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Elevated hepatocyte growth factor levels in osteoarthritis osteoblasts contribute to their altered response to bone morphogenetic protein-2 and reduced mineralization capacity

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ABSTRACT

Purpose: Clinical and *in vitro* studies suggest that subchondral bone sclerosis due to abnormal osteoblasts is involved in the progression of osteoarthritis (OA). Human osteoblasts isolated from sclerotic subchondral OA bone tissue show an altered phenotype, a decreased canonical Wnt/ß-catenin pathway, and a reduced mineralization *in vitro* as well as *in vivo*. These alterations were linked with an abnormal response to BMP-2. OA osteoblasts release factors such as the hepatocyte growth factor (HGF) that contribute to cartilage loss whereas chondrocytes do not express HGF. HGF can stimulate BMP-2 expression in human osteoblasts, however, the role of HGF and its effect in OA osteoblasts remains unknown. Here we investigated whether elevated endogenous HGF levels in OA osteoblasts are responsible for their altered response to BMP-2.
Methods: We prepared primary human subchondral osteoblasts using the sclerotic medial portion of the tibial plateaus of OA patients undergoing total knee arthroplasty, or from tibial plateaus of normal individuals obtained at autopsy. The expression of HGF was evaluated by qRT-PCR and the protein production by western blot analysis. HGF expression was reduced with siRNA technique whereas its activity was inhibited using the selective inhibitor PHA665752. Alkaline phosphatase activity (ALPase) and osteocalcin release were measured by substrate hydrolysis and EIA respectively. Canonical Wnt/β-catenin signaling (cWnt) was evaluated both by target gene expression using the TOPflash TCF/lef luciferase reporter assay and western blot analysis of β-catenin levels in response to Wnt3a stimulation. Mineralization in response to BMP-2 was evaluated by alizarin red staining.
Results: The expression of HGF was increased in OA osteoblasts compared to normal osteoblasts and was maintained during their *in vitro* differentiation. OA osteoblasts released more HGF than normal osteoblasts as assessed by western blot analysis. HGF stimulated the expression of TGF-β1. BMP-2 dose-dependently (1 to 100 ng/ml) stimulated both ALPase and osteocalcin in normal osteoblasts whereas, it inhibited them in OA osteoblasts. HGF–siRNA treatments reversed this response in OA osteoblasts and restored the BMP-2 response. cWnt is reduced in OA osteoblasts compared to normal, and HGF–siRNA treatments increased cWnt in OA osteoblasts almost to normal. Smad1/5/8 phosphorylation in response to BMP-2, which is reduced in OA osteoblasts, was corrected when these cells were treated with PHA665752. The BMP-2-dependent mineralization of OA osteoblasts, which is also reduced compared to normal, was only partially restored by PHA665752 treatment whereas 28 days treatment with HGF reduced the mineralization of normal osteoblasts.
Conclusion: OA osteoblasts expressed more HGF than normal osteoblasts. Increased endogenous HGF production in OA osteoblasts stimulated the expression of TGF-β1 and reduced their response to BMP-2. Inhibiting HGF expression or HGF signaling restored the response to BMP-2 and Smad1/5/8 signaling. In addition, decreased HGF signaling partly corrects the abnormal mineralization of OA osteoblasts while increased HGF prevents the normal mineralization of normal osteoblasts. In summary, we hypothesize that sustained elevated HGF levels in OA osteoblasts drive their abnormal phenotype and is implicated in OA pathophysiology.

Introduction

Osteoblasts from human osteoarthritis (OA) patients express an altered phenotype in bone tissue *in situ* [1–3], a situation which can still be observed *in vitro*. Indeed, OA osteoblasts have an abnormally

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high alkaline phosphatase activity (ALPase), show an increased osteocalcin secretion, yet express normal levels of specific cell surface makers CD73 and CD105 [4–7]. Bone tissue from OA patients has been described as undermineralized by a number of research groups [8–11]. More importantly, although presenting high expression of markers of osteoblast/osteocyte-like cells, OA osteoblasts fail to mineralize normally in response to BMP-2 stimulation [7]. We determined this was due to elevated levels of transforming growth factor β-1 (TGF-β1) in human OA osteoblasts [7], and we and others demonstrated previously that this was linked with an abnormal regulation of the ratio of type 1 collagen α1 to α2 chains both *in vivo* [8,9] and *in vitro* [7].

Abnormal production of growth factors has been described in OA osteoblasts, such as for the insulin-like growth factor-1 (IGF-1) [12], TGF-β1 [6,13], and the hepatocyte growth factor (HGF) [14]. Both TGF-β1 and HGF may be important for the initiation and progression of OA. Recent studies have indicated that overproduction of TGF-β1 specifically in mouse osteoblasts leads to OA-like features whereas inhibition of TGF-β1 in subchondral bone mesenchymal stem cells signaling reduced OA progression [15]. HGF, which contributes to the increased expression of metalloproteinase-13 (MMP-13) in human OA cartilage samples [16], is not produced by articular cartilage chondrocytes but likely originates from subchondral osteoblasts [14].

Interactions between HGF, TGF-β1 and BMP-2 have been observed in different cell systems. Indeed, TGF-β1 can stimulate HGF production in some cell types whereas the reverse has also been reported, suggesting that the interaction between TGF-β1 and HGF could be cell-specific [17–20]. TGF-β1 can inhibit the effect of BMP-2, which has a beneficial role on bone tissue homeostasis, *via* the SMAD signaling pathway [21], while HGF has also been described to play a similar role in the murine myeloid cell line C2C12 [22].

In general, TGF-β1 activates the ALK5/Smad2/3 pathway [23] whereas BMP-2 acts *via* the ALK1/Smad1/5/8 pathway [24]. The deleterious effect of TGF-β1 on the action of BMP-2 in bone tissue involves the ALK5 receptor pathway which triggers SMAD2/3 and inhibits the ALK1 receptor pathway activation [25]. Of note, recent studies have indicated that altered SMAD2/3 activity could be detrimental to the cartilage [26], and this triggers MMP-13 activity which is a key player for the loss of articular cartilage. A shift in ALK5 and ALK1 activity can also be observed during aging and in OA pathogenesis [27]. However, a role for HGF on the phenotype of osteoblasts in osteoarthritis has never been described, nor if there is a link between HGF and altered ALK1/Smad1/5/8 signaling in OA osteoblasts.

TGF-β1 and HGF also play important immunoregulatory role on OA mesenchymal stem cells (MSC) [28–30]. In addition, it is of note that cytokines and growth factors play key role in osteophyte formation [31], and that elevated TGF-β1 [6,13] and HGF [14] levels are observed in OA osteoblasts [32]. Osteophyte formation may be considered a repair response to stabilize the damaged joints, and it requires the local recruitment of specific MSC [33].

Hence, the present study was performed to unravel the potential role of HGF to alter the phenotype of OA osteoblasts, *via* their response to BMP-2 on ALPase activity and osteocalcin secretion, alterations of the cWnt and Smad1/5/8 pathways, and its role in bone mineralization in these cells.

Material and methods

Patients and clinical parameters

Tibial plateaus were obtained from OA patients undergoing knee replacement surgery and prepared as previously described [4–6]. A total of 37 patients (69 ± 9.4 years old, mean ± SD; 11 males/26 females) classified as having OA according to the criteria of the American College of Rheumatology were used [34]. No patients received medication that could interfere with bone metabolism. Specimens from 12 normal individuals (68.1 ± 15.7 years old, mean ± SD; 6 males/6 females) were obtained at autopsy within 12 h of death. Ethical approval was obtained for the use of all human material following a signed agreement by the patients undergoing knee surgery and for the autopsy specimens by their relatives, in accordance with the CHUM ethical committee guidelines.

Preparation of primary subchondral bone osteoblasts

Isolation of subchondral bone plate and the cell cultures was prepared as previously described [4]. Confluent normal and OA osteoblasts were maintained in culture media containing 10% FBS to promote their differentiation until they were switched to media containing 2% FBS for the last 48 h of culture. For the determination of biomarkers confluent cells were incubated in Ham's F12/DMEM culture media with 2% fetal bovine serum (FBS) for their last 48 h of culture in the presence of $1,25(OH)_2D_3$ (50 nM). In some cases, cells were stimulated with increasing doses of human recombinant BMP-2 (rhBMP-2), 1 to 100 ng/ml, or the vehicle for their last 48 h of culture in the presence of $1,25(OH)_2D_3$. Supernatants were collected at the end of the incubation for the determination of osteocalcin levels. Cells were prepared in either ALPase buffer for the phenotypic determination of ALPase activity, RIPA buffer for western blot analysis, or in TRIzol™ for qRT-PCR experiments. Protein determination was performed by the bicinchoninic acid method [35]. In some experiments, cells were maintained in culture 20 days post-confluence to determine the expression of HGF at specified time points.

Phenotypic characterization of human subchondral osteoblast cell cultures

ALPase activity was determined by substrate hydrolysis using p-nitrophenylphosphate of whole cell lysates whereas, osteocalcin release in cell supernatants was evaluated using an enzyme immunoassay (EIA) as previously described [4,6]. Determinations were performed in duplicate for each specimen.

Preparation of Wnt3a conditioned media (Wnt3a-CM)

Conditioned media (CM) were prepared from Murine L cell lines transfected with either an empty vector (Parental) or with Wnt3a (Wnt3a) obtained from the American Type Culture Collection (Cedarlane Laboratories, Ontario) as described [13]. CM were added to cells at a 20% final concentration.

Evaluation of mineralization

Confluent cells were incubated in BGJb media containing 10% fetal bovine serum (FBS), 50 μg/ml ascorbic acid, and 50 μg/ml β-glycerophosphate. This medium was changed every two days until day 28. Mineralization of cell cultures was measured by quantification of alizarin red staining (ARS) with the procedure of Gregory et al. [36].

Inhibition of HGF expression in OA osteoblasts by siRNA

HGF expression was inhibited in OA osteoblasts by specific siRNA using previously described methods [7,13,37,38]. Briefly, OA osteoblasts were split at 100,000 cells/ml. HGF–siRNA preparations (4 different siRNA constructs are provided by the manufacturer within the same sample) were obtained at Dharmacon (Lafayette, CO) as well as scrambled RNA, and they were added to OA osteoblasts at a final concentration of 100 ng/ml with 4.5 μl Hi-perfect (Quiagen, Ontario) per 100 μl total volume in BGJb media without serum for 1 h on day 0 and day 3. Controls were performed using scrambled RNA (siSCR) preparations provided by the same manufacturer, and cells were treated as per siHGF. The inhibition of HGF expression was followed using qRT-PCR.