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Bone sialoprotein as a potential key factor implicated in the pathophysiology of osteoarthritis

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SUMMARY

Objective: We previously identified an association between bone sialoprotein (BSP) and osteoarthritic (OA) chondrocyte hypertrophy but the precise role of BSP in ostearthritis (OA) has not been extensively studied. This study aimed to confirm the association between BSP and OA chondrocyte hypertrophy, to define its effect on molecules produced by chondrocytes and to analyse its association with cartilage degradation and vascular density at the osteochondral junction.

Method: Human OA chondrocytes were cultivated in order to increase hypertrophic differentiation. The effect of parathyroid hormone-related peptide (PTHrP), interleukin (IL)-1β or tumour necrosis factor (TNF)-α on BSP was analysed by real-time reverse transcription polymerase chain reaction (RT-PCR) and western blot. The effects of BSP on OA chondrocytes production of inflammatory response mediators (IL-6, nitric oxide), major matrix molecule (aggrecan), matrix metalloprotease-3 and angiogenic factors (vascular endothelial growth factor, basic fibroblast growth factor, IL-8, and thrombospondin-1) were investigated. BSP was detected by immunohistochemistry and was associated with cartilage lesions severity and vascular density.

Results: PTHrP significantly decreased BSP, confirming its association with chondrocyte hypertrophy. In presence of IL-1β, BSP stimulated IL-8 synthesis, a pro-angiogenic cytokine but decreased the production of TSP-1, an angiogenesis inhibitor. The presence of BSP-immunoreactive chondrocytes in cartilage was associated with the severity of histological cartilage lesions and with vascular density at the osteochondral junction.

Conclusion: This study supports the implication of BSP in the pathology of OA and suggests that it could be a key mediator of the hypertrophic chondrocytes-induced angiogenesis. To control chondrocyte hypertrophic differentiation is promising in the treatment of OA.

Introduction

Osteoarthritis (OA) is a pathology affecting every joint tissue, especially the cartilage that undergoes many structural and biochemical modifications leading to its final destruction. Many pathways are implicated in damages of cartilage, including the hypertrophic differentiation of chondrocytes. Indeed, in early and late stage OA, some differentiated chondrocytes in permanent articular cartilage are activated and acquire hypertrophy-like changes[1]. This phenotypic changes are associated with the calcification and neovascularisation of the extracellular matrix leading to an increase of cartilage stiffness (for review, see[2]). Consequently, chondrocyte hypertrophic differentiation has been hypothesized to be a key event in OA progression (for review, see[3]).

Pathological chondrocyte hypertrophy is considered as a reiteration of the endochondral ossification process normally absent in normal adult cartilage[4]. Hypertrophic differentiation of chondrocytes participates to cartilage degradation in OA because

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hypertrophic chondrocytes secrete large amounts of matrix metalloproteases (MMP) responsible for cartilage degradation in OA[5]. As observed in the growth plate of growing individuals, the change in cell phenotype is accompanied by invasion of articular cartilage by blood vessels coming from the subchondral bone. Previous studies have shown that in OA, channels containing vascular structures may break the tidemark and invade the articular cartilage[6–8]. Vascular structures are usually accompanied by neural invasion[9]. Moreover, vascular invasion, a process also called angiogenesis, is associated with calcification of the extracellular matrix.

The hypertrophic phenotype is characterized by a high expression of specific markers such as type X collagen[10–12], runt-related factor 2 (runx2)[13,14] or MMP-13[15–17]. A network of positive and negative regulatory factors controls chondrocyte hypertrophy, this includes transcription factors, growth factors, hormones, extracellular matrix molecules and proteases (for review, see[18]).

We previously showed that bone sialoprotein (BSP) was highly expressed by hypertrophic chondrocytes in comparison with non-hypertrophic chondrocytes and was correlated to specific markers of chondrocyte hypertrophy[19]. BSP is a major non-collagenous extracellular matrix protein that belongs to the small integrin-binding ligand N-linked glycoproteins (SIBLING) gene family[20]. In physiological conditions, BSP is expressed by mature osteoblasts, osteoclasts and hypertrophic chondrocytes of the growth plate[21–23]. In these tissues, BSP is involved in mineralization since its interaction with collagen promotes hydroxyapatite nucleation[24,25]. As a result, BSP is firstly known as a marker of bone formation and has been considered as a marker for subchondral bone for a long time[26]. In addition to its implication in tissue mineralization, BSP also promotes cell attachment and signalling through its Arg-Gly-Asp (RGD) sequence and alternate attachment mechanisms[27,28] and has been further described as an angiogenesis enhancer. Indeed, Bellahcène *et al.* showed that BSP mediates human endothelial cell attachment and migration through the interaction of its RGD domain with endothelial cell $\alpha_v\beta_3$ integrin receptors[29].

Hence, BSP constitutes a multifunctional protein with a large clinical interest. We previously identified a preliminary association between BSP and OA chondrocyte hypertrophy[19] but the precise role and regulation of BSP in OA remained to be extensively studied. Herein, we investigated the effects of parathyroid hormone-related peptide (PTHrP), interleukin (IL)-1β and tumour necrosis factor (TNF)-α on BSP production by OA hypertrophic chondrocytes. Further, we have investigated the effect of increased dose of BSP on OA chondrocytes production of inflammatory response mediators [IL-6, nitric oxide (NO)], a major matrix molecule [aggrecan (AGG)], MMP-3 and angiogenic factors [vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), IL-8, thrombospondin (TSP)-1]. Finally, an immunohistological study performed on a set of non-OA and OA cartilage sections demonstrated the association between BSP localization, cartilage lesions severity and vascularisation.

Material and methods

Chondrocytes culture in alginate beads

Culture settings

Human chondrocytes were isolated from cartilage of OA patients undergoing total knee replacement (TKR) surgery with the approval of the Ethic Committee of Medicine department of the University of Liege (number B70720108313, reference 2010/43). Alginate beads were prepared as previously described[30] and OA chondrocytes were embedded at a density of 4.2×10^6 cells per millilitre alginate solution. OA chondrocytes in alginate beads were cultivated for 3, 4, 8, 12, 21 or 28 days in Dulbecco's modified Eagle's medium (DMEM) (Lonza, Verviers, Belgium) either supplemented with 10 mM *4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid* (HEPES), 20 μg/ml proline (Sigma–Aldrich, Bornem, Belgium), 50 μg/ml ascorbic acid (Sigma–Aldrich, Bornem, Belgium), 2 nM glutamine (Lonza, Verviers, Belgium) and 10% (v/v) foetal bovine serum (FBS) (Lonza, Verviers, Belgium) in order to induce hypertrophic differentiation of OA chondrocyte, as previously validated[19] or with 1% of a preparation of insulin, transferrin, selenous acid, bovine serum albumin (BSA), and linoleic acid (ITS+) (ICN Biomedicals, Asse-Relegem, Belgium) in order to test the effect of recombinant BSP on OA chondrocyte. Media were changed twice a week. Some chondrocytes in alginate beads were cultivated with either 20 nM PTHrP, an inhibitor of hypertrophic differentiation (Abcam, Cambridge, United Kingdom) or 170 pg/ml IL-1β (10^{-11} M) or 25 ng/ml TNFα (Roche Diagnostic, Vilvoorde, Belgium), two key cytokines activating catabolic pathways of chondrocyte metabolism. These concentrations correspond to the maximal IL-1β concentration found in the synovial fluid of patients[31,32]. These compounds were added during the complete culture period of 28 days or only for the last 7 days of the culture (+7D), when OA chondrocytes expressed a highly hypertrophic phenotype.

To investigate the effects of BSP on chondrocytes metabolism and on the production of angiogenic factors, chondrocytes in alginate beads were cultivated 12 days in medium supplemented with 1% ITS+ and increased concentrations of recombinant BSP (R&D Systems, Abingdon, United Kingdom) that is 25, 100 and 400 ng/ml. Each chondrocyte culture was realized from a pool of three to six different OA patients that displayed a grade III or IV based on the classification tree for Collins grading of OA changes at the articular surface[33]. Each culture condition was done in triplicate. Cultures were repeated three times with different pools of chondrocytes coming from different donors.

Gene expression and assays

Real-time reverse transcription polymerase chain reaction (RT-PCR) of collagen type X and BSP

RNA from approximately 2×10^6 cells was isolated using the RNeasy minikit (Qiagen, Hilden, Germany), and PCR was performed using the LightCycler SYBR Premix Ex Taq system (Takara, Brussels, Belgium) as previously described[30]. The PCR template source was either 3 ng first-strand complementary DNA (cDNA) or purified DNA standard for calibration curve. The house-keeping gene hypoxanthine phosphoribosyltransferase (HPRT) was amplified and used as an internal control to standardize messenger RNA levels. Forward and reverse primer sequences used to amplify the desired cDNA are respectively the following: *HPRT*: TGTAATGACCAGTCAACAGGG and TGCCTGACCAAGGAAAGC; collagen type X (*col10a1*): GGGAGTGCCATCATCG and AGGGTGGGGTAGAGTT; *BSP*: GTGTCACTGGAGCCAA and ACCATCATAGCCATCGT.

Western blot analysis of BSP

Protein expression of BSP by chondrocytes was analysed by western blotting with a mouse monoclonal anti-BSP antibody (1:300) (LFMb-24, sc-73634; Santa Cruz Biotechnology Inc., Germany). Protein concentrations were measured using the Micro BCA Protein assay Kit (Thermo Scientific, Rockford, USA) according to the manufacturer's instructions. Equal amounts of protein were separated in a 7.5% sodium dodecyl sulfate (SDS)-polyacrylamide gel and were electrically transferred to polyvinylidene fluoride (PVDF) membrane. After incubation with goat anti-mouse