Bone 63 (2014) 29–35

Contents lists available at ScienceDirect

Bone

journal homepage: www.elsevier.com/locate/bone

Original Full Length Article

Type VII collagen is enriched in the enamel organic matrix associated with the dentin–enamel junction of mature human teeth

Jacob D. McGuire [a,*], Mary P. Walker [a,b], Ahmad Mousa [a], Yong Wang [a,b], Jeff P. Gorski [a,b]

[a] Department of Oral and Craniofacial Sciences, School of Dentistry, University of Missouri — Kansas City, Kansas City, MO 64108, USA
[b] Center of Excellence in Musculoskeletal and Dental Tissues, University of Missouri — Kansas City, Kansas City, MO 64108, USA

ARTICLE INFO

Article history:
Received 13 November 2013
Revised 20 February 2014
Accepted 21 February 2014
Available online 1 March 2014

Edited by: David Fyhrie

Keywords:
Mature human enamel
Dentin–enamel junction
Type VII collagen
Immunofluorescent confocal microscopy

ABSTRACT

The inner enamel region of erupted teeth is known to exhibit higher fracture toughness and crack growth resistance than bulk phase enamel. However, an explanation for this behavior has been hampered by the lack of compositional information for the residual enamel organic matrix. Since enamel-forming ameloblasts are known to express type VII collagen and type VII collagen null mice display abnormal amelogenesis, the aim of this study was to determine whether type VII collagen is a component of the enamel organic matrix at the dentin–enamel junction (DEJ) of mature human teeth. Immunofluorescent confocal microscopy of demineralized tooth sections localized type VII collagen to the organic matrix surrounding individual enamel rods near the DEJ. Morphologically, immunoreactive type VII collagen helical-bundles resembled the gnarled-pattern of enamel rods detected by Coomassie Blue staining. Western blotting of whole crown or enamel matrix extracts also identified characteristic Mr = 280 and 230 kDa type VII dimeric forms, which resolved into 75 and 25 kDa bands upon reduction. As expected, the collagenous domain of type VII collagen was resistant to pepsin digestion, but was susceptible to purified bacterial collagenase. These results demonstrate the inner enamel organic matrix in mature teeth contains macromolecular type VII collagen. Based on its physical association with the DEJ and its well-appreciated capacity to complex with other collagens, we hypothesize that enamel embedded type VII collagen fibrils may contribute not only to the structural resilience of enamel, but may also play a role in bonding enamel to dentin.

Introduction

The wear surface of mature teeth, enamel, is the most highly mineralized tissue in the body and is believed to be non-collagenous [1] and largely protein-free [2]. In contrast, the underlying dentin, which is similar to bone in composition [3], is a calcified, collagen-rich ectomesenchymal tissue that serves to support the outer, more brittle enamel [4]. The interfacial region coupling these dissimilar mineralized phases is known as the dentin–enamel junction (DEJ), which optically appears as an abrupt transition. However, a recent biomaterial study indicates the DEJ represents a broad functional zone that can, in part, be attributed to unidentified protein constituents at the dentin–enamel complex [5,6]. Embryologically, the innermost enamel at the DEJ represents the position of the dental basement membrane [7,8]. Composed largely of type IV/type VII collagen and laminin [9], these basement membrane constituents are widely believed to be removed by the time enamel secretion commences [10].

Although the extracellular organic matrix of enamel is largely removed, a small amount of protein remains in the inner region of the post-eruptive tissue [5]; distinct histologic features known as enamel tufts also extend vertically from the DEJ [11]. These hypomineralized fissures are believed to be a primary source of the fractures that develop in enamel during extensive function or overloading [12,13]. The residual enamel organic matrix layer is believed to either represent organic 'matter' that fills cracks formed within enamel [13] or biological remnants that toughen the inner enamel region [6]. For example, alteration or removal of enamel's residual organic matrix decreases its fracture toughness and resistance [14,15]. By analogy, the material properties of mature bone are dependent upon the quality of its organic matrix [16]. Hampered by its insolubility [17], the molecular composition of the enamel organic matrix at the DEJ of mature teeth has remained a mystery for over fifty years [18].

Inactivating mutations in type VII collagen cause dystrophic forms of epidermolysis bullosa which manifest as skin fragility [19] and malformed enamel [20]. This phenotype is very similar to that exhibited by type VII collagen null mice [21]. Interestingly, type VII collagen is restricted to the basement membrane separating epithelial layers from their underlying stroma. In teeth, type VII collagen is localized to the epithelial mesenchymal junction during mouse enamel development [21] and the basement membrane of developing human tooth

* Corresponding author at: Department of Oral and Craniofacial Sciences, School of Dentistry, Univ. of Missouri — Kansas City, 650 East 25th St., Room 3154, Kansas City, MO 64108, USA. Fax: +1 816 235 5524.
E-mail address: mcguireja@umkc.edu (J.D. McGuire).

http://dx.doi.org/10.1016/j.bone.2014.02.012
8756-3282/

germs [22]. Additionally, type VII collagen is also known to be expressed by enamel-forming murine ameloblasts [21]. Based on type VII collagen's requirement for anchoring the epidermis to the dermis in skin [23] and for normal amelogenesis [21], we sought to determine if type VII collagen is a component of the enamel organic matrix associated with the dentin–enamel junction of mature erupted teeth.

Methods

Collection of mature teeth and preparation

Mature erupted third molars without visual defect/caries, treatment planned for extraction, were acquired from local oral surgery clinics in the Kansas City area, according to a protocol approved by the University of Missouri Kansas City adult health science institutional review board (IRB#: 12-50-NHSR). Collected tooth specimens were washed using deionized water; and surface debris was removed using a scalpel and a stiff brush and stored at 4 °C in 0.9% phosphate buffered saline (PBS) containing 0.002% sodium azide (NaN_3; Sigma-Aldrich, Saint Louis, MO) prior to processing. Following partial or complete root removal, the remaining crowns were processed in total or sectioned sagitally creating 500–1000 μm thick sections with a low-speed water cooled diamond saw (Isomet 1000, Buehler, Lake Bluff, IL).

Time-lapse imaging of enamel demineralization and the residual enamel matrix layer

From three individual teeth, 500 μm sections were mounted in polymethyl methacrylate (Great Lakes Orthodontics, Tonawanda, New York). The sections were then immersed with the crown positioned down in 200 ml of 10% ethylenediaminetetraacetic acid (EDTA; Fischer Scientific, Hampton, NH) (pH 7.4), containing 0.002% NaN_3 and imaged under a fluorescent lamp with a Canon T2i digital SLR camera, and an EF 100 mm f/2.8 macro lens every 10 min for 120 h, using a multi-function timer remote control.

Morphological analysis of enamel tufts and the enamel matrix layer

Similarly, 500 μm sections from six individual teeth, from different patients, were polished sequentially with 600 and 1200 grit silicon carbide paper concluding with 1-micron diamond polishing paste (Buehler). Sections were stained for 1 min in 0.2% (w/v) Coomassie Brilliant Blue R-250 (Sigma-Aldrich) in 50% methanol (Sigma-Aldrich) and 10% acetic acid (Sigma-Aldrich), destained and imaged for analysis of histologic enamel tufts. The same sections were then demineralized in 5% EDTA (pH 7.4) for 4 h followed by staining in Coomassie blue for 1 min, destained and re-imaged for the same analysis. A light microscope (Nikon Eclipse ME600, Melville, NY) equipped with a digital camera (Nikon DXM1200) was used to image sections.

Confocal Immunofluorescence Studies

Sections (~700 μm) from third molars from eight different patients were mounted on #1 chambered borosilicate coverglass (Lab-Tek, Rochester, NY) followed by complete demineralization in 10% EDTA (pH 7.4), containing 0.002% NaN_3 for 14 days.

Sections were then washed to remove residual EDTA with Buffer A [PBS (pH 7.4) containing 0.1% bovine serum albumin (BSA) and 0.05% Tween-20] and immediately blocked for 2 h in Buffer B [PBS (pH 7.4) containing 1% BSA and 0.05% Tween-20] and avidin D blocking solution (Vector Labs, Burlingame, CA). Sections were then incubated overnight at room temperature with primary antibody (1:200, catalog #234192, rabbit polyclonal anti-collagen type VII, Millipore, San Diego, CA) diluted in Buffer B and biotin blocking solution (Vector Labs). Controls were treated with non-specific rabbit IgG (1:200, Sigma-Aldrich) under the same conditions.

For visualization, specimens were washed three times with Buffer A then incubated for 1 h with biotin conjugated-protein A (1:10,000, Millipore) in Buffer B; washed three times with Buffer A and then incubated with streptavidin labeled with AlexaFluor594 (1ug/ml, Jackson Laboratories, West Grove, PA) in Buffer B.

Serial z-focal plane images were captured on a confocal laser scanning microscope (CLSM; Leica TCS SP5 II, Wetzlar, Germany) at 405 and 594 nm using the resonant scanner and image formats of 1024 × 1024 pixels. Image J software was used to create z-stack and single plane images from the acquired data. Specific labeling was determined by subtracting the background from the 594 and 405 channels using the same software.

Collagenase treatment

Ten demineralized sections were treated with purified bacterial collagenase (0.075 mg/ml, catalog #C0773-3KV, Sigma-Aldrich) diluted in 50 mM TES buffer (pH 7.4) containing 0.36 mM CaCl, for 6 h, and then immunolabeled and imaged as described above. Control sections were treated similarly with buffer alone.

Whole crown and enamel protein extraction

Extraction of proteins from whole tooth crowns from different patients was adapted from a protocol developed for extracting protein from bone [24]. Briefly, the roots were removed above the cemento-enamel junction and the pulp tissue was extirpated and mechanically debrided. Each crown was flash-frozen in liquid nitrogen, pulverized, and mixed with extraction buffer containing 4 M guanidine hydrochloride, 0.5 M EDTA and a mixture of protease and phosphatase inhibitors for 72 h at 4 °C. Samples were then centrifuged and the supernatant was dialyzed three times each against water and then against 5% acetic acid at 4 °C using 6–8 kDa molecular weight cut-off dialysis tubing (Spectrum Laboratories, Rancho Dominguez, CA). Extracts were lyophilized and stored at −80 °C. Protein amounts were determined colorimetrically using the NI™ (Non-Interfering™) Protein Assay kit (G-Biosciences, Saint Louis, MO).

Extraction of enamel matrix proteins from six tooth crowns was accomplished by demineralizing whole crowns, enamel side up, in 40 ml of 10% EDTA (7.4 pH) containing 0.002% NaN_3 for 14 days at 20 °C. The residual enamel matrix layer was gently debrided from the dentin surface with a soft applicator brush (displayed in Fig. 1e), followed by removal of the intact, underlying dentin. Extracts were then dialyzed, lyophilized, stored, and protein amounts determined as described above.

Pepsin digestion

Extracted enamel matrix protein fractions from two tooth crowns were incubated separately with pepsin using a well-established protocol [25]. Protein weight after lyophilization was determined directly and each enamel sample was rehydrated at 1 mg/ml in 0.5 M acetic acid (Sigma-Aldrich). The specimens were then incubated with pepsin (100 μg/ml, Millipore) at 4 °C for 48 h. Additional enamel matrix fractions from each specimen were incubated similarly without pepsin (negative controls). Digestion reactions were inactivated by heat, lyophilized and stored frozen prior to analysis.

SDS-PAGE and immunoblotting

Pepsin digested and control protein extracts from whole crowns and enamel matrixes were dissociated in SDS/8 M urea sample buffer, heated at 95 °C, and electrophoresed as previously described using 4–20% linear gradient and 7.5% gels [26]. Blots were incubated with either primary mouse anti-type VII collagen monoclonal antibody recognizing the non-helical carboxy terminal region of the collagen VII dimer (1:500,