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Chondroitin sulfate is involved in the hypercalcification of the organic matrix of bovine peritubular dentin

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ABSTRACT

Apatitic mineral of dentin forms within the collagenous matrix (intertubular dentin, ITD) secreted from the odontoblastic processes (OP). Highly calcified mineral (peritubular dentin, PTD) is deposited at the interface between the ITD and each process membrane, creating a tubular system penetrating the dentin that extends from the dentino–enamel junction to the predentin–dentin junction. We focus on determining the composition of the PTD both with regard to its organic matrix and the inorganic phase. A laser capture technique has been adapted for the isolation of the mineralized PTD free from the ITD, and for the analysis of the PTD by SEM, TEM, and energy dispersive spectrometry (EDS), these data were subsequently compared with similar analyses of intact dentin slices containing ITD bounded-PTD annuli. Elemental line scans reveal clearly marked boundaries between ITD, PTD, and OP components, and illustrate the differences in composition, and topographical surface roughness. The organic matrix of the PTD was shown to be sulfur rich, and further antibody labeling showed the sulfated organic component to be chondroitin sulfate B. In this PTD organic matrix the S/Ca and Ca/P ratios were distinctly higher than in the ITD, indicating that polysaccharide bound S supplies the anionic counterion facilitating the formation of the apatitic PTD mineral.

1. Introduction

Tooth dentin begins to form at the dentinoenamel junction (DEJ), the junction of the polarized enamel-forming ameloblasts (AE) and the polarized dentin-forming odontoblasts (OD). As the dentin layer thickens, the OD cell bodies are driven inward in the direction of the dental pulp but each OD body remains connected to the DEJ via a major elongating odontoblastic process (OP). Thus the dentin extracellular mineralized collagenous matrix is not a solid body, but is penetrated (or fenestrated) by the OP which have a composition different from the mineralized matrix, and in dentin sections the cavities they once occupied have the appearance of tubules. Initially an OP is in direct contact with the surrounding wall of mineralizing secreted collagen fibrils, but as the cell body retracts in the direction of the pulp, its elongating OP also partially retracts and narrows, so that the membrane bound cell process leaves a space between the secreted mineralized dentin wall and the OP membrane. This space becomes filled with a hyper-mineralized collar (relative to the mineralized dentin) known as the peritubular dentin (PTD) (Orban's Oral Histology and Embryology. S.N. Bhaskar, ed., 10th edition, 1986, C.V. Mosby Co., Fig 4-4 p 105, or other standard text) (Orban & Bhaskar, 1986). The mineralized fibrillar collagen surrounding the tubules is called the intertubular dentin (ITD). Discussions of "dentin" usually consider the ITD to be the main component of the dentin but this is not entirely true because the packing of the dentinal tubules and spacing between tubules varies with the position of the tubules relative to the distance between the DEJ and pulp and their position relative to the cervical line (crown–root boundary) as well as tooth type and shape (Dutra-Correa, Anauate-Netto, & Arana-Chavez, 2007; Garberoglio & Brannstrom, 1976; Schilke, Lisson, Bauss, & Geurtsen, 1999). We have recently shown (Dorvee, Deymier-Black, Gerkowicz, & Veis, 2014), using careful ImageJ (Schneider, Rasband, & Eliceiri, 2012) analysis of undemineralized bovine molar slices cut at mid-length between DEJ and pulp, and perpendicular to the tubule long axis direction, that cross-section areas occupied by the ITD and PTD were nearly equal (in some sections, PTD >50 area%). Earlier, using time-of-flight secondary ion mass spectroscopy (TOF-SIMS), we had shown that the radial thicknesses of a PTD annulus was not uniform in all directions (Dorvee et al., 2014; Gotliv, Robach, & Veis, 2006) and that, while hypermineralized, the PTD organic matrix was essentially free of collagen (Gotliv & Veis, 2009; Gotliv & Veis, 2007) and showed

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evidence of a phospholipid–proteolipid complex. Bertassoni, Stankoska, and Swain (2012) confirmed the absence of collagen as a major component of the PTD, and showed that the PTD matrix contained a significant content of proteoglycans. Lu et al. (2014) have identified chondroitin sulfate within the tubule lumens and at the ITD-collagen interface.

It is clear that "dentin" is a heterogeneous material, and at least three components need to be considered: (1) The mineralized collagenous network of the ITD; (2) the hypermineralized PTD annulus; and (3) the cellular odontoblast process, including both membrane and cell contents. The structure and composition of the ITD is reasonably well known, but the composition, role, and function of the heterogeneous, hypermineralized, non-collagenous PTD remains enigmatic. In the present study we have used a variety of high resolution imaging and analysis procedures to determine the compositions of the dentin components in the context of their positions within the ITD and PTD, while also knowing exactly their position within the dentin. Our recently developed mineralized tissue Laser Capture technique (Dorvee et al., 2014) has been a major tool in this study and has allowed us to isolate both individual tubules and small groups of tubules and examine their PTD contents and structure to correlate along with other imaging and spectroscopic modalities.

2. Methods

2.1. Sample collection and preparation

Lower jaws were collected from 18 month old Black Angus cows immediately following their death (Aurora Packing Co., West Aurora, IL). The jaws were rinsed in cold water and kept on ice until transported to the laboratory. As soon as feasible, the unerupted molar teeth were freed from their bony crypts using bone saws. The large third molars were selected for study. As seen in Fig. 1(a-i) third molars have a complex folding of the enamel, and two to three roots.

Nevertheless clearly defined regions where a full clear tubule path from DEJ to PD-D were detected, Fig. 1(a-i,ii). The dental sacs surrounding the unerupted roots were rinsed away, then, as shown in Fig. 1, the teeth were cut into approximately 2.5 mm slices oriented perpendicular to the crown-root tooth axis. The cervical line of each tooth was easily identified and the sectioning was begun at 3 mm above that position. All cuts were made with a 1000 Precision Isomet Saw (Buehler, An ITW Company) using a 0.5 mm thick diamond blade lubricated with water. The slices were washed with deionized H_2O and frozen for storage until use. The position of each tooth slice relative to the cervical line was recorded. The presence of PTD in each slice was verified microscopically, then discrete $\sim 6 \times 3 \times 2.5$ mm blocks were excised from the tooth cross section slices, again using the 1000 Precision Isomet Saw with a 0.5 mm thick diamond blade, lubricated with water. Blocks were rinsed with DI H_2O, frozen and lyophilized for 6 h to completely dry the samples. The dry samples were then encased in Epon with no additional dehydration or infiltration. The Epon mixture was made with 13 mL Embed 812, 8 mL DDSA (Dodecenyl succinic anhydride), 7 mL NMA (NADIC methyl anhydride) and 0.45 mL DMAE (dimethylaminoethanol) (all from Electron Microscopy Sciences, Hatfield PA). The tooth sections were laid in the bottom of a shallow silicone mold and covered with the Epon mixture. The Epon encased tooth sections were then set in the oven at 60 °C overnight. Once cured, the Epon blocks were then trimmed, turned over in the mold, re-encased in a fresh Epon mixture set in the oven at 60 °C overnight. The result of this repeated encasement produced tooth sections embedded in the center of the Epon block. After general microscopic examination the orientation of the tubules between predentin–dentin junction and the DEJ was

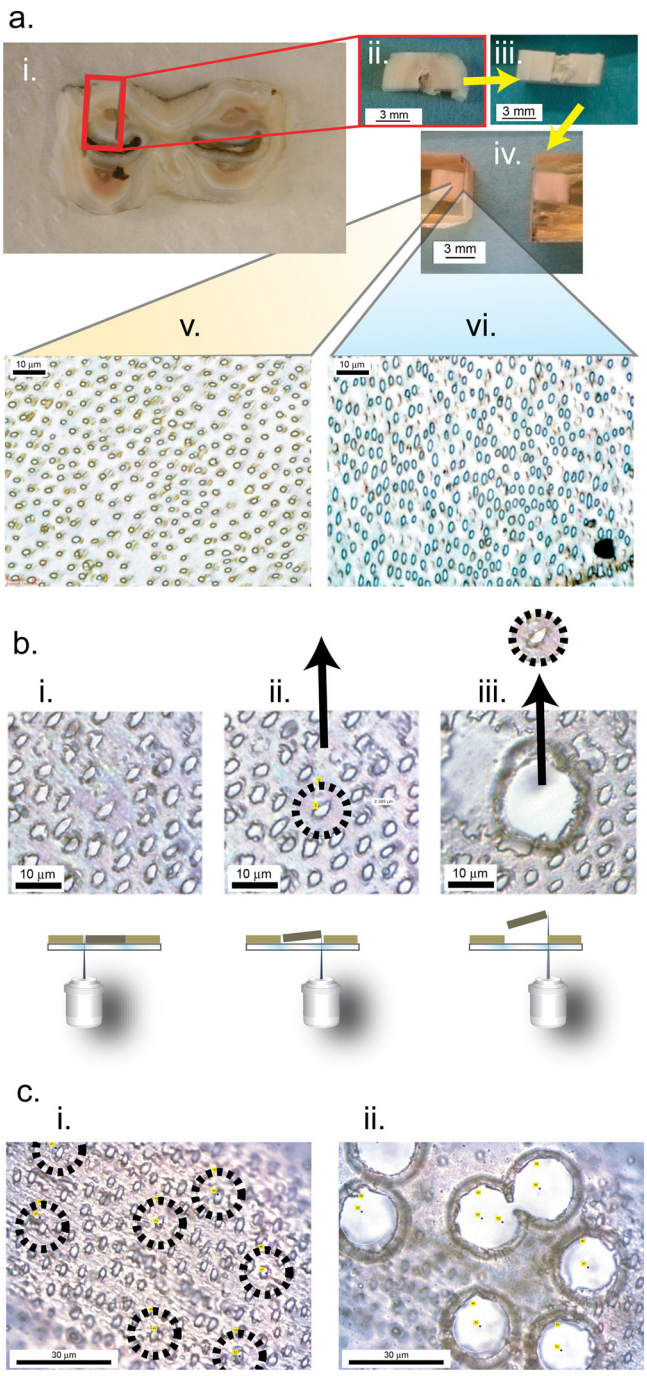

Fig. 1. Schematic of the targeting and isolation of dentin components for further analysis (a). Millimeter scale preparation: (a-i) A 2.5 mm thick slice from an intact bovine third molar tooth, collected 3 mm above the cervical line. (a-ii) A block cut as outlined from (a-i). Enamel, dentin and pulp cavity are all easily seen (a-iii). (a-ii) rotated 90°. The tubules running from the DEJ to the pulp cavity are essentially parallel to the a-iii surface horizontal plane. (a-iv) Block (a-iii) encased in Epon. This block was then sectioned by microtome from the predentin-dentin (PD–D) junction to the DEJ. (a-v) This is a 1 µm section from midway between the DEJ and PD–D while section (a-vi) is from a region near PD–D junction. A distinct mineralized PTD collar was observed around the tubules in (a-v), while PTD collars were not observed around the tubules present in (a-vi). (b & c) Micrometer preparation: (b-i) the microtomed 1 µm slices were examined to select areas where a tubule could be seen as essentially circular and free of contact with nearby tubules, and perpendicular to the plane of the section to assure that the tubule penetrated the full thickness of the slice. (b-ii) These slices were transferred to a Zeiss PALM PEN-MembraneSlide and the selected tubule was then cut with the appropriate circular laser path. The laser was then pulsed and the cut section catapulted to a TEM grid lying on second slide for analysis. (b-iii & c-i, ii) Using laser microdissection, multiple tubules can be targeted and removed.