TEM analysis of the early mineralization process of mantle dentin

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Proteoglycans (PGs) interact with hydroxyapatite and collagen fibrils during dentinogenesis and are proposed to play an important role in the regulation of the mineralization process. The aim of this study was to investigate the relationship between PGs and collagen fibrils at the early mineralization process of mantle dentin. Ten first molar dental germs of rats were removed and fixed in glutaraldehyde/formaldehyde in cacodylate buffer and post-fixed in osmium tetroxide. The samples were dehydrated and embedded in epoxy resin. Ultrathin sections were contrasted and analyzed in TEM before and after treatment with EDTA, chondroitinases AC and ABC. After EDTA treatment, a electrondense substance associated with collagen fibril was removed, and did not stain again. A high magnification of these areas showed globular structures with 15nm diameter surrounding collagen fibrils. In advanced mineralization areas, collagen fibrils showed a banded pattern and at high magnification the fibrils presented a light 10nm ring inside and a dark 10nm ring outside. After chondroitinase treatment, the electrondense substance associated with collagen fibrils was removed, showing a banded pattern of clear and dark areas along them. From morphological data, the authors proposed a model of interaction between PGs and collagen fibrils, where glicosaminoglycans chains are inside the fibrils, while the protein core remains outside. That stereochemical arrangement would start the crystal nucleation.

Key words: mantle dentin, mineralization, proteoglycans, collagen fibrils

Introduction

Dentine is a calcified connective tissue which provides a good model for the study of biomineralization, where the unmineralized predentine and mineralized dentine are easily discernible [1,2]. Two types of dentine were identified based on differences in properties of the mineral or the matrix: mantle dentine and circumpulpal dentine. Different mechanisms of initiating the mineralization and of organizing collagenous matrix have been described mandating separate analyses of these two kinds of dentine [3]. In the present study, was evaluated the early mineralization process of mantle dentine using Transmission Electron Microscopy analysis and selective enzymes for chondroitin 4-sulfate, chondroitin 6-sulfate and dermatan sulfate.

Briefly, dentine matrix is composed of type –I collagen fibrils, noncollagenous proteins (NCPs) and nanocrystalline apatite mineral [1,4]. The interaction of a range of acidic macromolecules (noncollagenous proteins), including phosphoproteins and proteoglycans (PGs) with collagen and minerals, has been proposed to explain the mineralization process [2,5,6,7].

PGs have been ascribed roles in collagen fibrillogenesis (Millan et al., 2005) and in the initiation of mineralization in the vicinity of the collagen gap zones for being able to bind the growing calcium phosphate surface, consequently influencing crystal growth and morphology [2]. Current theories of mineralization have proposed distinct pools of PGs during dentinogenesis [6,8], with a higher proportion

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of dermatan sulfate glycosaminoglycans (GAGs) within predentine, which were solely substituted with chondrointin sulfate in dentine matrix [7].
The modifications in PGs chemistry and conformation appear to be important in early mineralization process, since PGs chondroitin-sulfate rich have an increased affinity for hydroxyapatite (HAP) [8] and with dentine phosphoproteins are involved in the initiation, stabilization and control of HAP crystal growth [2]. In spite of studies clearly indicating that the interactions of these PGs, classified as small leucine rich proteins (SLRPs), with hydroxyapatite and calcium ions have been implicated in crystal nucleation [6,9], the interaction of these PGs with collagen fibrils are not yet fully understood. Therefore, the present study was done in order to investigate further the relationship between PGs, collagen fibrils and hydroxyapatite at early mineralization of mantle dentine.

Materials and Methods

Ten first molar dental germs were dissected from maxillas of five 4-days-old rats (*Rattus norvegicus*). The germs were sliced and fixed in solution of 4% glutaraldehyde and 4% formaldehyde in 0,1M sodium caccodylate buffer (ph 7.4) at room temperature for 6 h. The samples were stored in 0,1M sodium caccodilate buffer overnight. Next the samples were post-fixed in osmium tetroxide in 0,1M caccodilate buffer (pH 7.4) at 4^0C for 2 h, dehydrated in graded ethanol solutions and embedded in epoxy resin.
Semithin sections of 1μm thick were obtained, with glass knives on Reichert Ultracut microtome, and stained with toluidine blue. The sections were observed by light microscope to select the areas of mantle dentine. From these select areas, were obtained ultrathin sections (50 nm thick) with diamond knife. The sections were contrasted with uranyl acetate and lead citrate and examined in a Carl Zeiss EM-109 Transmission Electron Microscope (TEM). Moreover, electron micrographs at dentine mantle were obtained. After first analysis, the sections were treatment with 10% EDTA aqueous solution and chondroitinases in Tris-HCL buffer. Sections on nickel grids floated for 30 minutes on drop of EDTA or drop of distilled water as control in wet chamber for 60^o minutes at 30^0C. The same was done with chondroitinases AC (in order to remove chondroitin 4-sulfate and chondroitin 6-sulfate) and chondroitinases ABC (in order to remove chondroitin 4-sulfate and dermatan sulfate) using a drop of Tris-HCL buffer as control. After experimental treatment, the grids were washed with in distilled water and contrasted with uranyl acetate and lead citrate again. The electron micrograpy images before and after treatments were obtained at the same conditions and analysis by a single investigator.

Results

At the beginning mantle dentine mineralization, some collagen fibrils showed electrondense substance associated with them (Fig. 1A). After EDTA treatment, that substance was removed and did not stain again (Fig. 1B). At high magnification of these areas, were observed globular structures with 15 nm diameter surrounding fibrils collagen in cross section (1C). The longitudinal section showed similar globular structures arrangement along fibril axes.

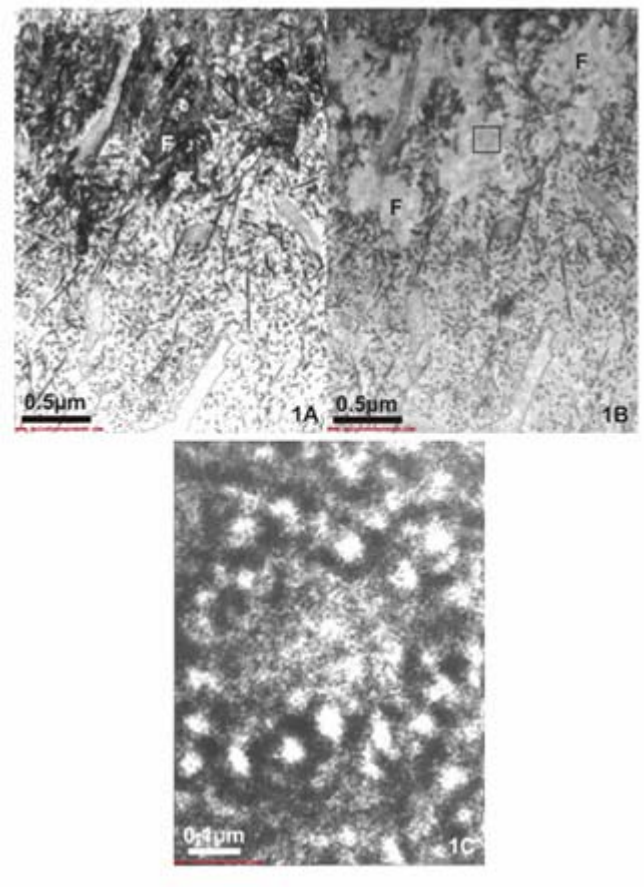

Fig. 1. Electron micrographs of mantle dentin. A-before EDTA treatment, collagen fibrils showed an electrondense substance associated with them. (F). B-After EDTA treatment, the electrondense substance was removed (F). C-High magnification of figure 1B at areas where the electrondense substance was associated before treatment. These areas showed globular nanospheres of 13-15 nm diameter surrouding the collagen fibrils in cross section (arrow).

In more advanced mineralization areas, collagen fibrils showed a banded pattern, after EDTA treatment (Fig. 2A). High magnification of these areas, in collagen fibrils cross section, showed a light 10nm ring surrounding central granular spots and a 10 nm dark ring outside (Fig 2B).
After chondroitinases treatment, the electrondense substance associated to collagen fibrils was removed, showing a banded pattern of clear and dark areas along them. Both chondroitinases (AC and ABC) showed the same results (Fig. 3A-B).

Based on images obtained with electronic micrographs before and after enzymatic treatment, was created a model of interaction between proteoglycans and collagen fibrils during the mineralization process (Fig 4).

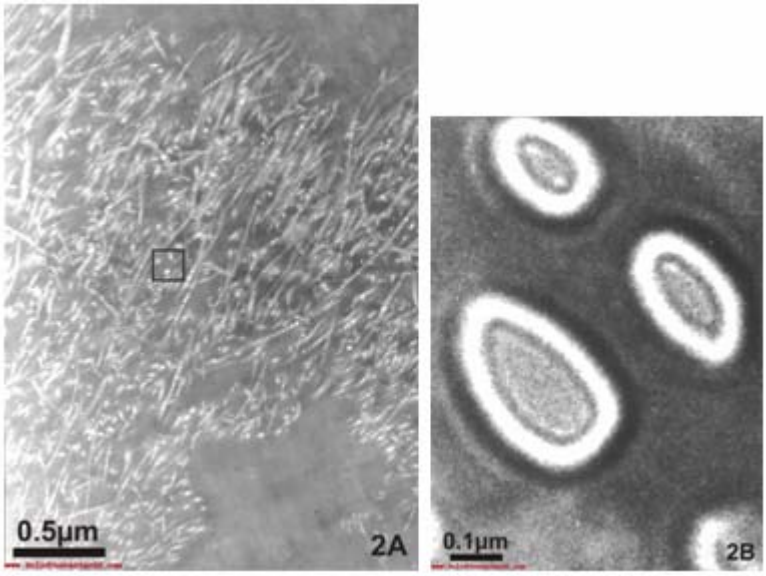

Fig. 2. Electron micrographs of mantle dentin after EDTA treatment. A-The collagen fibril showed a banded pattern. B- High magnification of 2 A showing collagen fibrils in cross section presenting a light 10 nm ring inside and a dark 10 nm ring outside.

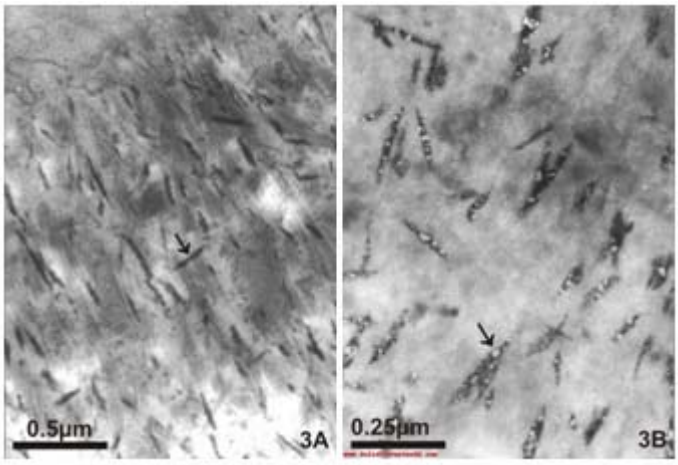

Fig. 3. Electron micrographs of mantle dentin. A- Areas with electrondense substance associated with collagen fibrils (arrow). B - after chondroitinases "ABC" treatment, the collagen fibrils showed a banded pattern (arrow).

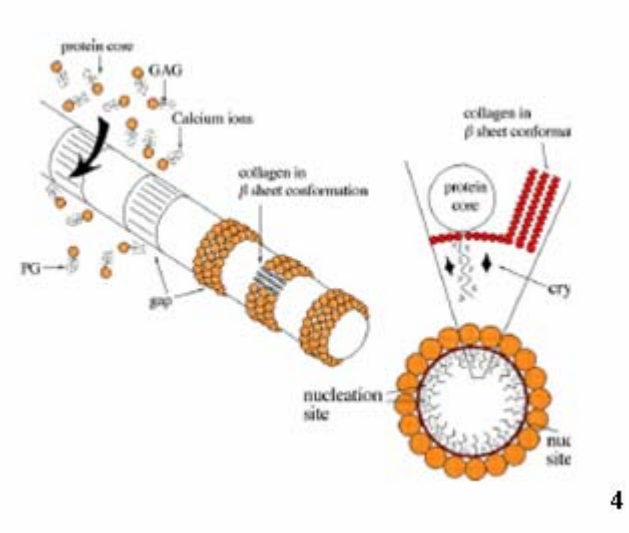

Fig.4. Cartoon of collagen fibrils-proteoglycans interaction.

Discussion

Previous studies using biochemical, histochemical and immunohistochemical techniques have identified and characterized the major proteoglycans (PGs) in predentine and dentine and their influence on the mineralization process [1,2,7,8,9,10]. Based on these studies, several hypotheses have been raised on the attempt of establishing a model of interaction between PGs-collagen fibrils-hydroxyapatite, although conflict views on the role of proteoglycans (SLRPs) in the initial mineral formation have been expressed.
In the present study was postulated the relevance of SLRPs in early mineralization process of mantle dentine. To confirm this idea, sections of the mantle dentine zone, selected from similar histological areas, were treated with chondrointinases AC or ABC. These enzymes promote protease digestion of PGs, removing the GAG chains [11]. Images obtained had showed an electrondense substance on collagen fibrils which was removed by specific chondroitinases confirming collagen fibril-GAG interactions (Fig 3). These observations associated with the results obtained from Rees *et al*. (2001) reinforce the hypothesis that in the intact PG molecule, the GAG chains and the protein core working together, producing a suitable conformation for hydroxyapatite nucleation. Supporting the present findings, Millan *et al*. (2004) demonstrated that the removal of the GAG chains from dentine PGs resulted in 76% reduction in HAP binding affinity. Therefore, both the protein core and GAG chains are able to bind to the HAP surface [8].
The absence of differences in the images obtained after chondroitinase AC or ABC treatment was expected. Previous studies had demonstrated a reduction of dermatan sulfate GAGs toward the mineralization front [2,8], with 100% of chondroitin sulfate PGs in mineralized dentine [7]. These results confirm the presence of the different pools of PGs during dentinogenesis, with different effects upon mineralization process [6,8,9]. However, dentine PGs are not just involved in mineralization, they are related to collagen fibrillogenesis, with the interactions mediated not only through the core protein but also through the GAG chains [7]. The proteoglycans ability to link to collagen is important for the formation and organization of collagen fibrils [12]. PGs influence in collagenous framework promotes a series of structural changes prior to the mineralization. The mechanism of such activity is probably by changing the assembly, structure, and charge of fibrils [7,12]. The relationship between collagen fibrils and PGs was evaluated in the present study after EDTA treatment.
In areas of the beginning of mantle dentine mineralization were observed 15 nm electrondense spheres around the cross-sectioned collagen fibrils (Fig 1), which were removed after EDTA treatment. These spheres were similar in size to globular proteins of PGs, biglican and decorin, which are SLRPs [13,14].

Considering that EDTA is a calcium chelant able to dissolve the mineral without altering dentine proteins [4], it would be expected that, after their use, only HAP would be removed. However, HAP shows high affinity to SLRPs [8]. Such affinity may attributed to their three dimensional structure and highly negative charge chains [11]. Therefore, it would be impossible to remove them separately.
These remarks, associated to literature data [11,15], suggest interection between collagen-PGs which could help the nucleation of hydroxyapatite crystals. Nevertheless, it is also important to reminder that the referred images could represent phosphoproteins, which are covalently bound to insoluble collagen [16] and may act as nucleators [2]. In order to confirm these, an association of techniques, such as a immunolocalization using specific antibodies, would be interesting. However, images obtained at more advanced areas (Fig. 2) allow the speculation of the PGs-collagen interaction.
The GAG chains and the protein cores may act synergistically exposing the anionic groups and binding tightly to cations such as calcium [7,9,11]. The results presented would support the theory that the interaction between GAG and calcium might create a 10 nm ring of light-stained substance inside the fibril. The globular core protein might stay at collagen fibrils superfaces forming a 10nm dark ring observed outside the collagen fibrils. Our data suggests that these collagen fibrils-PGs interactions establish sites of crystal nucleation. At the gap region, the collagen molecules would be disturbed by the presence of negatively charged GAG chains and by calcium ions concentration, which would favor β sheet conformation. These pleated sheets would maintain two-residues repeated at a distance of 7A° in a highly concentrated calcium area, allowing the mineralization to start. Calcium could be interacting with complementary domains of carboxylates in the adjacent gaps of the collagen fibrils. This stereochemical effect could be related to calcium fixation and to the formation of the first crystals [17,18].
The results of the present study associated to the results from the other researches which had used different analysis methods [2,7,8,15], allow the development of a model of interection that may be involved in the initiation of HAP crystal formation, as we propose in Figure 4. Further investigations will be needed to explore the model proposed to explain the interaction between PGs and collagen fibrils.

Final considerations: This paper is a reviewed format of A model of the early mineralization process of mantle dentin published in Micron 2007;38 (5):486-91.

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