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Identification of a protein-containing enamel matrix layer which bridges with the dentine–enamel junction of adult human teeth

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ABSTRACT

Objective: To investigate the ultrastructure and chemical composition of the dentine–enamel junction and adjacent enamel of minimally processed third molar tooth sections.
Design: Undecalcified human third molar erupted teeth were sectioned and etched with 4% EDTA or 37% phosphoric acid prior to visualization by scanning electron microscopy. Confocal Raman spectroscopy was carried out at 50 μm and more than 400 μm away from the dentine–enamel junction before and after mild etching.
Results: A novel organic protein-containing enamel matrix layer was identified for the first time using scanning electron microscopy of etched bucco-lingual sections of crowns. This layer resembles a three-dimensional fibrous meshwork that is visually distinct from enamel "tufts". Previous studies have generally used harsher solvent conditions which likely removed this layer and precluded its prior characterization. The shape of the organic enamel layer generally reflected that of sheath regions of enamel rods and extended from the dentine–enamel junction about 100–400 μm into the cuspal enamel. This layer exhibited a Raman C–H stretching peak at ~2931 cm^{-1} characteristic of proteins and this signal correlated directly with the presence and location of the matrix layer as identified by scanning electron microscopy.
Conclusions: The enamel protein layer was most prominent close to the dentine–enamel junction and was largely absent in cuspal enamel >400 μm away from the dentine enamel junction. We hypothesize that this protein containing matrix layer could provide an important biomechanical linkage between the enamel and the dentine–enamel junction and by extension, with the dentine, of the adult tooth (246 words).

1. Introduction

Developing enamel extracellular matrix initially contains no mineral crystals but is enriched in amelogenin, the predominant protein component of secretory stage enamel, along with enamelin. MMP-20 is believed responsible for catalyzing required processing of amelogenins needed to initiate mineralization of the enamel.[1,2] As the enamel matures, amelogenin is largely degraded by kallikrein-4 yielding mature

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Abbreviations: MMP-20, matrix metalloproteinase-20 or enamelysin; DEJ, dentine–enamel junction; SEM, scanning electron microscopy; E, enamel; OM, organic matrix; D, dentine; IM, interphase matrix; PSh, prism-like sheaths; PS, prism-like scallops; Et, etched; NEt, non-etched; Et/B, etched and bleached; PBS, phosphate buffered saline; BSE, backscattered electron; HMDS, hexamethyldisilazane; CPD, critical point drying; ANOVA, analysis of variance; EDTA, ethylene diamine tetracetic acid.

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mineralized enamel containing low contents of enamelin, ameloblastin, and an amelogenin fragment.[3–5] As a result, the most mature enamel in the mouse is estimated to contain only 25–30% the maximal protein content of developing enamel.[6]

Mature enamel is a brittle material composed predominantly of carbonated calcium hydroxyapatite (96%) with 3% water and ~1% organic matrix.[7] In contrast, tooth dentine is a collagenous tissue which is traversed by dentinal tubules which contain cytoplasmic extensions of pulpal neurons and odontoblasts. Situated between the enamel and dentine layers, the dentine–enamel junction[8] plays an important role in inhibiting crack propagation from enamel into dentine. This function stems from its complex, collagenous structure and biomaterial properties that are different from either dentine or enamel. Molecular structural differences in both mineral and organic matrix across the DEJ zone have been investigated by a two-dimensional confocal Raman micro spectroscopic mapping/imaging technique,[9] by micro hardness[10,11] and toughness,[12] auto-fluorescence scanning,[13] by FTIR imaging,[14] and phosphate content by Raman spectroscopy.[11] These results bolster the view, based on human and bovine teeth,[9–14] that the thickness of the DEJ is functionally greater than the 2 μm visible from microscopy. However, there appears to be a difference in the estimated thickness ranging from ~100 μm to 4.7 μm with micro hardness. Second, a review of Raman quantitation of phosphate data published by Gallagher et al.[10] reveals a biphasic curve describing the variation in dentine, DEJ, and enamel. This data indicates that the amount of mineral gradually increases moving away from the DEJ up to an additional 10 μm.

Several groups have investigated how experimentally induced cracks propagate in the cuspal enamel and through the DEJ.[15–17] The fractography revealed that the deviation of the crack path involved an area of enamel adjacent to the DEJ which was approximately 50–150 μm wide and displayed altered material properties. Collagen bundles with diameters of 80–120 nm which are oriented parallel to the DEJ zone, intertwine with dentine collagen fibrils, and penetrate 2–10 μm into the enamel[18] also likely play a significant role in resisting crack propagation from the enamel into the dentine layer. This reflects the fact that in the intact tooth multiple full thickness cracks commonly found in enamel do not typically result in catastrophic failure via crack extension into the dentine.[13,14]

Gallagher et al.[13] used a 351-nm laser excitation source to perform auto fluorescence microscopy of dentine, enamel, and the DEJ to obtain information regarding their morphology and spectral characteristics. The emission spectra of these calcified dental tissues were different from one another, enabling the DEJ to be imaged and dimensions estimated. The DEJ displayed sharp and clearly delineated borders at both its enamel and dentine margins. The dentinal tubules and the enamel prisms appeared to terminate abruptly at the DEJ. The average median DEJ width was 10 microns, ranging from 7 to 15 μm, and it did not appear to depend on intra-tooth position. Depending upon which method is used, the thickness of the DEJ is estimated to be a graduated transitional region of 5 and 150 μm in width—distinctly broader than the 2 micron thick sharp morphological interface evident by optical microscopy. Thus, the DEJ can be appropriately defined as an interphase region of functionally graded properties intermediate between those of bulk phase enamel and dentine. These findings suggest that the functional properties of the DEJ extend beyond the interfacial boundary visible microscopically, however, it is unclear what the structural explanation is for these functional effects.

There is a long history of using scanning electron microscopy to investigate enamel structure. The first work[19] was carried out even before the introduction of commercial instruments and was performed on a prototype SEM microscope. Soon after the introduction of commercial instruments, numerous papers appeared describing the ultrastructure of etched enamel.[20,21] Etching of enamel is needed for both clinical reasons[22] and for revealing ultrastructural details of the enamel layer. Therefore, enamel etching is now a widely accepted technique in dental research, particularly in combination with SEM, and a number of papers containing SEM micrographs of etched enamel have been published.[23–26] Nevertheless, none of these previous studies observed an organic matrix in enamel from mature healthy human teeth despite the fact that Tichenor and Taher[27] and Taher et al.[28] attempted to infiltrate de-proteinized human enamel with resin for the purpose of observing resin casts of the etched matrix. Resin infiltration was apparently unsuccessful because the micrographs of etched embedded enamel did not differ from those of published images of non-embedded enamel.

Since the enamel region closest to the optically thin DEJ is less mineralized and softer than bulk phase enamel, we speculate this is due to a higher protein content in the latter. However, little proof exists to support this rationale, nor evidence as to which protein(s) may be enriched in this region. We present results of a combined SEM and Raman spectroscopic approach on etched and non-etched full thickness human enamel sections which provides direct evidence for an organic protein containing layer extending ~100–400 μm away from the DEJ into the cuspal enamel. The appearance of this layer in SEM is distinctly different than that of enamel "tufts".[29] Since its limits resemble the DEJ interphase region defined functionally, we hypothesize that this organic layer may be responsible in part for the increased toughness and inhibition of crack propagation through the dentine–enamel junction.

2. Materials and methods

2.1. *Source of human teeth*

Teeth are de-identified and were collected according to protocol #03-06e approved by the University of Missouri-Kansas City Adult Health Sciences Institutional review board.

2.2. *Specimen processing*

Ten non-carious human third molars from different individuals were used in the study. The extracted teeth were stored from 3 days to 2 months at 4 °C in 0.9% phosphate buffered saline with 0.002% sodium azide prior to processing. After removal of the roots, the crowns were sectioned buccolingually with a low speed diamond saw (Buehler Ltd., Lake Bluff, IL) to obtain 1.5 mm thick slices. Some of the slices were