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Mineralization defects in cementum and craniofacial bone from loss of bone sialoprotein

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ABSTRACT

Bone sialoprotein (BSP) is a multifunctional extracellular matrix protein found in mineralized tissues, including bone, cartilage, tooth root cementum (both acellular and cellular types), and dentin. In order to define the role BSP plays in the process of biomineralization of these tissues, we analyzed cementogenesis, dentinogenesis, and osteogenesis (intramembranous and endochondral) in craniofacial bone in *Bsp* null mice and wild-type (WT) controls over a developmental period (1–60 days post natal; dpn) by histology, immunohistochemistry, undecalcified histochemistry, microcomputed tomography (microCT), scanning electron microscopy (SEM), transmission electron microscopy (TEM), and quantitative PCR (qPCR). Regions of intramembranous ossification in the alveolus, mandible, and calvaria presented delayed mineralization and osteoid accumulation, assessed by von Kossa and Goldner's trichrome stains at 1 and 14 dpn. Moreover, $Bsp^{-/-}$ mice featured increased cranial suture size at the early time point, 1 dpn. Immunostaining and PCR demonstrated that osteoblast markers, osterix, alkaline phosphatase, and osteopontin were unchanged in *Bsp* null mandibles compared to WT. $Bsp^{-/-}$ mouse molars featured a lack of functional acellular cementum formation by histology, SEM, and TEM, and subsequent loss of Sharpey's collagen fiber insertion into the tooth root structure. $Bsp^{-/-}$ mouse alveolar and mandibular bone featured equivalent or fewer osteoclasts at early ages (1 and 14 dpn), however, increased RANKL immunostaining and mRNA, and significantly increased number of osteoclast-like cells (2–5 fold) were found at later ages (26 and 60 dpn), corresponding to periodontal breakdown and severe alveolar bone resorption observed following molar teeth entering occlusion. Dentin formation was unperturbed in $Bsp^{-/-}$ mouse molars, with no delay in mineralization, no alteration in dentin dimensions, and no differences in odontoblast markers analyzed. No defects were identified in endochondral ossification in the cranial base, and craniofacial morphology was unaffected in $Bsp^{-/-}$ mice. These analyses confirm a critical role for BSP in processes of cementogenesis and intramembranous ossification of craniofacial bone, whereas endochondral ossification in the cranial base was minimally affected and dentinogenesis was normal in $Bsp^{-/-}$ molar teeth. Dissimilar effects of loss of BSP on mineralization of dental and craniofacial tissues suggest local differences in the role of BSP and/or yet to be defined interactions with site-specific factors.

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Introduction

Bone sialoprotein (BSP) is an anionic extracellular matrix (ECM) protein associated with mineralized tissues of the skeleton and dentition [15]. BSP is a member of the Small Integrin Binding Ligand N-linked Glycoprotein (SIBLING) family of multifunctional proteins, also including osteopontin (OPN), dentin matrix protein 1 (DMP1), dentin sialoprotein (DSP), dentin phosphoprotein (DPP), and matrix extracellular phosphoglycoprotein (MEPE) [21,69,77]. Like other SIBLING proteins, BSP contains several highly conserved functional domains. These include an N-terminal collagen-binding domain, an integrin-binding arginine-glycine-aspartic acid (RGD) motif (involved in cell attachment, migration, and cell signaling), and two polyglutamic acid repeats capable of in vitro hydroxyapatite (HA) nucleation in coordination with several phosphorylated serine residues [1,2,33,34,85]. These functional domains have been confirmed using in vitro approaches, however, the physiological role(s) of BSP in mineralized tissue formation in vivo remain elusive and difficult to define.

The functional importance for BSP in the axial skeleton has been the focus of most published in vivo studies to date. Mice null for the *Bsp* gene ($Bsp^{-/-}$) feature delayed long bone growth and mineralization, as well as low bone turnover as a consequence of reduced osteoclast formation and activity [11,58,59]. In a femur cortical bone defect model, $Bsp^{-/-}$ mice displayed delayed bone repair [60,63], and in a femur bone marrow ablation model, absence of BSP caused both reduced medullary trabecular bone formation and delayed osteoclastic resorption [83]. Investigations focusing on early stages of long bone development identified alterations in the growth plate, delayed initiation of mineralization, and a reduction in expression of some osteogenic markers in $Bsp^{-/-}$ vs. WT mice [12,40].

BSP has been identified in the ECM of tooth cementum and dentin as well as in cartilage and bone [15,16,19,26,28,43,57,74]. Previously we identified a developmental defect in cementum in teeth of $Bsp^{-/-}$ mice, resulting in the progressive loss of periodontal attachment, disorganization of the periodontal ligament (PDL), and alveolar bone loss at later ages [26]. However, the potential importance of BSP in dentin mineralization remains unclear. While the functional importance of BSP in endochondral bone formation and repair has been confirmed by analysis of long bones, examination of the craniofacial complex can provide insights into the importance of BSP in bone formed by endochondral ossification (e.g. portions of the neurocranium, including synchondroses of the cranial base that contribute to midfacial shape) versus intramembranous ossification (e.g. viscerocranium, including the mandible, alveolus, and frontal calvarial bone).

Our aim in these experiments was to define the functional importance of BSP in dental and craniofacial development, focusing on intramembranous versus endochondral processes of ossification, periodontal ligament (PDL) attachment to the alveolar bone and cementum, and dentin formation.

Materials and methods

Animals

Animal procedures were performed in accordance with guidelines of the Canadian Council on Animal Care and Animal Care and Veterinary Services, University of Western Ontario (London, ON, Canada) and National Institutes of Health (Bethesda, MD, USA). Preparation and genotyping of $Bsp^{-/-}$ and wild-type (WT) mice were described previously [26,58], and mice were maintained on a mixed 129/CD1 background. After weaning at three weeks of age, mice were provided both a standard pelleted mouse diet (2018 Tekland Global 18% protein diet, Harlan Laboratories, USA), as well as a soft gel diet (Diet Gel 31M, Clear H_2O, Portland, ME) to reduce incisor malocclusion in homozygous $Bsp^{-/-}$ mice. Homozygous WT and $Bsp^{-/-}$ littermates were analyzed, with three to six mice analyzed per genotype at ages 1, 5, 14, 26–30, and 60 days postnatal (dpn).

Histology

Procedures for histology and immunohistochemistry (IHC) were described previously for Bouin's fixed, decalcified, paraffin-embedded samples [23,24]. For undecalcified histology, tissues were processed and methylmethacrylate-embedded for automated microtome sectioning (6 μm) for Goldner's trichrome and von Kossa staining [46].

IHC employed biotinylated secondary antibodies and peroxidase substrate. Primary antibodies included polyclonal rabbit anti-BSP (1:200; Renny Franceschi, University of Michigan, Ann Arbor) [26], monoclonal rat IgG anti-tissue nonspecific alkaline phosphatase (1:200; R&D Systems, Minneapolis, MN) [88], polyclonal LF-175 rabbit anti-osteopontin (1:200; Larry Fisher, NIDCR) [25], polyclonal rabbit IgG anti-osterix/SP7 (1:100; Abcam, Cambridge, MA), polyclonal rabbit anti-osteocalcin (1:1000; Clontech Laboratories Inc., Mountain View, CA), and polyclonal goat IgG anti-RANKL (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) [26]. Tartrate resistant acid phosphatase (TRAP) staining was performed on decalcified and deparaffinized tissues according to manufacturer's instructions (Wako Chemical, Japan).

Transmission electron microscopy (TEM)

TEM was performed at two different facilities (14 dpn at NIH and 27–30 dpn at University of Toronto). For 14 dpn samples, mandibles were fixed in 2% paraformaldehyde, 0.5% glutaraldehyde in phosphate buffered saline (PBS) for 48 h, and washed in cacodylate buffer. Next, tissues were serially dehydrated in alcohol, embedded in LR white resin (Electron Microscopy Sciences, Hatfield, PA) and UV polymerized for 8 h. Thin sections (80 nm) were obtained from buccal and lingual aspects of the first maxillary and mandibular molars using a Leica Ultracut-UCT ultramicrotome (Leica Microsystems, Deerfield, IL), placed onto 300-mesh copper grids, and stained with saturated uranyl acetate in 50% methanol followed by lead citrate. Samples were viewed with a JEM-1200EXII electron microscope (JEOL, Tokyo, Japan) at 80 kV, and images were captured using a XR611M, mid-mounted, 10.5 megapixel CCD camera (Advanced Microscopy Techniques, Danvers, MA).

For 27–30 dpn samples, freshly dissected mandibles were fixed overnight at 4 °C in 0.8% formaldehyde and 0.2% glutaraldehyde in PBS. For both mineralized and demineralized tissues, sections were obtained from the lingual aspect of the first molar. For examination of natively mineralized tissues, samples from 27 dpn mice were washed with water, dehydrated in an ethanol gradient followed by propylene oxide, and embedded in Embed 812 resin (Electron Microscopy Sciences). Sections (~90 nm thick) were cut using a Leica Ultracut ultramicrotome, transferred to carbon-coated formvar Ni grids and examined unstained. For examination of demineralized tissues, fixed mandibles from 30 dpn mice were demineralized for 7 days in PBS containing 12.5% EDTA (pH 7.4), 0.2% paraformaldehyde, and 0.05% glutaraldehyde at 4 °C with rocking and daily solution change. The mandibles were then infiltrated with several changes of 2.3 M sucrose, frozen, and sectioned on a Leica EM UC6-NT ultracryomicrotome at −90 °C. Sections (~210 nm thick) were transferred to Ni grids supported by carbon-coated formvar and stained with 2% uranyl acetate in water for 5 min. and washed in water. For both mineralized and demineralized sections, grids were imaged on an FEI Technai 20 TEM operating at 200 kV with an AMT 16000-S CCD camera.

Microcomputed tomography (microCT)

For dentoalveolar imaging, dissected and formalin-fixed mandibles were scanned on a Scanco Medical microCT 50 (Scanco Medical AG, Brüttisellen, Switzerland) with parameters of 9 μm voxel size, 55 kVp, 145 mA, with 0.36 degree rotation step (180 degree angular range)