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Loss of bone sialoprotein leads to impaired endochondral bone development and mineralization

Erik Holm [a], Jane E. Aubin [b], Graeme K. Hunter [a,c], Frank Beier [d], Harvey A. Goldberg [a,c,*]

[a] Department of Biochemistry, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada N6A 5C1
[b] Department of Molecular Genetics, University of Toronto, Toronto, Ontario, Canada M5S 1A8
[c] School of Dentistry, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada N6A 5C1
[d] Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada, N6A 5C1

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ABSTRACT

Bone sialoprotein (BSP) is an anionic phosphoprotein in the extracellular matrix of mineralized tissues, and a promoter of biomineralization and osteoblast development. Previous studies on the *Bsp*-deficient mouse ($Bsp^{-/-}$) have demonstrated a significant bone and periodontal tissue phenotype in adulthood. However, the role of BSP during early long bone development is not known. To address this, early endochondral ossification in the $Bsp^{-/-}$ mouse was studied. Embryonic day 15.5 (E15.5) wild-type (WT) tibiae showed early stages of ossification that were absent in $Bsp^{-/-}$ mice. At E16.5, mineralization had commenced in the $Bsp^{-/-}$ mice, but staining for mineral was less intense and more dispersed compared with that in WT controls. Tibiae from $Bsp^{-/-}$ mice also demonstrated decreased mineralization and shortened length at postnatal day 0.5 (P0.5) compared to WT bones. There was no detectable difference in the number of tartrate-resistant acid phosphatase-positive foci at P0.5, although the P0.5 $Bsp^{-/-}$ tibiae had decreased *Vegfα* expression compared with WT tissue. Due to the shortened tibiae the growth plates were examined and determined to be of normal overall length. However, the length of the resting zone was increased in P0.5 $Bsp^{-/-}$ tibiae whereas that of the proliferative zone was decreased, with no change in the hypertrophic zone length of $Bsp^{-/-}$ mice. A reduction in cells positive for Ki-67, an S-phase cell-cycle marker, was noted in the proliferative zone. Decreased numbers of TUNEL-positive hypertrophic chondrocytes were also apparent in the $Bsp^{-/-}$ tibial growth plates, suggesting decreased apoptosis. Expression of the osteogenic markers *Alp1*, *Col1a1*, *Sp7*, *Runx2*, and *Bglap* was reduced in the endochondral bone of the neonatal $Bsp^{-/-}$ compared to WT tibiae. These results suggest that BSP is an important and multifaceted protein that regulates both chondrocyte proliferation and apoptosis as well as transition from cartilage to bone during development of endochondral bone.

1. Introduction

Mammalian long bones develop through a complex and intricately regulated process called endochondral ossification. The formation of bone initiates as a condensation of pluripotent mesenchymal stem cells to form the template of the bone [1]. These precursor cells differentiate into chondroblasts under the regulation of the transcription factors Sox5, 6, and 9 [2,3]. The chondroblasts deposit a matrix composed mainly of type II collagen and aggrecan before maturing into chondrocytes [4]. These chondrocytes are highly proliferative and develop the early growth plate [5]. The cells within the growth plate terminally differentiate into hypertrophic chondrocytes at the centre of the bone while secreting type X collagen. At this point the cartilaginous anlage calcifies around the late hypertrophic chondrocytes that secrete vascular endothelial growth factor in order to promote blood vessel invasion, and then proceed to undergo apoptosis [6]. This allows recruitment of osteoclasts that resorb the mineralized cartilaginous matrix, as well as secretion of osteoid followed by its mineralization by osteoblasts [7]. The chondrocytes at the ends of the bones continue to proliferate and hypertrophy to regulate longitudinal bone growth, while the osteoblasts terminally differentiate by either becoming trapped in mature bone and becoming osteocytes, or undergoing apoptosis.

Bone sialoprotein (BSP) is an anionic phosphoprotein believed to be one of the primary regulators of mineralization in bone and teeth. It is highly expressed by mineralizing cells such as hypertrophic chondrocytes and osteoblasts [8–10]. BSP is a member of the Small Integrin-Binding Ligand N-linked Glycoprotein (SIBLING) family of proteins. This family includes osteopontin (OPN) and dentin matrix phosphoprotein (DMP1), both of which are derived from a common ancestor that is shared with

Abbreviations: HEB, Hypertrophic zone of growth plate and endochondral bone; RP, Resting and proliferative chondrocyte zones of growth plate.

* Corresponding author at: School of Dentistry, Schulich School of Medicine and Dentistry, University of Western Ontario, London, Ontario, Canada, N6A 5C1.
E-mail addresses: eholm@uwo.ca (E. Holm), jane.aubin@utoronto.ca (J.E. Aubin), graeme.hunter@schulich.uwo.ca (G.K. Hunter), fbeier@uwo.ca (F. Beier), hagoldbe@uwo.ca (H.A. Goldberg).

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other calcium-binding proteins found in enamel, milk and saliva [11]. The members of the SIBLING family are located in a syntenic gene locus that has been named the "bone gene cluster" located on murine chromosome 5 [12]. BSP shares common features with other members of this group, which are intrinsically disordered proteins with little or no secondary or tertiary structure [13–15]. These proteins also contain integrin-binding RGD (Arginine-Glycine-Aspartate) sequences and highly acidic regions composed of (depending on the protein) poly-glutamate or poly-aspartate sequences. In BSP, the poly-glutamate sequences, which are highly conserved [16,17], are critical for hydroxyapatite (HA) nucleation activity based on *in vitro* studies [18]. The RGD sequence of BSP mediates cell attachment [19,20] but also has been demonstrated to promote osteoblastic cell differentiation and mineralization *in vitro* through cellular signaling pathways involving focal adhesion kinase and extracellular signal-regulated kinases [21]. As such, BSP is postulated to be an important mediator of bone mineralization.

BSP is highly expressed in developing bones during endochondral ossification [22] and is also deposited at the mineralization front of bone and in the cement lines [23]. Overexpression of BSP downstream of a CMV promoter (CMV-BSP) *in vivo* resulted in mice that are significantly smaller than their wild-type controls [24]. There are also defects in their endochondral bones, with aberrant growth plate formation and development. The growth plates of the CMV-BSP mice have no change in overall length, although there was an increase in hypertrophic zone size and number of hypertrophic chondrocytes. At 8 weeks of age, the mice also show a decrease in the proliferative zone length. This suggests that the overexpression of BSP promotes terminal differentiation in these chondrocytic cells. In addition, these CMV-BSP mice have increased numbers of osteoclasts in their trabecular bone, suggesting that BSP promotes osteoclast formation [24]. These differences suggest that BSP is also responsible for promoting terminal differentiation in other types of skeletal cells.

Mice deficient in BSP ($Bsp^{-/-}$) also have decreased long bone length and cortical bone thickness relative to wild-type (WT) mice at 4 months of age [25]. Conversely, they have a higher trabecular bone density than WT mice, but an apparent lower rate of bone turnover. This decrease in turnover could be due, in part, to a decrease in osteoclast numbers. However, Wade-Gueye et al. demonstrated that ovarectomized $Bsp^{-/-}$ mice are still susceptible to significant bone loss or osteoporosis, suggesting that BSP is not a critical requirement for osteoclast attachment and bone resorption *in vivo* [26]. $Bsp^{-/-}$ mice have decreased mineralization of cortical bone and a significant delay in cortical bone repair following injury [27]. This process of repair in many ways mirrors bone development and suggests a defect in endochondral bone development. Malaval et al. noted only a minor decrease in mineralization of bone from $Bsp^{-/-}$ neonatal mice, and did not report any other changes to skeletal tissues in these mice [25]. To date, there has been no characterization of BSP's role in early mineralization and bone development processes.

In this study we sought to determine the role of BSP in the development of endochondral bone. We demonstrate that lack of BSP results in delayed patterns of bone development, which can be attributed, in part, to the reduction in the proliferation and apoptosis of the chondrocytes within the growth plate. Additionally, we show that expression of osteoblastic markers is reduced in the $Bsp^{-/-}$ endochondral bone at postnatal day (P0.5). Together, these data demonstrate an important role of BSP in normal bone development and mineralization.

2. Materials and methods

2.1. Animal protocol

Animal procedures were performed in accordance with guidelines of the Canadian Council on Animal Care (CCAC) and Animal Care and Veterinary Services (ACVS), University of Western Ontario, under protocol number 2008–092. Preparation and genotyping of *Bsp* homozygous knock-out ($Bsp^{-/-}$) and WT mice were described previously [28]. Mice were maintained on a mixed 129/CD1 background and were fed a standard pelleted mouse diet (2018 Tekland Global 18% protein diet, Harlan Laboratories, USA) and tap water *ad libitum*. Animals used in this study were generated by breeding heterozygous parents to produce litter-matched offspring. For statistical analyses, the number of litter-matched animals used of each time point was as follows: E15.5 n = 6, E16.5 n = 13, P0.5 n = 10, P10.5 n = 5.

2.2. Histology and immunohistochemistry

$Bsp^{-/-}$ and WT tibiae were harvested and prepared for histology as previously described [29–31]. Briefly, animals were euthanized and tibiae dissected and fixed in 10% formalin in PBS at 4 °C overnight. Tissues were prepared for paraffin embedding using standard histological processing. Longitudinal sections of the tibiae were collected at 5 μm thickness by rotary microtome and mounted on positively charged glass slides. Slides were then deparaffinised in xylene and rehydrated for histological analyses.

Safranin O staining for the cartilage was performed as previously described [29]. Tartrate-resistant acid phosphatase (TRAP) stain was performed to identify osteoclast activity using a commercial kit (TRAP kit, product # 387A, Sigma, Oakville, Canada) as previously described [30]. TRAP-positive foci per scaled unit area (pixels) were determined. Von Kossa stain, to detect mineral, was performed using 1% silver nitrate under ultraviolet light for 20 min. Unbound stain was removed using 5% sodium thiosulfate for 5 min. The slides were then stained with 1% w/v alcian blue in 3% acetic acid for 20 min, and counter-stained with 0.1% nuclear fast red for 5 min. All chemicals used for staining were acquired from Sigma. Absolute quantification of von Kossa staining was performed by pixel counting of total positively stained area from each section including both trabecular and cortical bone at time points E15.5, E16.5, and P0.5. P10.5 quantification measured the stained trabecular bone or secondary ossification centre relative to total area. Statistical analyses were performed using independent samples *t*-test (GraphPad Prism software, v4.00).

Immunohistochemistry (IHC) and immunofluorescence (IF) were performed as previously described with some modifications [28]. Antigen retrieval was done by heating samples to 121 °C for 30 sec in 1 M sodium citrate (pH = 6) in a decloaking chamber (Biocare Medical, Concord, CA, USA). Samples were slowly cooled to 80 °C then removed. Slides were then blocked with 5% goat serum in PBS for 1 h. Samples were probed with primary antibody overnight at 4 °C at dilutions noted below, or following manufacturer's guidelines. After washing, secondary antibody was used according to manufacturer's protocol then developed using peroxidase substrate. Slides were dehydrated through graded ethanol and cleared in xylene prior to mounting.

Reagents for IF and IHC were: rabbit anti-mouse BSP (1:200, courtesy of Renny Franceschi, University of Michigan, Ann Arbor, MI, USA); rabbit anti-Ki-67 (1:200, ab15580, Abcam, Toronto, Canada); I*n Situ* Cell Death Detection Kit (11684795910, version 16, Roche, Laval, Canada); rabbit anti-mouse PECAM-1 (sc-1506-R, Santa Cruz, Dallas, TX, USA); and rabbit anti-p57 (sc-8298, Abcam). The secondary probe and development was performed using ABC staining system (sc-2018, Santa Cruz) according to manufacturer directions.

2.3. Histomorphometry

Proximal growth plates of $Bsp^{-/-}$ and WT tibiae were evaluated by two independent, blinded observers. Ki-67-positive cells were quantified in a 100× 100 μm box along the midline of the growth plate at a distance of 300 μm from the end of the hypertrophic zone. Ki-67-positive cells were quantified and normalized to total number of cells per area by ImageJ (v1.46r, NIH, USA). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) quantification was performed by enumerating total numbers of TUNEL-positive cells in the hypertrophic zone using ImageJ. Overall bone length was measured using images of tibiae taken