The role of bone sialoprotein in the tendon–bone insertion

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http://dx.doi.org/10.1016/j.matbio.2016.01.016
Edited by Marian Young

Abstract

Tendons/ligaments insert into bone *via* a transitional structure, the enthesis, which is susceptible to injury and difficult to repair. Fibrocartilaginous entheses contain fibrocartilage in their transitional zone, part of which is mineralized. Mineral-associated proteins within this zone have not been adequately characterized. Members of the Small Integrin Binding Ligand N-linked Glycoprotein (SIBLING) family are acidic phosphoproteins expressed in mineralized tissues. Here we show that two SIBLING proteins, bone sialoprotein (BSP) and osteopontin (OPN), are present in the mouse enthesis. Histological analyses indicate that the calcified zone of the quadriceps tendon enthesis is longer in $Bsp^{-/-}$ mice, however no difference is apparent in the supraspinatus tendon enthesis. In an analysis of mineral content within the calcified zone, micro-CT and Raman spectroscopy reveal that the mineral content in the calcified fibrocartilage of the quadriceps tendon enthesis are similar between wild type and $Bsp^{-/-}$ mice. Mechanical testing of the patellar tendon shows that while the tendons fail under similar loads, the $Bsp^{-/-}$ patellar tendon is 7.5% larger in cross sectional area than wild type tendons, resulting in a 16.5% reduction in failure stress. However, Picrosirius Red staining shows no difference in collagen organization. Data collected here indicate that BSP is present in the calcified fibrocartilage of murine entheses and suggest that BSP plays a regulatory role in this structure, influencing the growth of the calcified fibrocartilage in addition to the weakening of the tendon mechanical properties. Based on the phenotype of the $Bsp^{-/-}$ mouse enthesis, and the known *in vitro* functional properties of the protein, BSP may be a useful therapeutic molecule in the reattachment of tendons and ligaments to bone.

Introduction

Members of the Small Integrin Binding Ligand N-linked Glycoprotein (SIBLING) protein family are associated with the mineralized tissues of the skeleton and dentition [1]. SIBLINGs are anionic phosphoproteins with a flexible structure and extensive post-translational modifications. The family consists of 5 members: bone sialoprotein (BSP), osteopontin (OPN), dentin matrix protein 1 (DMP1),

dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE), with the encoding genes located in a syntenic gene locus on murine chromosome five [1].

BSP is a heavily glycosylated and phosphorylated protein that is expressed at the onset of mineralization in hard connective tissues [2]. In cell-free systems, BSP has been demonstrated to be a potent nucleator of hydroxyapatite (HA), the principal mineral component of bone [3]. Nucleation and binding to HA by BSP is conferred by polyglutamic acid sequences found in the central region of the protein [3,4]. BSP also contains an N-terminal collagen-binding domain and it is proposed that binding of BSP to collagen promotes HA nucleation [5,6]. Finally, BSP contains an integrin-binding RGD motif, located towards the C-terminus, which promotes osteoblast differentiation and matrix mineralization [7].

Mice in which the *Bsp* gene has been ablated (*Bsp*$^{-/-}$) have a variety of skeletal and dental defects [8]. At 4 months of age, these mice display reduced long-bone length and cortical thickness, and a lower rate of bone formation, but have higher trabecular bone density than wild type mice [9]. However, due to incisor malocclusion phenotype on hard diet, some of these defects are ameliorated if *Bsp*$^{-/-}$ mice are fed a soft food diet [10]. Further studies by the Malaval group showed that the *Bsp*$^{-/-}$ mouse displays a delay of bone repair in cortical injury models [11] and impaired bone formation and resorption in marrow ablation models [12].

More recently, a significant periodontal phenotype has been uncovered in the *Bsp*$^{-/-}$ mouse. Acellular cementum is a thin mineralized tissue found along the cervical portion of the root of the tooth, into which the periodontal ligament (PDL) inserts. Immunohistochemistry shows that acellular cementum is rich in BSP, and in the *Bsp*$^{-/-}$ mouse there is a striking reduction in cementum deposition [13]. Additionally, the *Bsp*$^{-/-}$ mouse PDL is disorganized, with poorly aligned collagen fibers that do not properly insert into the tooth root [10,13,14]. However, insertion of the PDL collagen into alveolar bone appears normal [14].

The defects observed in the PDL of the *Bsp*$^{-/-}$ mouse have spurred us to investigate other junctions between soft and mineralized tissue. Of particular interest is the enthesis, the transition site of tendon and ligament insertion into bone. Fibrocartilaginous entheses are found where tendons or ligaments attach to the epiphysis or apophysis of long bones [15]. As such, they are present at several key sites involved in locomotion, such as where the supraspinatus tendon (SST) of the rotator cuff meets the epiphysis of the humerus, and where the quadriceps tendon (QCT) inserts into the back of the patella.

Fibrocartilaginous entheses contain a fibrocartilage zone containing types II, IX and X collagen and fibrochondrocytes at the interface between tendon and bone [16]. Fibrocartilaginous entheses display 4 transitional zones: the dense connective tissue of the tendon, uncalcified fibrocartilage (UFC), calcified fibrocartilage (CFC), and bone. A sharp boundary occurs between the calcified and uncalcified fibrocartilage which is known as the tidemark. Entheses are largely avascular and as such, have a limited potential for regeneration [15,17]. Once torn away from bone, reattachment of a tendon or ligament is difficult. Indeed, the failure rates for repairs of massive rotator cuff tears remain high despite advances in surgical technique [18].

Given its HA nucleating and collagen binding properties, and the phenotype observed in the *Bsp*$^{-/-}$ mouse PDL, we hypothesize that BSP is present in fibrocartilaginous entheses and is involved in directing the mineralization and organization of these structures. In this study we show for the first time that the mineralized zones of murine fibrocartilaginous entheses contain the SIBLING proteins BSP and OPN, and that the loss of BSP results in a morphological abnormality of the QCT enthesis as well as a mechanical defect in the patellar tendon.

Results

BSP and OPN are present in the mineralized tissues of fibrocartilaginous entheses

Immunohistochemistry was performed to identify BSP in the murine fibrocartilaginous entheses. BSP was detected in the calcified fibrocartilage (CFC) of the QCT and SST entheses, as well as the adjacent bone (Fig. 1). Additionally the presence of OPN was identified in the enthesis, and its tissue distribution mimics that of BSP. The absence of BSP and OPN immunostaining in the mineralized tissues in *Bsp*$^{-/-}$ and *Opn*$^{-/-}$ mice, respectively was confirmed (Fig. 1).

Bsp$^{-/-}$ mice exhibit abnormalities in the calcified fibrocartilage of the enthesis

In order to study BSP's role in the murine fibrocartilaginous enthesis, histological characterization of two selected entheses was performed. The QCT enthesis was chosen due to its large size, as well as ease of access. It was thought that differences between wildtype and *Bsp*$^{-/-}$ animals would be more pronounced in a larger enthesis. The SST enthesis was chosen due to its medical relevance, as avulsion of the SST from the humeral head is a common rotator cuff injury.

Tidemark to bone lengths (corresponding to the CFC) in 15 week-old *Bsp*$^{-/-}$ entheses was measured (Fig. 2A, B, E, F, Table 1, Supplemental Fig. 1). At