The Influence of Fluoride on Proteoglycan Structure Using a Rat Odontoblast In Vitro System

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Summary. Using an in vitro rat incisor odontoblast system, the effect of fluoride on proteoglycans was investigated at both the metabolic and structural level. Incisors were removed from 4-week-old rats, split longitudinally, and the pulps removed. Teeth were incubated at 37°C, 5% CO2 in Eagle's Minimum Essential Medium containing 35S-sulfate for 7 hours in the presence of 0 mM, 3 mM, or 6 mM sodium fluoride. Teeth were demineralized in EDTA, proteoglycan was extracted from the residue with 4 M guanidinium chloride, and further purified by anion exchange chromatography. Uptake of radiolabel was monitored by liquid scintillation counting. The resultant products were examined by cellulose acetate electrophoresis, SDS-PAGE, chondroitinase for 7 hours in the presence of 0 mM, 3 mM, or 6 mM sodium fluoride. Teeth were demineralized in EDTA, proteoglycan was extracted from the residue with 4 M guanidinium chloride, and further purified by anion exchange chromatography. Uptake of radiolabel was monitored by liquid scintillation counting. The resultant products were examined by cellulose acetate electrophoresis, SDS-PAGE, chondroitinase digestion, and amino acid analysis. Differential effects of fluoride were observed in both metabolism and biochemical characterization of proteoglycans following incubation at the two concentrations. Fluoride decreased uptake of the radiolabel but led to an accumulation of glycosaminoglycan within the proteoglycan of the matrix. Chondroitin sulfate was the predominant glycosaminoglycan identified, with the additional presence of dermatan sulfate and heparan sulfate identified. Dermatan sulfate levels increased in 3 mM-treated teeth. Fluoride-treated proteoglycans had a reduced molecular weight (200-90K to 180-79K); this reduction is primarily a result of smaller glycosaminoglycan chains, with limited reduction in the size of the core protein of 6 mM-treated teeth occurring. Such alterations in the biochemical metabolism and hence structure and function of proteoglycan may be implicated in the hypomineralization seen in fluorosis.

Key words: Fluoride – Proteoglycans – Odontoblasts.

Fluoride administered at low concentrations is known to be a potent stimulator of bone formation and is often used as a therapeutic agent (10^-5 M serum concentrations) in diseases such as osteoporosis. However, the prolonged ingestion of excessively high amounts of fluoride leads to both skeletal and dental fluorosis, characterized by hypomineralization of calcified tissues. Both collagenous and noncollagenous components are known to undergo structural alteration during fluorosis. However, the precise mechanisms of fluoride toxicity with respect to biochemical changes of the organic matrix within the mineralized tissues, including synthetic events, have not been fully elucidated.

The organic matrix of all connective tissues, both mineralized and nonmineralized, can be generally described as a collagenous fibrous network embedded in a ground substance, an important component of which are proteoglycans. These nonfibrillar macromolecules are composed of a central protein core, the amino acid composition of which are characteristically enriched in aspartic acid, glutamic acid, serine, and glycine. Attached to this protein core are highly anionic glycosaminoglycan (GAG) chains, covalently bound via the serine residue. The nature of the proteoglycan is such that it is considered to play possible roles in hydration, compressibility, collagen fibril formation, and mineralization. Chondroitin 4-sulfate has been identified as the predominant GAG present in mineralized tissues studied from various sources and dental fluorosis, characterized by hypomineralization of fluorotic tissues may be partly responsible for the irregular mineralization leading to reduced bone strength. Using an in vitro rat incisor odontoblast system, the present study was carried out to examine the effect of various concentrations of fluoride within the culture medium on the metabolism and biochemical structure of proteoglycan within the mineralized matrix.

Materials and Methods

Preparation of Teeth and Incubation Procedure

The procedure used for the isolation and exposure of the incisor
fluoride. To serve as a control, the remaining incisor preparation and incubated in 10 ml of media, as described above, containing 200

Effect of Fluoride on Incorporation of $^{35}$S-Sulfate into Proteoglycans

The tooth preparations were split into six equal groups, into a six-well plastic culture tray, each well containing 5 ml of Eagle's Minimum Essential Medium supplemented with 1% non-essential amino acids, 1% D-glucose, 2 mM L-glutamine (Sigma Chemical), and 100 μCi of Na$_2^{35}$SO$_4$ (Amersham International, UK) per culture well. The incisor preparations were incubated at 37°C, in 5% CO$_2$, for 1, 3, 5, 7, 9, and 11 hours. After each time interval, the incubation was stopped by removal of the media. Teeth were stored at -20°C until required for extraction, purification of proteoglycan, and assay of incorporation of the radiolabel, as described below.

The results from this preliminary time course study indicated maximum incorporation of the radiolabel into the dentine/predentine proteoglycans was determined to be 7 hours (Fig. 2). On the basis of this profile, an incubation time of 7 hours was chosen to investigate the influence of fluoride on $^{35}$S-sulfate incorporation. In a separate study, the incisor preparations were split into three equal groups, and incubated in 10 ml of media, as described above, containing 200 μCi Na$_2^{35}$SO$_4$, supplemented with either 3 mM or 6 mM sodium fluoride. To serve as a control, the remaining incisor preparation was incubated in media in the absence of fluoride. Teeth were incubated for 7 hours at 37°C in 5% CO$_2$. All biochemical analyses were performed on the proteoglycan preparation obtained from this latter study.

Extraction and Purification of Proteoglycan from Dentine

Following incorporation of $^{35}$S-sulfate, the teeth were frozen in liquid nitrogen and powdered in a ball mill prior to demineralization with 10% EDTA (trisodium salt), pH 7.45 for 5 days at 4°C; the sample was held within dialysis sacs (Mr cut-off 12,000). The EDTA was removed by exhaustive dialysis against double-distilled water containing protease inhibitors (1 mM iodoacetic acid, 5 mM n-ethylmaleimide, and 5 mM benzamidine HCl) for 3 days at 4°C plus 1 day against double-distilled water. Decalcified samples were recovered by lyophilization. To release proteoglycan, samples were extracted with 4 M guanidinium chloride, 0.05 M sodium acetate, pH 5.9 for 48 hours at 4°C in the presence of the above proteolytic inhibitors. The extract was dialyzed against double-distilled water, including proteolytic inhibitors at 4°C followed by lyophilization. Proteoglycan was further purified by anion exchange chromatography using FPLC (Pharmacia LKB Biotechnology) on Mono Q (1 ml bed volume). Unbound material was eluted from the column with 7 M urea, 0.05 M tris-HCl, pH 6.5. A linear gradient, increasing in sodium chloride concentration from 0–1 M in the above buffer, over 24 ml, selectively eluted bound material from the column. Absorbance at 280 nm was recorded, the profile of which is shown in Figure 1, together with information on the protein fractions obtained.

Protein fractions were dialyzed against distilled water, including proteolytic inhibitors followed by lyophilization. Duplicate 0.5-μg samples were dissolved in 3 ml Optiphase "Hisafe" II liquid scintillation fluid (Pharmacia LKB Scintillation Products), and levels of incorporation of $^{35}$S-Sulfate into proteoglycan were assessed by liquid scintillation counting. The uptake in radioactivity was measured as counts/minute/μg of glycosaminoglycan, as determined by cellulose acetate electrophoresis (see analytical procedures). Proteoglycan was detected in fraction V only as confirmed by the detection of Alcian blue staining material following cellulose acetate electrophoresis and liquid scintillation counting.

Analytical Procedures

Cellulose Acetate Electrophoresis. Samples (100 μg) of the purified proteoglycan extract were treated with 10 μl of a nonspecific protease (Sigma Chemical Company, type XIV) in 0.1 M tris-HCl, 5 mM CaCl$_2$, pH 7.5 (5 mg/ml) to liberate the glycosaminoglycan constituents. The digests were examined by cellulose acetate electrophoresis in 0.2 M calcium acetate, pH 7.2 for 4 hours at 0.6 mA/cm width of sheet [10]; the separated components were stained with...