [Rodríguez et al., 1995] or by the presence of different concentrations of fetal calf serum in the culture medium [Rodríguez, 1995]. It has also been demonstrated that the structure of PGs changes with, among other factors, the developmental stages of the cell [Hutchison and Yassin, 1986], with the presence of growth factors [Rapraeger, 1989], or minerals like zinc [Rodríguez and Rosselot, 2001].

According to the results present here, we can speculate that the stimulatory effect of fluoride on bone mineralization may be mediated in part by changes in the rate of synthesis or of the structural characteristics of bone PGs. The changes produced by fluoride in PGs suggest that these molecules play an inhibitory role on the mineralization of bone.

It is interesting to note that the effects observed in the rate of synthesis of PGs, in the composition of GAG chains associated to the core protein as well as in the proliferation rate of avian osteroblasts, were evidenced at a narrow and low range of NaF concentrations. This is in agreement with observations that elevated concentrations of NaF cause negative effects in mineralized tissues (bones and teeth) [Meunier, 1992]. In vitro studies such as those described here may be important to determine the range of fluoride concentrations that produce beneficial effects on bone cells. This is of special importance since NaF is used as therapeutic agent in the treatment of osteoporosis. It has been observed that at low concentrations NaF induces an increase in bone mass by stimulating bone cell proliferation. The effects of prolonged, high concentrations of fluoride on mineralization can be seen in clinical and pathological situations such as skeletal and dental fluorosis, where hypomineralization is evident, and results in bone weakness increasing the rate of fractures. [Fejerskov et al., 1988]. Knowledge of the optimal concentrations of fluoride that produce the greatest beneficial effects on mineralized tissues is also important to undertake adequate fluoridation of drinking water.

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