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Original Article

Analysis of calcium, phosphorus, and carbon concentrations during developmental calcification of dentin and enamel in rat incisors using scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX)

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ABSTRACT

Objective: The study was designed to investigate the concentrations of calcium (Ca), phosphorus (P), and carbon (C) during developmental calcification of dentin and enamel in rat incisors.
Methods: Mandibular incisors from eight 2-week-old male Wistar rats were analyzed by scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX). We analyzed data on the elements in the course of developmental processes in dentin and enamel and along the vertical line of the matrix between odontoblasts and ameloblasts.
Results: The dentin concentrations of Ca and P and the Ca/P ratio were the lowest, while the C concentration was the highest in initial dentin. The Ca and P concentrations were the lowest, whereas the C concentration was the highest in predentin along the vertical line; the Ca/P ratio did not show any differences. The concentrations of Ca and P increased, while the C concentration decreased during early maturation and more so in late maturation in developing enamel, while the Ca/P ratio increased during late maturation. The Ca and P concentrations and the Ca/P ratio were the highest, while the C concentration was the lowest in enamel adjacent to the junction with dentin on the vertical line.
Conclusions: During tooth development, the initial dentin matrix may possess distinctive mineral characteristics as compared with other parts of dentin and predentin. Elemental composition of the mineral in enamel may change during late maturation. Our results are suggestive of degradation of organic components during developmental calcification in dentin and enamel.

1. Introduction

Dentin and enamel are characteristic tissues of teeth and are known to develop with reciprocal interaction of the respective matrix-producing cells, i.e., odontoblasts and ameloblasts [1,2]. The tissues are calcified during development, but how the calcification proceeds is not well understood.

Odontoblasts deposit initial dentin and calcify the matrix before enamel is secreted by ameloblasts [1]. Matrix vesicles derived from odontoblasts are thought to provide nucleation sites for calcification of the initial dentin to form the outer layer of dentin: mantle dentin. In contrast, calcification of the inner layer of dentin—circumpulpal dentin—that is formed after the outer layer, proceeds via spreading of mineral deposition from the pre-existing calcified dentin matrix [1,3]. Little is known about the differences among matrices of initial dentin, dentin in the outer layer, and dentin of the inner layer in terms of concentrations of calcium (Ca) and phosphorus (P)—representing minerals—and carbon (C), representing organic matrices [4,5].

Ameloblasts deposit the enamel matrix onto the outer layer of dentin. Characteristic noncrystalline mineral ribbons are deposited by a calcification front closely associated with the secretory surface of the ameloblast plasma membrane [6], and thereafter, enamel

Abbreviations: SEM-EDX, scanning electron microscopy with energy dispersive X-ray spectroscopy; DC, central layer of dentin; EC, central layer of enamel; PD, predentin; DP, dentin adjacent to predentin; DE, dentin adjacent to the junction with enamel; EA, enamel adjacent to ameloblasts; ED, enamel adjacent to the junction with dentin; ACP, amorphous calcium phosphate; HA, hydroxyapatite; MMPs, metalloproteinases; DEJ, dentin-enamel junction

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calcification proceeds. A study on the process of enamel calcification in rats revealed the concentrations of Ca and P in developing incisors by scanning electron microscopy with energy dispersive X-ray spectroscopy (SEM-EDX) [7]. Nonetheless, how the concentration of C is involved in the process of calcification of enamel is not known.

The present study was designed to investigate the concentrations of Ca, P, and C in dentin and C together with Ca and P in enamel during the developmental process of calcification in rat incisors using SEM-EDX.

2. Materials and methods

2.1. Tissue preparation

Eight 2-week-old male Wistar rats were used. They were obtained from the SLC Corporation (Kotoh, Shizuoka, Japan) and kept on a standard light-dark schedule and standard relative humidity. A stock diet and tap water were available *ad libitum*. All procedures were approved by the Animal Research Committee of Tohoku University.

The rats were anaesthetized with sodium pentobarbital (50 mg/kg) supplemented by isoflurane inhalation, and perfused through the aorta with 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M phosphate buffer. The mandibles were removed, divided in the middle, and kept in the same fixative overnight at 4 °C and then in 20% sucrose in 0.1 M phosphate buffer overnight at 4 °C. Each resected mandible was frozen with 4% carboxymethyl cellulose sodium salt (CMC; Leica Microsystems Japan, Tokyo, Japan) in hexane with dry ice. Serial 20-μm frozen sections were prepared with the cryo transfer kit without decalcification [8,9]. The slices were cut from a frozen specimen serially until the sagittal midplane of the mandibular incisor was exposed.

Some of the fixed mandibles were decalcified and embedded in paraffin. The sagittal sections of an incisor were prepared and stained with hematoxylin-eosin.

2.2. SEM-EDX

Each frozen specimen in which the midplane of the mandibular incisor was exposed was rinsed with 0.1 M phosphate buffer, dehydrated in a graded series of ethanol solutions and t-butyl alcohol, and freeze-dried. The midplane of the incisor was examined and analyzed by SEM-EDX (JSM-6390LA, EX-2300, JEOL, Tokyo, Japan). The examination and analysis were performed at 15-kV voltage, 10-mm working distance, and under low pressure (60 Pa). The tissues were visualized in backscattered mode. The distributions and concentrations of Ca, P, and C in the tissue were examined, and the ratio of the concentrations, Ca/P, was calculated [4,5]. The concentration was assessed as the ratio of the number of atoms of each element to that of C, N, O, Na, Mg, P, S, and Ca.

2.3. Analysis of element concentrations in dentin and enamel

The analysis of the concentrations of Ca, P, and C in dentin was performed for data from the onset of calcification, identified by SEM-EDX mapping analysis for Ca and P, through maturation. The analysis of enamel was performed on data from the formation of enamel rods through maturation. This is because enamel formation occurred after dentin calcification and, in the SEM-EDX mapping analysis, the initial calcified enamel was undistinguishable from calcified dentin. The analysis was performed for the matrix of the central layer of dentin (DC) and enamel (EC) along the developing mandibular incisor. The following five developmental points were selected for analysis of dentin and enamel (Fig. 1).

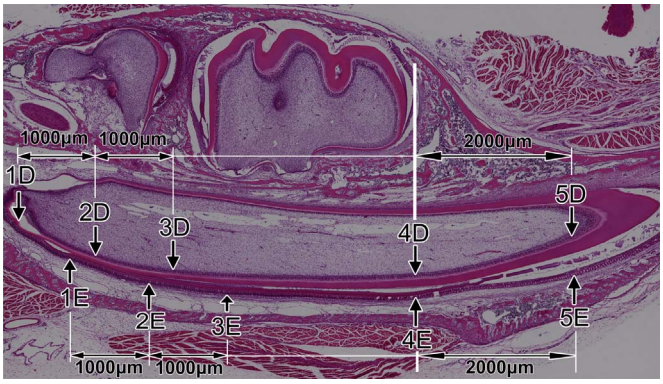

Fig. 1. A developing mandibular incisor of a 2-week-old rat, stained with hematoxylin-eosin. Five points were selected for analysis in developing dentin (1D, 2D, 3D, 4D, and 5D) and enamel (1E, 2E, 3E, 4E, and 5E).

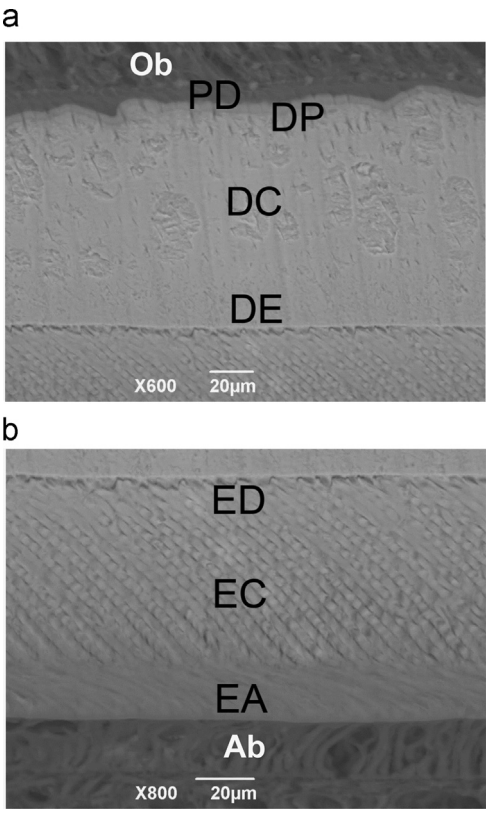

Fig. 2. Scanning electron micrographs of dentin at 4D (a) and enamel at 4E (b). Four points were selected for analysis in dentin (PD, DP, DC, and DE) and three points were selected in enamel (EA, EC, and ED). Ob: odontoblasts; Ab: ameloblasts.

1D: A point where dentin calcification takes place.
2D: A point 1000 μm distal to 1D along developing dentin.
3D: A point 2000 μm distal to 1D along the developing dentin.
4D: A point where the tangent to the mesial surface of the first molar meets the developing dentin of the incisor.
5D: A point 2000 μm distal to 4D along the developing dentin.
1E: A point where enamel rod formation occurs.
2E: A point 1000 μm distal to 1E along developing enamel.
3E: A point 2000 μm distal to 1E along the developing enamel.
4E: A point where the tangent to the mesial surface of the first molar meets the developing enamel of the incisor.
5E: A point 2000 μm distal to 4E along the developing enamel.

The dentin and enamel analyses were also conducted on data along the vertical line of the matrix between odontoblasts and ameloblasts at 4D and 4E at the following points (Fig. 2).

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