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Proteomic analysis of human dental cementum and alveolar bone

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Abstract

Dental cementum (DC) is a bone-like tissue covering the tooth root and responsible for attaching the tooth to the alveolar bone (AB) via the periodontal ligament (PDL). Studies have unsuccessfully tried to identify factors specific to DC versus AB, in an effort to better understand DC development and regeneration. The present study aimed to use matched human DC and AB samples (n=7) to generate their proteomes for comparative analysis. Bone samples were harvested from tooth extraction sites, whereas DC samples were obtained from the apical root portion of extracted third molars. Samples were denatured, followed by protein extraction reduction, alkylation and digestion for analysis by nanoAcquity HPLC system and LTQ-FT Ultra. Data analysis demonstrated that a total of 318 proteins were identified in AB and DC. In addition to shared proteins between these tissues, 105 and 83 proteins exclusive to AB or DC were identified, respectively. This is the first report analyzing the proteomic composition of human DC matrix and identifying putative unique and enriched proteins in comparison to alveolar bone. These findings may provide novel insights into developmental differences between DC and AB, and identify candidate biomarkers that may lead to more efficient and predictable therapies for periodontal regeneration.

Keywords

alveolar bone; dental cementum; proteomic analysis; periodontal ligament; dentin; superoxide dismutase 3

1. Introduction

Dental cementum (DC) is a mineralized tissue covering the tooth root, critical for anchoring the tooth to the surrounding alveolar bone (AB) via the periodontal ligament (PDL) (Figure 1A) [1–3]. DC and AB share a common progenitor cell population in the ectomesenchymal

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Conflict of interest statement

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dental follicle, and DC is often described as bone-like, though questions remain whether cementoblasts are merely positional osteoblasts [4, 5]. Despite many similarities in morphology and matrix composition, these two mineralized tissues differ in several important respects. Unlike bone, DC is avascular, non-innervated, and grows by apposition with no significant role for turnover or remodeling [1, 2]. The process of cementogenesis remains poorly understood at present, though key developmental differences in DC versus AB have been identified through knock-out mouse approaches [2, 6–8]. Additionally, AB and DC may respond quite differently to therapeutic interventions in cases where periodontal tissues are lost as a consequence of disease; AB repair occurs more rapidly and predictably, while cementum regeneration is often difficult and unpredictable [9].

Although it is well established that there is overlap between extracellular matrices (ECM) of DC and AB, it has been hypothesized that each matrix contains unique proteins that may provide insight as to their physiologic differences. For both tissues, type I collagen is the primary ECM component, with the remaining organic matrix being composed of varying amounts of noncollagenous proteins (NCPs). These include proteoglycans (e.g., versican, decorin, and biglycan), glycoproteins that are often phosphorylated and sulfated (e.g., osteonectin and arginine-glycine-aspartic acid (RGD) integrin-binding proteins), and gamma-carboxyglutamic acid (gla)-containing proteins (e.g., matrix gla protein, protein S, and osteocalcin). Together, these proteins most likely participate in regulation of cell metabolism, matrix deposition and mineralization, and may contribute to determining the structure and biomechanical properties of the tissue [10, 11]. However, the relationship of NCPs to the collagenous framework, the significance of their patterns of distribution, and, particularly, the function of the individual proteins in the presence of various other matrix constituents remains to be determined, and therefore, it is critical to further understand matrix composition of these two mineralized tissues.

The goal of this study was to identify putative unique candidate markers in each tissue using a comparative proteomic analysis of human DC and AB. We hypothesized that physiologic differences and unique development regulation of DC versus AB would be reflected by selectively expressed and unique proteomic profiles for these two tissues. A more comprehensive understanding of the physiology and ECM composition of these two tissues is expected to contribute to more predictable and reliable regenerative approaches.

2. Materials and methods

2.1. Human subjects and sample collection

Dental cementum (DC) and alveolar bone (AB) were harvested from seven clinically healthy human subjects (5 females and 2 males) ranging from 20 to 30 years old. Additional inclusion criteria were a minimum of three semi-included or erupted functional third molars presenting a completely formed root, where extraction was clinically indicated. Human subjects studies were approved by the UNICAMP School of Dentistry IRB (008/2011), and all subjects provided a signed written consent, in compliance with the World Medical Association Declaration of Helsinki, Ethical Principles for Medical Research Involving Human Subjects. Following tooth extraction, soft connective tissues adhering to the tooth surfaces were carefully scraped off using a sterile curette, and discarded. Teeth were rinsed in sterile phosphate buffered saline (PBS) several times and DC samples were collected from the apical region of the root using a curette under stereomicroscope. AB fragments were collected from the tooth extraction sites when osteotomy was indicated. After several rinses in PBS to remove potential contaminants, DC and AB samples were ground using a chisel and stored in sterile PBS at -80°C .

2.2. Sample preparation

Thawed DC and AB samples were denatured by incubation in 100 μ L of 0.2% of *RapiGest*TM (Waters Corporation, Milford, MA, USA) and vortexed for 5 min, followed by agitation with 0.5 mm zircon/silica beads in a Mini BeadBeaterTM (Marconi, Piracicaba, SP, Brazil) for 1 min. Homogenized samples were then incubated at 99°C for 5 min, cooled to room temperature, centrifuged for 1 min at 13,000 rpm to pellet undigested solids, and supernatants were transferred to clean tubes for total protein concentration determination by Bradford's method, using bovine serum albumin (BSA) as a standard. Extracted proteins were reduced by incubation in 5 mM dithiothreitol at 60°C for 30 min, alkylated by incubation in 15 mM iodoacetamide at room temperature for 30 min, and finally digested with sequencing grade-modified trypsin (Promega, Madison, WI, USA) at 37°C for 3 h, followed by centrifugation at 14,000 rpm for 10 min. Supernatants were lyophilized and stored at -80°C prior to analysis.

2.3. Liquid chromatography-high resolution mass spectrometry (LC-MS/MS) analysis

Tryptic peptide mixture samples were reconstituted in a 2% acetonitrile/0.1% formic acid solution, and subsequently applied in cartridges of solid phase extraction by ion exchange (MCX, Waters – Milford, MA, USA) for exclusion of non-ionized small molecules and impurities, following the manufacturer's instructions. Eluents were dried and reconstituted in a 2% acetonitrile/0.1% formic acid solution to a final concentration of 0.4 μ g/ μ L. For nano-LC-MS/MS, 5 μ L of each resulting peptide mixture were analyzed in triplicate on a high resolution and high accuracy LTQ-FT Ultra (Thermo Scientific, San Jose, CA, USA) coupled to a nanoAcquity HPLC system (Waters, Milford, MA, USA). Peptides were separated by a 2–90% acetonitrile gradient in 0.1% formic acid using a capillary column prepared and packaged in-house. A laser-puller was used to obtain the tip of the column from a silica capillary of 75 μ m of internal diameter and 30 cm in length. The capillary column was packed using reverse phase Jupiter C-12 particles (Phenomenex, Torrance, CA, USA). The nanoelectrospray voltage was set to 2.2 kV and the source temperature was 275°C. For LTQ-FT analysis, the equipment was set up in the data-dependent acquisition mode. The full scan MS spectra (m/z 400–1,400) were acquired in the FT-ICR analyzer after accumulation to a target value of 1e6. Resolution in the FT was set to $r=50,000$ and the 6 most intense peptide ions with charge states ≥ 2 were sequentially isolated to a target value of 2,000 and fragmented in the linear ion trap by low-energy CID (normalized collision energy of 35%). The signal threshold for triggering an MS/MS event was set to 500 counts. Dynamic exclusion was enabled with an exclusion size list of 500, exclusion duration of 30 sec, and repeat count of 1. An activation $q=0.25$ and activation time of 30 msec were used.

2.4. Data analysis

Peak lists (msf) were generated from the raw data files using Proteome Discover version 1.3 (Thermo Fisher Scientific) with Sequest search engine and searched against Human International Protein Database, version 3.86 (91,522 sequences; 36,630,302 residues, release July 2011) with carbamidomethylation (+57.021 Da) as fixed modifications; oxidation of methionine (+ 15.995 Da); phosphorylation of serine, threonine, and tyrosine (+79.966 Da) as variable modifications; one trypsin missed cleavage and a tolerance of 10 ppm for precursor and 1 Da for fragment ions. For protein quantification, the data files were analyzed in Scaffold Q+ (version 3.3.1, Proteome Software, Inc., Portland, OR, USA) and the quantitative value (normalized spectral counts) was obtained with the protein thresholds established at a minimum 90.0% probability and at least 1 peptide with thresholds set up to minimum 60.0% probability and filtered using XCorr cutoffs (+1 > 1.8, +2 > 2.2, +3 > 2.5, and +4 > 3.5) to have less than 1% FDR. Only peptides with a minimum of five amino acid residues which showed significant threshold ($p<0.05$) in Sequest-based score were

considered as a product of peptide cleavage. The peptide was considered unique when it differed in at least 1 amino acid residue; covalently modified peptides, including N- or C-terminal elongation (i.e. missed cleavages) were counted as unique, and different charge states of the same peptide and modifications were not counted as unique. For label-free quantitation of endogenous peptides, the spectral count and the number of unique peptides were assessed. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (<http://proteomecentral.proteomexchange.org/>) via the PRIDE partner Repository [12] with the dataset identifier PXD000420. Resulting spectrum count values were used to analyze the distribution of identified proteins throughout the samples. Statistically different differential expression of proteins common to DC and AB was determined using the paired Student's *t*-test. Only proteins with quantitative values that showed significant threshold ($p < 0.05$) were considered in the results. The fold-change between proteins significantly expressed in DC and AB was also calculated. The spectrum count values of the full list of differentially expressed proteins are displayed in Table 5 in the Supplemental Material. Among proteins identified as exclusive to DC or AB, only proteins found in at least 50% of the subjects in the same tissue were considered in the results and are shown in Table 4.

2.5. Histology and immunohistochemistry

To collect tissues for immunohistochemistry or quantitative PCR (see below), mice at ages 15, 26, 30, and 60 days post natal (after root formation is complete and DC is present) with background of 129S1/SvImJ or C57BL/6 were euthanized by cervical dislocation, in accordance with the American Veterinary Medical Association Panel on Euthanasia, and approved by the Institutional Animal Care and Use Committee (IACUC), University of Washington (Seattle, Washington, USA). Hanford miniature pigs were employed to harvest first mandibular molar tissues at 13–16 weeks of age, as described previously [13], and approved by University of Washington IACUC. Deidentified human teeth used in this study were extracted for orthodontic reasons. Use of de-identified discarded human dental tissues for research activities was approved by the National Institutes of Health (Bethesda, MD, USA). Tissue processing and immunohistochemistry was performed as previously described [14, 15]. Briefly, mouse mandibles were removed and fixed in Bouin's solution for 24 h and decalcified AFS solution (acetic acid, formalin, and sodium chloride) at 4 °C. Pig molars were decalcified sequentially in 8% ethylenediaminetetraacetic acid (EDTA) and then 25% formic acid, as previously described [13]. Human teeth were fixed in 10% neutral buffered formalin and decalcified in 20% EDTA at 4 °C for approximately 5 weeks. Paraffin-embedded tissues were cut by microtome to five-micrometer buccal-lingual (coronal) or sagittal sections. Immunohistochemistry (IHC) reactions were performed using the following primary antibodies: polyclonal rabbit anti-biglycan (BGN) LF-159 (courtesy provided by Dr. Larry Fisher, NIH, Bethesda, MD, USA); polyclonal rabbit anti-decorin (DCN) LF-113 (provided by Dr. Larry Fisher); polyclonal rabbit anti-fibromodulin (FMOD) LF-150 (provided by Dr. Larry Fisher); polyclonal rabbit anti-periostin (POSTN) ab14041 (Abcam Inc., Cambridge, MA, USA); polyclonal rabbit anti-osteopontin (OPN) LF-175 (provided by Dr. Larry Fisher); rabbit anti-superoxide dismutase 3 (SOD3) (Abcam Inc.). Biotinylated secondary antibodies were incubated with an avidin–biotinylated peroxidase enzyme complex, then developed to a red product (indicating positive immunolocalization) using a 3-amino-9-ethylcarbazole (AEC) substrate kit (Vector Laboratories, Inc., Burlingame, CA, USA). Counterstaining was performed in Mayer's hematoxylin. Negative controls, lacking primary antibody, were performed.

2.6. Quantitative polymerase chain reaction (Q-PCR)

First molar teeth, femurs, calvaria, and brains ($n=3$ for all) were removed from 15 dpn mice and stored in RNA Later nuclease inhibitor (Ambion, Life Technologies, Grand Island, NY

USA). Total RNA was isolated by mortar and pestle grinding of tissues in liquid nitrogen, followed by extraction with Trizol reagent (Invitrogen, Life Technologies), according to manufacturer's instructions. Quantitative real time polymerase chain reaction (Q-PCR) was performed using two-step RT-PCR with Transcriptor First Strand Synthesis kit and LightCycler 480 SYBR Green I master kit on the Lightcycler 480 II real-time PCR system (Roche Applied Science, Indianapolis, IN, USA) for mouse superoxide dismutase 3 (*SOD3*) with primers 5-ggggaggcaactcagagg-3' and 5-tggctgaggttctctgcac-3' and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA expression using primers 5-accacagtccatgccatcac-3' and 5'-tccaccaccctgttctgta-3'. Statistical analysis for Q-PCR results was performed by one-way ANOVA using Prism 6.0 (GraphPad, La Jolla, CA, USA).

3. Results and discussion

3.1. Proteomic profiles of human alveolar bone and dental cementum

The proteomes of human dental cementum (DC) and alveolar bone (AB) were analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). Proteins were identified by Sequest algorithm against the IPI human protein database, and validated using Scaffold software by peptide hits of one or greater. Peptide mixtures generated from each human subject were analyzed in triplicate and the cumulative results were derived from the data combination of all seven subjects. The Venn diagram in Figure 1B displays the tissue distribution of 318 proteins identified in the present study. A total of 235 proteins were identified in AB and 213 proteins were identified in DC. Of the total proteins identified, 105 were exclusive to AB samples, whereas 83 were exclusive to DC. The complete list of proteins exclusively identified for each tissue is shown in Table 1, with further categorization by their predicted biological processes.

Predicted cellular localization and molecular function were assigned for identified proteins (Figures 1C and 1D, respectively) by the Database for Annotation, Visualization and Integrated Discovery program (DAVID, version v6.7), using the functional annotation tool for gene ontology (GO) terms enrichment analysis (<http://david.abcc.ncifcrf.gov/home.jsp>) [16, 17]. Enriched GO terms with a significant p-value ($p < 0.05$) generated by a modified Fisher Exact test followed by the Bonferroni test were considered in the results. This analysis demonstrated that, for the three dimensions used (cellular distribution, molecular function and biological processes) AB and DC shared similarities regarding their content, and areas of quantitative differences. For instance, AB had significant values for some GO terms such as intracellular organelle, cytoskeletal part, and cytoplasmic vesicle (cellular distribution); and calcium ion binding, enzyme inhibitor activity and growth factor binding (molecular function), whereas DC presented significant values for the GO term binding (molecular function) ($p < 0.05$) (Figure 1C and 1D).

In addition, analysis of proteomic profiles by predicted biological processes characterized according to the Human Protein Reference Database (HPRD; <http://www.hprd.org>), demonstrated some degree of similarity between AB and DC, with most proteins involved in cellular process, biological regulation, and metabolic process in both groups. In contrast, most proteins exclusive to AB were involved in cell growth and/or maintenance, while most proteins limited to DC were involved with cell communication and signal transduction (Table 1).

3.2. Proteins common to alveolar bone and dental cementum

Many of the proteins identified in AB and DC samples have been previously reported, serving to confirm and validate our approach for sample preparation and analysis. Type I collagen was by far the most predominant overlapping protein in the two tissues. Several

previously reported non-collagenous extracellular matrix (ECM) proteins, with localization to bone and dental cementum [1, 10, 11, 18–20], were detected in both tissues, including fibronectin (FN), tenascin (TNN), osteocalcin (BGLAP), vitronectin (VTN), vimentin (VIM), lumican (LUM), and chondroadherin (CHAD) (Supplemental Material). Interestingly, osteopontin (OPN), previously demonstrated to be present in both tissues, was found only at low levels in DC, but not in AB, whereas tissue nonspecific alkaline phosphatase (TNAP) was found only in AB (Table 1). Some molecules reported in both tissues, including ECM proteins osteonectin (ON) and bone sialoprotein (BSP), and growth factors such as epidermal growth factor (EGF) and insulin-like growth factor I (IGF1), were not detected by the methods used in the current study. Lack of detection of these proteins may point to limitations of the protein extraction protocol and possibly incomplete release of low-expressed proteins from the ECM of AB and DC. ECM proteins BSP and OPN strongly interact with the inorganic phase hydroxyapatite [21] of mineralized tissues such as bone and cementum, possibly affecting extraction efficiency. In the present study, denaturant bound-bead-based agitation was used to facilitate release of proteins from ground tissues, without employing sample demineralization. Previous studies analyzing the human dentin proteome have included tissue demineralization, and the resulting total number of proteins detected was found to be 233–269 proteins [22, 23], very similar to the number of proteins detected in the present study, with a total of 235 and 213 proteins for AB and DC samples, respectively.

DC samples were extracted from the apical third of the root, the thickest area of DC, in order to avoid contamination by dentin. In the present study, two selective dentin markers, dentin phosphoprotein (DPP) and dentin sialoprotein (DSP) [22, 23] were not detected indicating that cementum samples were not likely contaminated with dentin. However, periostin (POSTN) was found in AB and DC samples, and its presence might result from traces of PDL contamination to both samples [24]. Although attention was paid to remove soft tissue adhering to the root surface by carefully scraping tissues along the root surface, it is not possible to remove embedded Sharpey's fibers from the PDL, which insert into the interior of both tooth root and bone.

To the authors' knowledge, this is the first study to generate a proteome profile for human DC, therefore, there is no reference available for direct comparison of the current approach and the data set generated. To date, this also is the first comprehensive proteomic study to include patient-matched AB and DC samples. While there is not enough space for a detailed exhaustive analysis of the complete proteome inventories identified here, some differentially expressed and novel proteins were selected for further discussion below.

3.3. Proteins differentially localized to alveolar bone or dental cementum

A total of 130 proteins were found to overlap between AB and DC. Table 2 includes a list of the 33 proteins identified in both tissues, but present at significantly different relative quantitative values (paired Student's *t*-test, $p < 0.05$). Of the differentially expressed proteins, 18 and 15 were more abundant in AB and DC, respectively ($p < 0.05$). In AB, these included ECM proteins fibronectin (FN), versican (VCAN), asporin (ASPN) and collagen alpha-3(VI) chain (COL6A3). In DC, selectively increased proteins included biglycan (BGN), osteomodulin (OMD), insulin-like growth factor II (IGF2), POSTN, and pigment epithelium-derived factor (SERPINF1), an anti-angiogenic factor. Fold-change values for selected significantly differentially localized proteins are listed in Table 3, and these factors and their functions are described in more detail below.

FN is a multifunctional protein involved in cell-matrix interactions, including bridging to matrix components such as fibrillar collagen and heparin [25], and regulating processes of importance in hemostasis. Because of these important roles, FN has been evaluated for use

in periodontal regenerative therapies [26, 27] and bone repair processes [28, 29]. FN has been reported in bone, predentin, and dentin [22], and differentially distributed in the cementum matrix [30]. In the present investigation, FN was found at a 33-fold greater concentration in AB versus DC, perhaps suggesting a different or more critical role for FN in AB. As with FN, the glycoprotein VCAN was detected more abundantly in AB samples than in DC (5-fold increase). This protein is present at early stages of bone formation, and by association with hyaluronan, may demarcate space that will ultimately become mineralized [10]. It was reported that VCAN is subsequently degraded, releasing fragments that are homologous to EGF that may influence osteoblastic metabolism, and replaced by two small proteoglycans, DCN and BGN that are able to bind to other matrix constituents or growth factors [10].

Several proteoglycans were identified in the current study. DCN and BGN are members of the small leucine rich proteoglycan (SLRP) family that also includes ASPN, CHAD, and OMD [31], that are all ECM proteoglycans suspected to play a role in the mineralization process [10]. BGN, ASPN and OMD were differentially detected in AB and DC in the present study. ASPN was found in higher levels in AB than in DC with approximately 5-fold increase in AB. BGN and OMD were found with higher spectrum counts in DC with 2-fold and more than 11-fold increase compared to AB, respectively. BGN and DCN are closely related proteins in structure [32], and found in a variety of extracellular matrices, but also show divergent localization in tissues [33, 34] as they may compete for the same binding site on collagen [35]. These observations may explain a higher expression of one of these proteins to a specific tissue, as in DC compared to AB. Previous studies showed the presence of BGN in dentin [22, 23], mandibular bone cells [22, 36], and dental cementum [37]. The osteoporosis-like phenotype in BGN knock-out mice [36], and its interaction with growth factors including bone morphogenetic protein 4 (BMP-4) [38, 39], underscores the importance of this proteoglycan in the modulation of mineralized tissues. ASPN, a class II SLRP, also binds collagen and calcium and may play a role in controlling mineralization. Previous reports indicate that ASPN is present in dentin but not in mandibular bone [22], and are in contrast to our findings that identified ASPN in AB with higher levels than in DC. Current data support a role for ASPN in regulating chondrogenesis, and support the importance of ASPN in bone and joint diseases, including osteoarthritis [40–42], rheumatoid arthritis, and lumbar disc disease [43]. In the oral tissues, ASPN has been reported in the periodontal ligament (PDL), possibly playing a role as a negative regulator of periodontal ligament mineralization [44, 45].

Type VI collagen (COL6), a major structural component of microfibrils and involved in cell adhesion, was identified in AB with significant higher spectrum counts than in DC (1.25 fold-increases). COL6 was previously reported in gingival connective tissue, periodontal ligament, alveolar bone (pericellular localization) and cementum [24, 25]. Mutations in genes that code for the collagen VI subunits result in the autosomal dominant disorders as Bethlem myopathy and Ullrich congenital muscular dystrophy, both characterized by early childhood onset, muscle weakness and joint contractures [46, 47].

Although the abundances of SERPINH1 were not significantly differentially localized ($p=0.053$), this collagen chaperon-like protein [26] was found with low spectrum counts in AB, and only a few peptides were found in DC, resulting in a 12-fold increase of this protein in AB in relation to DC. Although reported in human dentin [21], there are no previous reports of the presence of this important chaperone protein of type I collagen in DC. This protein is localized to the endoplasmic reticulum and plays a role in the collagen biosynthesis pathway as a collagen-specific molecular chaperone important in osteogenesis. Alterations in SERPINH1, in addition to other genes involved in post-translational modification of type I collagen molecules, cause severe osteogenesis imperfecta, a

connective tissue disorder characterized by bone fragility, low bone mass, and fractures, which may be accompanied by bone deformity, dentinogenesis imperfecta, short stature, and shortened life span [26, 27].

Other proteins found to be more abundant in DC versus AB included IGF2 (3 -fold), POSTN (almost 2-fold), and SERPINF1 (approximately 2-fold). IGF2 is a member of the insulin family of polypeptide growth factors, involved in development and growth [48]. POSTN is a matricellular protein known to be present in the PDL, while BGLAP (also known as osteocalcin, OCN) is an ECM protein with a high affinity for hydroxyapatite by virtue of its gla-residues [10]. These two proteins are implicated in regulating cell adhesion and extracellular matrix mineralization [10]. Although the differential expression of BGLAP was not significant ($p=0.08$), this protein was more than 4-fold higher in DC versus AB. SERPINF1, implicated as an inhibitor of angiogenesis [49] is present in dentin [22] and bone [50], and now identified in DC. Defects in SERPINF1 cause osteogenesis imperfecta type 12 (OI12), which features fractures of long bones and vertebrae, and bone deformities observed as early as the first year of life [51].

In AB, 6 proteins appeared in at least 4 subjects or more (Table 4). Among abundant protein components involved in blood coagulation, FGB, FGA, and FGG, also isoform 1 of collagen alpha-1 (XIV) chain (COL14A1), were identified exclusively in bone in 5 of 7 subjects. COL14A1, also known as undulin, is a glycoprotein of the interstitial ECM associated with the surface of interstitial collagen fibrils and plays a role in the supramolecular organization of collagen by interconnecting mature fibrils to fibril bundles [52, 53]. In musculoskeletal tissues, COL14A1 has been localized to the extracellular matrix (i.e. on the collagen fibers) of the tendon and epimysium, co-distributed with fibronectin and tenascin-C [54], other family members of large ECM glycoproteins.

Immunohistochemistry (IHC) was used to confirm the presence and localization of selected proteins identified in DC or AB (Figure 2). DC enriched proteins assayed included OPN, BGN, POSTN, fibromodulin (FMOD), and SOD3 (also discussed in more detail below), while DCN was probed in AB. OPN was concentrated in DC matrix, as well as being present in AB, especially at reversal lines (Figure 2A, B). BGN was richly present in the PDL, and also localized to cementocytes embedded in DC (Figure 2C, D). POSTN localized heavily to PDL, and Sharpey's fibers entering into DC and AB were observed to be positive for POSTN (Figure 2E, F). FMOD was found in high concentrations in the PDL matrix, and also in cementocytes (Figure 2G, H). SOD3 was found in PDL and DC matrix, with cementocytes staining positive (Figure 2I, J). Thus, while BGN, FMOD, and SOD3 were not heavily stained by IHC in the DC matrix per se, their specific localization to cementocytes argues for local production of these proteins, validating their identification by proteomic analysis. DCN was found in PDL matrix, and also labeled Sharpey's fibers entering AB matrix (Figure 2K, L).

3.4. Superoxide dismutase 3 (SOD3) is a marker for dental cementum

SOD3, also called extracellular superoxide dismutase (ecSOD), was found to be exclusive to DC in this study, and was identified in DC samples from all subjects tested (Table 4). This protein is one of a group of three oxidoreductases known as SODs (SOD1 [CuZnSOD]; SOD2 [MnSOD]; SOD3 [ecSOD]) [55]. These superoxide dismutases are the major cellular defense against O_2^- and peroxynitrite produced as a consequence of aerobic respiration and substrate oxidation [55]. Each SOD is the product of a distinct gene and holds a specific subcellular localization, but catalyzes the same reaction of dismutation of O_2^- into oxygen and H_2O_2 [55]. High dose and/or inadequate removal of reactive oxygen species (ROS), especially superoxide anion, results in oxidative stress, which has been implicated in the pathogenesis of numerous diseases [56]. Studies using genetically altered mice and viral-

mediated gene transfer indicate that SOD3 plays an important role in various oxidative stress-dependent pathophysiologies, including hypertension, heart failure, ischemia-reperfusion injury, and lung injury [57–59]. SOD3 is secreted by cells and anchored to the extracellular matrix via binding to heparan sulfate proteoglycans (HSPGs), collagen, or fibulin-5 [58, 60–62]. SOD3 is found in plasma, lymph, and synovial fluids, as well as in selective tissues [55]. Among the mineralized tissues, SOD3 has been detected in human articular cartilage, and has been associated with the pathophysiology of osteoarthritis [63]. SOD3 has been reported to be present in tooth dentin, particularly along dentinal tubules, however the histological data presented by Park *et al.* clearly also shows SOD3 localized to DC, though this point was not discussed by the authors [22]. No reference could be found in the published literature for the presence of SOD3 in bone matrix.

SOD3 was identified exclusively in DC by proteomic analysis, and present in all DC samples analyzed, and thus was identified as a protein of interest requiring more detailed IHC analysis (Figure 3). In addition to localization in DC matrix and cementocytes in first molars of mice at 60 days post natal (dpm) (Figure 2I, J), SOD3 was localized to cellular DC in sagittally sectioned first and second mouse molars, and in the acellular form of DC on the continuously erupting incisor tooth in mice at 26 dpm (Figure 3A–C). In mice, light SOD3 staining could be identified in PDL, as well as AB. Mouse femurs were IHC stained for comparison, and while SOD3 was found in epiphysis and metaphysis around the growth plate, little SOD3 was observed in the trabecular or cortical bone distal to the growth plate (Figure 3D–F). Real-time Q-PCR revealed that SOD3 mRNA was found in significantly higher quantities in mouse molars compared to femur and calvarial bones, where low SOD3 expression was not statistically different from levels found in brain tissue (Figure 3G). In human teeth, SOD3 was strongly expressed in cementoblasts and adjacent PDL lining the cervical tooth root, and was identified around cementocytes of the apical DC (Figure 3H, I), as well as in odontoblasts and dentin tubules (Figure 3J), similar to the pattern described in a previous study [22]. This pattern was also confirmed in pig molars, where strongest expression of SOD3 was found in cementoblasts and cementocytes of the DC, but where the protein was also expressed in cells of the adjacent bone (Figure 3K). Considering SOD3 localization and proposed function as an endogenous antioxidant, these results suggest that SOD3 may play an important role in protecting cementum from the effects of oxidative stress during cementum formation and/or maintenance.

4. Conclusions

This study provides the first analysis of the human dental cementum proteome, and identifies proteins unique and selectively identified in dental cementum versus alveolar bone. Using the LC-MS/MS approach, we confirmed the presence of previously reported protein constituents in cementum and bone matrices, identified proteins common to both tissues, and for the first time report the presence of differentially localized novel proteins in the dental cementum (SERFINF1 and SOD3) and alveolar bone (COL14A1). Immunohistochemistry confirmed the presence SOD3 in dental cementum and related cells of mouse, pig, and human teeth. While these findings provide important insights into the extracellular matrix composition of dental cementum and alveolar bone matrices, ultimately identifying many similarities between the two, the identified differences may represent the basis for improved tissue biomarkers. Continuing studies may contribute to the development of more efficient and predictable therapies for periodontal regeneration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Biological significance

Periodontal disease is a highly prevalent disease affecting the world population, which involves breakdown of the tooth supporting tissues, the periodontal ligament, alveolar bone, and dental cementum. The lack of knowledge on specific factors that differentiate alveolar bone and dental cementum limits the development of more efficient and predictable reconstructive therapies. In order to better understand cementum development and potentially identify factors to improve therapeutic outcomes, we took the unique approach of using matched patient samples of dental cementum and alveolar bone to generate and compare a proteome list for each tissue. A potential biomarker for dental cementum was identified, superoxide dismutase 3 (SOD3), which is found in cementum and cementum-associated cells in mouse, pig, and human tissues. These findings may provide novel insights into developmental differences between alveolar bone and dental cementum, and represent the basis for improved and more predictable therapies.

Highlights

- We report the first comparative proteome analysis of alveolar bone and cementum.
- 318 proteins were identified by LC-MS/MS without previous sample demineralization.
- 33 proteins were significantly differentially expressed in both tissues.
- We confirmed SOD3 as a new potential biomarker for dental cementum.

Figure 1. Proteomic profiles of human dental cementum and alveolar bone

(A) Diagram of tooth indicating dental cementum (DC) and alveolar bone (AB), as well as periodontal ligament (PDL), dentin (DENT), and enamel (ENAM). (B) Venn diagram of proteins identified by LC-MS/MS exclusive to alveolar bone (105) versus dental cementum (83), and those found in both tissues (130). Predicted (C) cellular distributions and (D) molecular function of proteins identified in human alveolar bone and dental cementum, generated by DAVID software using GOTERM_CC_3 and GOTERM_MF_ALL databases. Gene Ontology (GO) terms are represented as different color wedges in the pie charts, with the number of proteins per group shown in parentheses.

Figure 2. Localization of dental cementum and alveolar bone enriched proteins in mouse tissues Immunohistochemistry was used to confirm presence of selected proteins enriched in cementum (A–J) or bone (K–L) based on proteomics analysis. (A, B) Osteopontin (OPN) is noted in the dental cementum (DC) matrix and alveolar bone (AB), especially at reversal lines. (C, D) Biglycan is richly present in the periodontal ligament (PDL), and also localized to cementocytes (Ccy; inset) of the DC. (E, F) Periostin (POSTN) localizes heavily to PDL, and Sharpey's fibers (SF; inset) entering into DC are observed to be positive for POSTN. (G, H) Fibromodulin (FMOD) is found in high concentrations in the PDL matrix, and also produced by cementocytes (inset). (I, J) Superoxide dismutase 3 (SOD3) is found in PDL and cementum matrix, with cementocytes staining positive (inset). (K, L) Decorin (DCN) is found in PDL matrix and Sharpey's fibers (SF; inset) entering alveolar bone. (M, N) Negative control slides display lack of staining in the absence of primary antibody. All images are from 60 dpn mouse mandibular molar teeth and surrounding tissues. Yellow dotted lines are used to mark the cementum-dentin interface.

Table 1
Proteins exclusive to human alveolar bone or dental cementum

Listed proteins were present exclusively in only AB or DC samples. Proteins are separated by biological process according to the Human Protein Reference Database (HPRD).

Biological Process	Alveolar Bone	Dental Cementum
Cell communication/Signal transduction	ANXA6; NUCB2; ARHGEF2; ITPR2; MPZ; ANK1; DGKA; ANXA3	PPP1R10; GNAZ; MFGE8; ALS2; TGFB1; RAB5B; TGFB2; DEPDC5; SORCS1; TMEM9B; SPARCL1; GPIBB; OR5M3; RAB1C; GAS6; ITGAV
Cell growth and/or maintenance	WDR5; HSPG2; MYH9; COL14A1; LAMA4; COL21A1; KRT72; MYL6; DNAH1; MAP4; LAMC1; MYH11; DMD; GYPC; FN1; TPM1; KLC3; SPTA1; NID1; FBLN2; SPTB; NID2	COL25A1; PCOLCE; SPP1; FMOD; C1QTNF5; EMILIN1; COL11A2; COL5A3; STRIP2; KIF13B; COL11A1; VIL1; DBF4
Cell adhesion	MAEA	
Cytoskeletal anchoring	XIRP2	
Metabolism; Energy pathway	ACOX3; ATP5B; GGT5; PRDX2; TM7SF2; SDHA; GSTM1; NKX1-1; ALPL	SOD3; GALNT12; MCCC2; GAPDH; FAXDC2; ENO1; HCG2026193
Protein metabolism	PPIA; FGB; FGG; CPA4; FGA; PSMB4; ITIH2; RPN1; ITIH1; A2M; RPLP0; CPA3; PSMC6; RPL18; APCS; RPN2; RPL30	THOC4; PROC; ADAMTS19; EEF1G; USP34; PRSS1; USP44; USP28; RPS10; SPP2
DNA replication		RFC2
Muscle contraction		TTN
Nervous system development		AHNAK
Regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	CHD7; HIST1H3A; GMEB2; CABIN1; PRDM1; ZC3H13; H2AFY; LIG1	HLX; GPATCH1; BAZ2A; ZNF512; ZFP64; ASXL2; SIX6OS1
Regulation of gene expression	MBNL1	
Transcription		TBX18
Apoptosis	CASP8; WWOX; WIP1	
Transport	SYT13; RYR1; CACNA1F; SLC2A1; RHD; APOH; SLC25A6; RLBP1; CNGB1; SCFD2	GRIK2; CTHRC1; TTR; ATP1B3; SLC41A3
Ion transport		CACNA1H
Cytoskeletal anchoring	XIRP2	
Immune response	C3; PTX3; DMBT1; HLA-C	FCGBP; MXRA8
Unknown	FLJ42569; 23 kDa protein; FTO; Conserved hypothetical protein; TMEM51; CCSMST1; NUP205; 64 kDa protein; 33 kDa protein; SEZ6L2; KIF21B; 26 kDa protein; C12orf63; CEP128; RHCE; RUFY3; 68 kDa protein; AUTS2; TMEM109; LOC100508795; NLRC5	49 kDa protein; ALS2CR11; ANKIB1; SLC25A42; RNF165; CLEC3B; ZNF362; PRR12; SRPX2; NAA25; MCPH1; AAT; FNBPI; ZG16B; CILP2; KIAA1731; RBFA; cDNA FLJ77419

Table 2
Overlapping but differentially expressed proteins in human alveolar bone and dental cementum

Listed proteins were identified in both AB and DC, but determined to be present at significantly different concentrations (by paired Student's *t*-test, $p < 0.05$). Proteins are separated by predicted biological process according to the Human Protein Reference Database (HPRD).

Biological Process	Alveolar Bone	Dental Cementum
Cell communication/Signal transduction	ANXA2; GNA13	AHSG; COL1A2; IGF2; OMD; POSTN; POSTN; POSTN; SERPINF1
Cell growth and/or maintenance	ACTA2; ACTG1; ASPN; COL6A3; FN1; GSN; LMNA; TUBA1C; TUBB; VCAN	BGN;
Protein metabolism		F9; F10
Regulation of nucleobase	HIST1H2BE; HIST1H4A; HIST2H2BE	
Transport	AE1; HBB	
Unknown	POTEE	CCDC68; FAM49B; IPI00922262; SRPX

Table 3
Fold-difference in significantly differentially expressed proteins of human alveolar bone versus dental cementum

Fold-change values and p-values (paired Student's *t*-test) are shown for selected proteins found to be significantly differentially expressed in AB versus DC. See Table 2 and Table 5 (in Supplement material) for full list of overlapping but differentially expressed proteins.

Accession Number	Description	Fold change AB/DC	p-Value
IPI00791534	Solute carrier family 4, anion exchanger member 1	49.91	0.033
IPI00022418	Isoform 1 of Fibronectin	33.71	0.006
IPI00020101	Histone H2B type 1-C/E/F/G/I	5.47	0.013
IPI00026314	Isoform 1 of Gelsolin	5.33	0.048
IPI00418431	ASPN protein	5.21	0.009
IPI00021405	Isoform A of Prelamin-A/C	5.19	0.009
IPI00009802	Isoform V0 of Versican core protein	5.07	0.003
IPI00418169	Isoform 2 of Annexin A2	4.98	0.2E-03
IPI00453473	Histone H4	4.46	0.024
IPI00022200	Isoform 1 of Collagen alpha-3(VI) chain	1.25	0.036
IPI00410241	Periostin isoform thy6	-1.67	0.047
IPI00218585	Isoform 2 of Periostin	-1.71	0.034
IPI00006114	Pigment epithelium-derived factor	-1.71	0.044
IPI00007960	Isoform 1 of Periostin	-1.83	0.012
IPI00304962	Collagen alpha-2(I) chain	-1.91	0.021
IPI00643384	cDNA FLJ36740 fis. highly similar to Biglycan	-2.00	0.008
IPI00922262	Highly similar to Alpha-2-HS-glycoprotein	-2.17	0.008
IPI00001611	Isoform 1 of Insulin-like growth factor II	-2.98	0.004
IPI00020990	Osteomodulin	-11.21	0.011
IPI00289795	Isoform 1 of Sushi repeat-containing protein SRPX	-12.98	0.004

Table 4
List of proteins identified exclusively in alveolar bone and dental cementum by Scaffold software according to quantitative values
Spectrum counts of proteins identified in at least 4 of 7 patients (PT) and their averages are shown. See Table 1 for full list of exclusive proteins.

Accession Number	Description	Spectral Counts						
		PT1	PT2	PT3	PT4	PT5	PT6	PT7 Average
Alveolar Bone								
IPI00298497	Fibrinogen beta chain	3.98	0.00	9.84	15.00	3.14	23.15	0.00 7.87
IPI00021891	Isoform Gamma-B of Fibrinogen gamma chain	5.00	1.44	9.09	11.98	0.00	18.62	0.00 6.59
IPI00922213	cDNA FLJ53292, highly similar to fibronectin, transcript variant 5	2.99	0.00	5.28	7.50	3.14	16.75	2.64 5.47
IPI00021885	Isoform 1 of Fibrinogen alpha chain	1.97	2.96	3.76	3.25	0.00	8.57	0.00 2.93
IPI00176193	Isoform 1 of Collagen alpha-1(XIV) chain	0.00	4.58	3.01	0.00	4.22	5.54	2.64 2.86
IPI00220194	Solute carrier family 2, facilitated glucose transporter member 1	0.99	4.40	2.27	1.15	0.00	0.99	0.00 1.40
Dental cementum								
IPI00027827	Extracellular superoxide dismutase [Cu-Zn]	2.48	4.50	13.29	3.11	2.58	5.58	5.42 5.28
IPI00967067	Osteopontin (Fragment)	1.24	0.00	3.59	0.00	2.57	0.00	7.61 2.14
IPI00009028	Tetranectin	0.00	0.00	1.21	3.11	1.04	0.00	7.37 1.82
IPI00002236	cDNA FLJ59612, highly similar to Lactadherin	1.24	5.95	0.00	0.00	2.05	0.00	2.07 1.62
IPI00021817	Vitamin K-dependent protein C	2.56	1.46	1.06	3.11	0.00	0.00	1.04 1.32
IPI00186946	Isoform 1 of Collagen alpha-1(XXV) chain	0.00	0.00	1.21	1.59	0.00	1.78	1.16 0.82