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The glycosylation profile of osteoadherin alters during endochondral bone formation

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ABSTRACT

Endochondral bone formation involves the dynamic interplay between the cells and their extracellular environment to facilitate the deposition of a calcified matrix. Numerous molecules are involved within this process, including collagens and non-collagenous proteins, and their post-translational modifications have been shown to effect their biomolecular interactions. Osteoadherin (OSAD), a keratin sulfate (KS)-substituted small leucine-rich proteoglycan has been isolated from mineralized tissues and is considered to be a mineralized tissue-specific protein. However, to date, information is limited concerning the dynamic expression and role of this proteoglycan during bone formation and the biomineralization process. The current study aimed to examine the dynamic expression of this protein throughout mouse metatarsal long bone development, from the cartilage anlagen (E15) to the fully formed bone (Adult). Using quantitative gene expression analysis we observed that OSAD was produced with the onset of mineralization and the formation of the ossification center. This finding was reflected in the localization studies, using both light and electron microscopy, and showed that initial OSAD localization was restricted to the endosteal surfaces of the diaphysis and forming metaphysis. Furthermore, we analyzed protein extracts, both mineral and non-mineral associated fractions, and showed that OSAD was substituted with varying patterns of glycosylation during bone development. Sequential enzymatic digestions of the non-mineral bound protein extracts demonstrated that OSAD lacked any KS chains throughout all development stages. Whereas, in the mineral bound fractions, with long bone maturation the substitution with KS became more apparent with development. Therefore, it can be concluded that different pools of OSAD are produced during endochondral bone formation and these may have specific roles in directing the mineralization process.

Introduction

Endochondral bone formation has been a well-characterized process, and it is recognized that a significant number of molecules contribute to dynamic bone formation, including transcription factors, cell signaling molecules and extracellular matrix (ECM) proteins facilitating mineral deposition [1–5]. Collagen type I, is the main ECM component whose structure/function is organized by non-collagenous proteins, including bone sialoprotein (BSP), osteocalcin and the family of small leucine-rich proteoglycans (SLRPs). Non-collagenous proteins have been shown to have multiple functions in the regulation of mineralization, from cell attachment and migration to modulators of matrix synthesis and degradation, to regulators of HAP binding and crystal growth (as reviewed by [6]).

The expression pattern of SLRPs during endochondral bone formation is generally not well understood. The SLRP family is currently composed of five classes that contain at least 17 genes [7]. Initially proposed to function as structural components, SLRPs are now recognized as key molecules across a broad range of functions, including cell signaling, and influencing cell functions such as proliferation, differentiation, adhesion and migration [7–9]. The interactive nature of SLRPs is complicated; they can exert their influence via their protein core (40–60 kDa) or through their extensive patterns of post-translational modifications, which are known to significantly affect the proteoglycan's structural and functional relationships. Glycosoaminoglycan (GAG) chains, N-linked glycosylations and/or tyrosine sulfation have been identified associated with SLRPs in the ECM. The protein core possesses a characteristic leucine-rich motif consisting of six to ten repeats flanked by cysteine residues at the N- and C-terminals. Twelve different SLRPs have been associated with mineralized tissues but classes I and II have been extensively studied. Canonical class I members, decorin (DCN) and biglycan (BGN) have both been isolated from bone [10,11] and in mineralized tissues are substituted with chondroitin sulfate (CS) GAG chains. During endochondral bone development DCN was not detected in bone whereas BGN was located to developing growth plates [12]. DCN has a principle role in collagen type I fibril formation, binds to calcium and regulates hydroxyapatite (HAP) crystal growth [13–17], whereas BGN-collagen interactions remain a matter of controversy and debate (reviewed [18]). The generation of transgenic knockout

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mouse models has revealed key information regarding the roles of these SLRPs in bone, for example DCN does not exhibit a skeletal phenotype but large irregular collagen fibrils causing skin fragility reminiscent of Ehler–Danlos syndrome [19]. In comparison, the BGN knockout presents with a severe osteoporotic phenotype [20], and in turn the DCN/BGN double knockout displays an even more pronounced skeletal phenotype [21]. The class II SLRPs, fibromodulin (FMD), lumican (LUM) and osteoadherin (OSAD) are substituted with keratin sulfate (KS) I GAG chains, and have also been detected in bone [22–24]. During endochondral ossification FMD was localized to the developing ossification center and LUM detected in pre-osteogenic tissue [12]. The FMD and LUM knock-out transgenic models do not present with a predominant bone phenotype but with alterations to the collagen fibrils [25,26]. The FMD/LUM double knockout phenotype is more severe than the single knockouts [27], and the BGN/FMD knockout provides a valuable model for spontaneous osteoarthritis [28].

OSAD is of particular interest, since it is exclusively expressed by mineralizing tissues and was first identified and purified from bovine long bone [22,29]. In bone, OSAD was localized to the primary spongiosa of bovine fetal rib growth plate [22], and ultrastructural localization studies have additionally shown OSAD to have a similar distribution to BSP in rat long bone and calvaria [30]. OSAD mRNA has been detected within mature osteoblasts and a role has been proposed in the regulation of cell proliferation and migration [22,29,31]. To date however, the exact function of OSAD in mineralized tissues remains to be elucidated. It is proposed to have a high affinity for HAP via the extended acidic C-terminal and in cell attachment via the integrin $\alpha_v\beta_3$ [22,29].

Despite these investigations, information regarding the distribution and contribution of OSAD during development is limited. The current investigation aimed to clarify the localization of OSAD in the developing mouse metatarsal as a model of endochondral bone formation, with a view to provide further evidence for the role of OSAD during bone formation. Furthermore, as we know that the glycosylation substitution of proteoglycans can significantly affect their bimolecular interactions and in an attempt to elucidate the putative function of ODAD in mineralization, we examined the glycosylation pattern of OSAD during the developmental process, specifically examining the mineral-bound and non-mineral bound fractions of OSAD.

Materials and methods

Animals

Central metatarsals were dissected out from the NMR1 strain of mice (Scanbur BK AB, Stockholm, Sweden) at various developmental time stages, from embryonic (E) days 15, 18/19, new born (NB), 5 days (5d) and adult (Ad (>3 months)). We have previously used the metatarsal model to study the global gene transcriptome during endochondral bone formation and found that OSAD as significantly up-regulated at the onset of mineralization [5]. The experiments were performed in accordance with the current legislation in Sweden, and after approval from the Karolinska Institutet Ethical Research Board.

RNA isolation, reverse transcription and quantitative real-time PCR (q-PCR)

Total RNA was extracted from the long bones at the indicated time points using the RNeasy Kit (Qiagen, VWR International AB, Stockholm, Sweden) as described by the manufacturer. First-strand cDNA was reverse transcribed from 1 μg DNAse I-treated total RNA using 100 U SuperScript™ III reverse transcriptase (both from Invitrogen, Life Technologies Europe BV, Stockholm, Sweden) according to the standard protocols.

Quantitative real-time PCR (q-PCR) was performed using 40 ng cDNA, Syber® Green PCR Master Mix (Applied Biosystems, Life Technologies Europe BV, Stockholm, Sweden) and mouse primers against glyceraldehyde-3-phosphate dehydrogenase (GAPDH NM_008084) (forward CAA TGT GTC CGT CGT GGA TCT; reverse CGT GCC GCC TGG AGA AAC CTG CC), OSAD (NM_012050) (forward ATG GTG TAT TCG CTA AAC TTT CAA; reverse AGT CAC ATT CAC CAG CCC GTC CAT) [5], DCN (NM_007833) (forward GTT GGA AAG GCT TTA CCT GT; reverse AGA GAC TCT TCA GTC CCT GG), BGN (NM_007542) (forward GTC CAG GAA AGG GTT CAT AC; reverse CAA CTG ACC ATC ACC TCC TA) and FMD (NM_021355) (forward TCT GCT CAT CCT TTG GTC TA; reverse GAG GAA GCC AGG TCT AGT GT) (CyberGene AB, Stockholm Sweden). The reactions were run at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C 15 s, 53 °C 30 s and 60 °C for 1 min on an Applied Biosystems 7500 Fast Real-Time PCR system. The comparative cycle threshold method was used to analyze the results using the software DataAssist™ from Applied Biosystems. GAPDH was used to standardize the comparative Ct ($\Delta\Delta C_t$) values and E15 tissues to calibrate the values of the samples of interest.

Immunohistochemical staining — light microscopy

For histological analyses the tissues were fixed in 4% paraformaldehyde for 3–24 h at 4 °C. The samples were then decalcified in 12.5% (w/v) ethylenediamineteraacetic acid (EDTA) solution, pH 7.0 for 2–14 days, dehydrated and embedded in paraffin. Seven micrometer thin sections were mounted on Super Frost Plus slides (Menzel-Gläser, Braunschweig, Germany). The sections were deparaffinized in xylene and rehydrated through a graded series of alcohols and rinsed in Tris buffered saline (TBS 50 mM Tris, 150 mM sodium chloride, pH 7.6).

For immunohistochemical staining, the rehydrated sections were pre-treated with 0.2 M HCl for 15 min, rinsed in TBS. All sections were then treated with 3% H_2O_2 for 5 min at room temperature. Non-specific binding was blocked using 4% normal rabbit or goat serum (DAKO Glostrup, Denmark), followed by incubation with the primary antibody against mouse OSAD (1:300 R&D Systems Inc., Minneapolis, USA) or BSP (1:500 Millipore AB, Stockholm, Sweden), prepared in blocking solution. Detection of KS was performed using 5D4 (1:200 Seikagaku Corp., Japan) and the Mouse on Mouse Kit (Vector Laboratories, Burlingame, USA), according to the manufacturer's instructions. Control experiments were performed with a 1:300 dilution of the anti-OSAD antibody (0.2 μg/ml) in the presence of 10× excess of recombinant mouse OSAD (0.1 μg/μl R&D Systems Inc.) or with the omission of the primary antibody, and incubated overnight at 4 °C. The sections were rinsed three times in TBS for 5 min and subsequently probed with the secondary antibody; horseradish-peroxidase (HRP)-conjugated rabbit anti-goat IgG for OSAD, HRP-conjugated goat anti-rabbit for BSP and HRP-conjugated anti-streptavidin for 5D4 (1:300, DAKO) for 1 h at room temperature. Bound antibody was detected using the DAB substrate (DAKO) and sections were counterstained for 10 s with Mayer's hematoxylin, followed by dehydration and mounting in Pertex® (Histolab Products AB, Gothenberg, Sweden).

Immunohistochemical staining — electron microscopy

Mouse paws from the corresponding time points were fixed in 3% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4 overnight. The tissues were demineralized for 2–14 days in 12.5% EDTA prior to dehydration in methanol and embedding in Lowicryl K11M (Polysciences Inc., Warrington) at low temperature, as described by Hultenby et al. [32]. Ultrathin sections were cut and placed on formvar coated one-hole nickel grids. Immunolabeling was performed by first blocking with normal rabbit serum (DAKO) for 30 min, followed by incubation with the primary antibody (goat anti-mouse OSAD) at 1:30 in 0.1 M phosphate buffer containing 0.1% normal rabbit serum at 4 °C overnight. Grids were washed in phosphate buffer and the primary antibody detected with a rabbit anti-goat antibody conjugated with 10 nm gold particles (BB International England) for 2 h. The grids were washed