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# Osteoblasts from the sclerotic subchondral bone downregulate aggrecan but upregulate metalloproteinases expression by chondrocytes. This effect is mimicked by interleukin-6, -1β and oncostatin M pre-treated non-sclerotic osteoblasts

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## Summary

*Objective*: To determine the effects of osteoarthritic (OA) subchondral osteoblasts on the metabolism of human OA chondrocytes in alginate beads.

*Methods*: Human chondrocytes were isolated from OA cartilage and cultured in alginate beads for 4 days in the absence or in the presence of osteoblasts isolated from non-sclerotic (N) or sclerotic (SC) zones of human OA subchondral bone in monolayer (co-culture system). Before co-culture, osteoblasts were incubated for 72 h with or without 1.7 ng/ml interleukin (IL)-1β, 100 ng/ml IL-6 with its soluble receptor (50 ng/ml) or 10 ng/ml oncostatin M (OSM). Aggrecan (AGG) and matrix metalloproteases (MMP)-3 and -13 mRNA levels in chondrocytes were quantified by real-time polymerase chain reaction. AGG production was assayed by a specific enzyme amplified sensitivity immunoassay.

*Results*: SC, but not N, osteoblasts induced a significant inhibition of AGG production and AGG gene expression by human OA chondrocytes in alginate beads, and significantly increased MMP-3 and MMP-13 gene expression by chondrocytes. When they were pre-incubated with IL-1β, IL-6 or OSM, N osteoblasts inhibited AGG synthesis and increased MMP-3 and -13 gene expression by chondrocytes in alginate beads in a same order of magnitude as SC osteoblasts.

*Conclusions*: These results demonstrate that SC OA subchondral osteoblasts could contribute to cartilage degradation by stimulating chondrocytes to produce more MMP and also by inhibiting AGG synthesis.
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*Key words*: Osteoblasts, Cartilage, Interleukin-1, Interleukin-6, Osteoarthritis.

There is consensus that osteoarthritis (OA) is characterized by subchondral bone thickening, accompanied by an increased osteoid volume and a low mineralization[1]. These histomorphological modifications indicate that a dysregulation of bone remodeling may be part of OA. Indeed, osteoblasts isolated from subchondral OA bone demonstrate an altered phenotype[2]. In comparison to normal osteoblasts, they produce more alkaline phosphatase (ALP), osteocalcin (OC), insulin-like growth factor (IGF)-1 and urokinase (uPA), while IGF-binding protein (IGFBP) and plasminogen activator inhibitor (PAI)-1 levels remain unchanged[3,4]. The imbalance between PAI-1 and uPA may favor the hydrolysis of IGFBP, resulting in the freeing of IGF-1 locally, which can then act via an autocrine/paracrine pathway to enhance bone matrix formation[2]. Further, OA osteoblasts are resistant to parathyroid hormone (PTH) stimulation, a situation that can also contribute to abnormal bone remodeling and bone sclerosis in OA[3]. Based on the production of interleukin (IL)-6 and prostaglandin (PG) $E_2$, two groups of OA individuals have been identified. Osteoblasts from one group of individuals produced levels of $PGE_2$ and IL-6 very similar to normal cells, whereas another group of OA individuals always showed an increased $PGE_2$ and IL-6 production[5]. Interestingly, all OA osteoblasts produce higher level of transforming growth factor (TGF)-β1 compared to normal cells, whereas IL-1β production is similar[5]. Because microcracks, vascular channels or neovascularization provide a link between subchondral bone tissue and cartilage, it was hypothesized that IL-6, TGF-β and probably some other unexplored factors could also contribute to the abnormal remodeling of OA cartilage[6–9]. Westacott *et al.*[10] have shown that OA osteoblasts in monolayer increased glycosaminoglycan release from cartilage explants, whereas normal cells did not. However, no single effector responsible for osteoblast-induced cartilage degradation has been yet identified.

Until now, the influence of OA osteoblasts phenotype on cartilage matrix formation and metalloproteinases synthesis remains unexplored. Herein, we describe an original co-culture model in which osteoblasts in monolayer, isolated from non-sclerotic (N) or sclerotic (SC) zones of human subchondral bone, are cultured together with OA human

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chondrocytes. We have also co-cultured human OA chondrocytes with N or SC osteoblasts pre-incubated with IL-1β, -6 or oncostatin M (OSM). These cytokines play an important role in bone remodeling and could be involved in bone sclerosis process that occurs in OA. IL-6, in the presence of its soluble receptor (IL-6sR), increases collagenase-3 and IGF-1 expression in osteoblasts from fetal rat calvariae[11,12]. IL-6 also stimulates bone resorption by increasing osteoclasts recruitment and differentiation. The effects of IL-6 on osteoclasts recruitment require the presence of osteoblasts and depend on IL-6 receptors and receptor activator of NF-κB (RANK)/RANK ligand[13]. In primary neonatal murine or fetal rat calvariae osteoblasts culture, OSM induces proliferation, collagen synthesis, and IL-6 secretion, whereas it inhibits ALP[14]. In primary culture of rat osteoblast cells, IL-1β decreases OC synthesis but has no effect on ALP activity[15]. IL-1β also stimulates PTH-related protein, $PGE_2$, IL-6 and plasminogen activator secretion and decreases TGF-β1 production by human osteoblast-like cells[16–18].

In this work, we demonstrate that SC OA osteoblasts phenotype contributes to cartilage degradation by inhibiting cartilage matrix component synthesis and by increasing metalloproteinases synthesis by human chondrocytes. Further, we show that N osteoblasts incubated with IL-1β, IL-6 and OSM have similar effect to that of SC osteoblasts on chondrocytes in alginate beads. These findings suggest that these cytokines could be responsible for the modifications of osteoblasts phenotype in OA.

## Materials and methods

### SUBCHONDRAL OA OSTEOBLASTS IN MONOLAYER CULTURE

Tibial and femoral subchondral bone plates were collected from the knees of cadavers (three men aged 48, 57 and 65 years) with severe OA within 2 h after death. After careful elimination of trabecular bone and articular cartilage, OA subchondral bone was dissected under a microscope to separate SC from N zones. N and OA SC bone zones were identified by a marked difference in thickness and intermediate zones were not used. We have considered as SC bone only the subchondral bone zone with a thickness greater than 2 mm and either cartilage denuded or overlaid by fibrillated cartilage. The plates were then cut into small fragments of approximately 2 $mm^3$ with wire cutters, washed with Dulbecco's Modified Eagle Medium (DMEM) and then submitted to enzymatic digestions following the protocol of Hilal *et al.*[2]. Briefly, small pieces of bone (2 $mm^3$) were sequentially incubated with 0.5 mg/ml hyaluronidase type IV S (Sigma–Aldrich, Bornem, Belgium) for 20 min, and 0.6 mg/ml clostridial collagenase IA (Sigma–Aldrich, Bornem, Belgium) for 30 and 240 min successively (2 g of bone in 20 ml of enzymatic solution). The digested bone pieces were placed in T-75 flasks and cultured in DMEM supplemented with 15% fetal calf serum (FCS), 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin, until osteoblasts migrated out of bone explants. At this point, the medium was replaced with fresh media containing 10% FCS, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin. At confluence, cells were collected by trypsinization, seeded (50,000 cells/$cm^2$) in 12-well plates (12-well companion plates, Falcon, BD Biosciences, Erembodegem, Belgium) and grown for 3 days in DMEM containing 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES. After washings, osteoblasts were maintained for 12 days in differentiation media, composed of DMEM containing 100 U/ml penicillin, 100 μg/ml streptomycin, 10 mM HEPES, 2% Ultroser G, a serum substitute, $10^{-8}$ M 1,25$(OH)_2$vitamin $D_3$ (Sigma–Aldrich, Bornem, Belgium), 50 μg/ml ascorbic acid (Sigma–Aldrich, Bornem, Belgium) and 2 mM proline (Invitrogen, Merelbeke, Belgium). At the end of this differentiation period, cells showed an osteoblastic phenotype characterized by the production of OC and ALP. The cell population was homogenous and did not contain fibroblastic or adipocytic cell types as shown by morphological observations and Fast Red or Oil Red O staining. N osteoblasts were isolated from normal areas of subchondral bone whereas SC osteoblasts were isolated from the SC areas of subchondral bone. After washings, cells were then cultured for 72 h in the absence or in the presence of human recombinant IL-1β (1.7 ng/ml or 85 U/ml, Roche, Brussels, Belgium), 100 ng/ml IL-6 in combination with 50 ng/ml IL-6sR (Biosource Europe, Fleurus, Belgium) or 10 ng/ml OSM (Sigma–Aldrich, Bornem, Belgium). These concentrations corresponded to the maximal IL-1β, OSM and IL-6 concentrations found in the synovial fluid of OA patients[19–21]. The nutrient medium used in this incubation phase was DMEM supplemented with 1% ITS+ (ICN Biomedicals, Asse-Relegem, Belgium), 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid (Sigma–Aldrich, Bornem, Belgium), 2 mM proline (Invitrogen, Merelbeke, Belgium). ITS+ is a premixed cell growth system containing in 1 ml: 0.625 mg insulin, 0.625 mg transferrin, 0.625 μg selenious acid, 0.125 g bovine serum albumin and 0.535 mg linoleic acid.

### CHONDROCYTES CULTURE IN ALGINATE BEADS

Cartilage was obtained from the knees of cadavers (three men aged 50, 52 and 63 years) with OA within the 2 h after death, being excised from the superficial and medium layers of cartilage and avoiding the calcified layer. Upon dissection, the femoral, patellar and tibial articular surfaces were evaluated for gross pathological cartilage modifications according to a personal scale[22]. The severity of pitting was recorded for each donor. Four different grades were considered: 0, normal white cartilage in all areas examined; I, the presence of a yellow–gray area with some superficial fibrillations on one or more articular surfaces; II, irregular surface with deep fibrillations on one or more articular surfaces; and III, ulcers penetrating to the subchondral bone on one or more articular surfaces. Experiments were performed with cartilage specimen showing OA cartilage lesions of grade II or III. Cartilage was cut into small fragments and then subjected to enzymatic digestions sequentially with hyaluronidase, pronase and collagenase (3 g of cartilage per 10 ml of enzyme solution) as previously described[23]. The cells were filtered through a nylon mesh with a pore diameter of 70 μm, and then washed three times with sterile water (0.9% NaCl). Cell viability was estimated by trypan blue exclusion test and in all cases was greater than 95%. Chondrocytes were suspended in 1.2% low viscosity alginate (Sigma–Aldrich, Bornem, Belgium) in 0.9% NaCl solution at a density of $4 \times 10^6$ cells/ml, which was slowly passed through a 25 gauge needle in a dropwise fashion into 102 mM $CaCl_2$ solution (Sigma–Aldrich, Bornem, Belgium). After instantaneous gelation, the beads were allowed to polymerize further for 10 min in this $CaCl_2$ solution. Ten alginate beads containing OA chondrocytes were placed in porous inserts (with 1 μm pore size; Falcon,

BD Biosciences, Erembodegem, Belgium) and placed in a nutrient culture medium (DMEM supplemented with 2% Ultroser G) for the next 48 h (wash-out period). Then, the beads were washed in phosphate buffered saline (PBS) and placed in co-culture with N or SC OA subchondral osteoblasts or with normal skin fibroblasts in monolayer. Normal skin fibroblasts obtained by outgrowh from biopsy as previously described[24] were provided by Lambert Ch (Liège, Belgium).

### OA OSTEOBLASTS/CHONDROCYTES CO-CULTURE

After three PBS washes of osteoblasts or alginate beads, the inserts (with a pore size of 1 μm; Falcon, BD Biosciences, Erembodegem, Belgium) containing 10 alginate beads were co-cultured for 4 days with osteoblasts in monolayer pre-incubated or not with cytokines (Fig. 1). Coculture medium was DMEM supplemented with 1% ITS+, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml ascorbic acid, 2 mM proline. Six wells of a 12-well plate were used for each culture conditions. The experiment was repeated three times, each time using subchondral osteoblasts and chondrocytes coming from different donors, and six wells were used for each culture condition. As controls, OA chondrocytes in alginate beads were cultured in mono-culture or in co-culture with normal skin fibroblasts in monolayer. At the end of the co-culture period, cells conditioned culture medium (S) was carefully collected and kept at −20°C until analysis. The alginate beads were dissolved in 1 ml of 0.1 M citrate for 10 min. The resulting suspension was centrifuged at 1200 rpm for 10 min. By this method, two fractions were collected: the supernatant containing macromolecules originated from the further-removed matrix (FRM) compartment, and a pellet, containing cells with their associated matrix (CM). Osteoblasts in monolayer were collected after 5 min treatment with a solution containing 0.125% trypsin−0.5 mM EDTA (Invitrogen, Merelbeke, Belgium). The cell pellets of chondrocytes, osteoblasts or fibroblasts were washed three times with PBS and then either homogenized in 1 ml PBS by ultrasonic dissociation for DNA and aggrecan (AGG) quantification or in 175 μl of cell lysis buffer (Promega, Leiden, The Netherlands) for RNA isolation. Cell extracts were kept at −70°C until analysis.

### DNA ASSAY

The DNA content of the cultures was measured according to the fluorimetric method of Labarca and Paigen[25].

### IMMUNOASSAYS FOR AGG, MMP-3, OC, IL-1β, IL-6 AND TGF-β1

AGG, matrix metalloprotease (MMP)-3, OC, IL-1β, IL-6 and TGF-β1 were directly measured in conditioned culture media by specific enzyme amplified sensitivity immunoassays (EASIA, Biosource Europe, Fleurus, Belgium[26]). AGG was measured in the different compartments of alginate bead and in culture supernatants by a specific EASIA in which a monoclonal antibody raised against the keratan sulphate region of AGG as a capture antibody, and another monoclonal antibody recognizes the hyaluronic acid binding region (HABR) as a detector. The AGG HABR is captured by the anti-KS antibody irrespective of whether cytokine-induced proteolysis occurs in the AGG interglobular domain, so permitting accurate quantification of AGG synthesis. Human purified AGG was used as standard to quantification. The limit of detection of these immunoassays was 5.5 ng/ml for AGG, 1.25 ng/ml for MMP-3, 1.9 ng/ml for OC, 10 pg/ml for IL-1β, 8 pg/ml for IL-6 and 30 pg/ml for TGF-β1. The intra- and inter-assay coefficients of variation were less than 5% for all immunoassays.

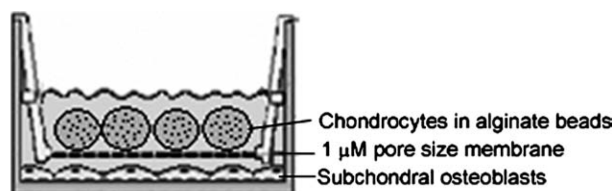



Fig. 1. Schematic representation of the subchondral osteoblasts/chondrocytes co-culture model.

### ALP ASSAY

ALP activity was quantified in the cellular fraction of the osteoblasts culture. Cell extract (50 μl) was incubated with 100 μl of *p*-nitrophenylphosphate (liquid *p*-NPP, ready to use, KEM-EN-TEC, Kobenhavn, Denmark). In the presence of ALP, *p*-NPP is transformed to *p*-nitrophenol and inorganic phosphate. *p*-Nitrophenol absorbance is measured at 405 nm after 10 min of incubation at 37°C. A standard preparation of *p*-nitrophenol was used for calibration. Results were expressed in nanomoles of *p*-nitrophenol released per min and per μg of DNA.

### QUANTITATIVE REAL-TIME RT-PCR

RNA from $3 \times 10^6$ cells was isolated by SV total RNA isolation system (Promega, Leiden, The Netherlands). RNA (0.5 μg) was reverse transcribed in 30 μl buffer containing 10 mM DTT, 1 mM dNTPs, 500 ng $(dT)_{15}$ primer, 140 U M-MLV Reverse Transcriptase and 40 U RNaseOUT (Life Technologies, Merelbeke, Belgium) for 60 min at 37°C. The reaction was stopped by incubating the samples at 95°C for 5 min and by adding 100 μl of $H_2O$. Polymerase chain reaction (PCR) was performed by using the LightCycler-FastStart DNA Master Sybr Green I (Roche Diagnostics, Brussels, Belgium). The PCR template source was either 3 ng first-strand cDNA or purified DNA standard. Primer sequences used to amplify the desired cDNA are as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward and reverse primers: 5′-TTGGTATCGTGGAAGGACTCA-3′ and 5′-TGTCATCA TATTTGGCAG GTTT-3′; AGG forward and reverse primers: 5′-GCACCATGCCTTCTGCTTCCGAG-3′ and 5′-CTCCACTGCCTGTGAAGTCACCAC-3′; MMP-3 forward and reverse primers: 5′-ATGAGGTACGAGCTGG-3′ and 5′-TCACGCTCAAGTTCCC-3′; MMP-13 forward and reverse primers: 5′-CAACGGACCCATACAG-3′ and 5′-ACAGACCATGTGTCCC-3′. Amplification was performed with a spectrofluorometric thermal cycler (LightCycler, Roche Diagnostics, Brussels, Belgium). For each gene under study, a standard curve was generated from purified cDNA of the gene concerned ranging from $10^6$ to 10 copies. The efficiency of reverse transcription is similar for one particular messenger in all our culture conditions, so we can state that the cDNA levels reflect the mRNA levels for the concerned gene. To standardize mRNA levels, we amplified GAPDH, a house keeping gene, as an internal control. Gene expression was normalized by calculating the ratio

between the number of cDNA copies of AGG, MMP-3, MMP-13, and that of GAPDH. After amplification, a final melting curve was recorded by cooling the PCR mixture to 65°C for 30 s and then slowly heating it to 95°C at 0.1°/s. Fluorescence was measured continuously during the slow temperature rise to monitor the dissociation of dsDNA. Specificity of the expected LightCycler products was demonstrated by melting curve analysis. Amplification products performed in the LightCycler were checked by electrophoresis on 1.5% ethidium bromide stained agarose gel. The estimated size of the amplified fragments matched the calculated size.

#### CALCULATION AND STATISTICAL ANALYSIS

The results (mean ± S.E.M.) were expressed as sum of the concentration of AGG found in the culture supernatant, CM and FRM per μg of DNA, or as GADPH-normalized gene expression. A multivariate repeated measures ANOVA model with calculation of contrasts was performed on all the experiments.

## Results

#### INFLUENCE OF OA OSTEOBLASTS PHENOTYPE ON HUMAN CHONDROCYTE METABOLISM

In comparison with N zones isolated osteoblasts, SC subchondral OA osteoblasts in monolayer produced higher amounts of ALP (+95%), OC (+98%), IL-6 (+134%) and TGF-β1 (+41%) whereas IL-1β concentration was similar (Table I). These findings indicate that SC osteoblasts had an altered phenotype. We have then cultured N or SC osteoblasts in monolayer with OA chondrocytes in alginate beads. In parallel, skin fibroblasts in monolayer and chondrocytes in alginate beads were co-cultured as a control condition. At the beginning of the co-culture period, 3.5 ± 0.5 μg of DNA was measured in alginate beads, osteoblasts and fibroblasts monolayer culture, indicating that a similar number of chondrocytes, osteoblasts or fibroblasts were introduced in the co-culture model. Further, co-culture conditions did not significantly modify DNA content in the different compartments of the co-culture, demonstrating that cell number remained stable over the co-culture period (data not shown).

When they are cultured alone (mono-culture), human OA chondrocytes in alginate beads spontaneously produced 8.5 μg of AGG per μg of DNA. As previously described, newly-synthesized AGG were essentially accumulated in alginate bead[26]. Seventy-three percent was found in the FRM, 26% in the CM and only 1% was released in the supernatant. When alginate beads were co-cultured with SC osteoblasts, AGG content in alginate bead was decreased by 27 ± 7% ($P < 0.001$) whereas N osteoblasts or normal skin fibroblasts did not modify AGG content significantly [Fig. 2(A)]. The decrease of AGG content was significantly more marked in the CM compartment of alginate bead than in FRM. AGG content was decreased by 45 ± 8% in CM ($P < 0.01$), but only by 20 ± 7% in FRM ($P < 0.05$). The ratio of AGG released into the supernatant was unchanged in co-culture (data not shown). In mono-culture, OA chondrocytes in alginate beads spontaneously expressed a mean value of 22 copies of AGG per 1000 copies of GAPDH. When they were co-cultured with osteoblasts, AGG mRNA level in chondrocytes was significantly reduced by SC ($P < 0.001$) but not significantly by N osteoblasts [Fig. 2(B)].

In mono-culture, OA chondrocytes in alginate beads spontaneously secreted a mean value of 752 ng of MMP-3 per μg DNA and expressed a mean value of 1400 copies of MMP-3 and 5 copies of MMP-13 per 1000 copies of GAPDH. In co-culture with SC osteoblasts, MMP-3 production and MMP-3 and -13 mRNA levels in chondrocytes were significantly increased by 1.6, 1.65 and 2 times, respectively ($P < 0.001$). In comparison, N osteoblasts or skin fibroblasts did not modify MMP-3 production or mRNA level but decreased that of MMP-13 mRNA ($P < 0.01$) (Fig. 3).

#### EFFECTS OF PRE-TREATMENT OF OSTEOBLASTS WITH IL-1β ON HUMAN CHONDROCYTE METABOLISM

IL-1β (1.7 ng/ml) significantly stimulated ALP and IL-6 ($0.01 > P > 0.001$) production, but inhibited those of OC and TGF-β1 ($P < 0.001$) in both N and SC osteoblasts in monolayer (Table I). IL-1-treated osteoblasts were then

Table I
*Effects of IL-1β, IL-6 or OSM on human subchondral osteoblasts in monolayer*

| | | Control | IL-1β | IL-6/IL-6sR | OSM |
|---|---|---|---|---|---|
| DNA (μg/wells) | N | 3.1 ± 0.5 | 3.0 ± 0.6 | 3.5 ± 0.3 | 3.2 ± 0.4 |
| | SC | 3.3 ± 0.3 | 3.1 ± 0.4 | 3.6 ± 0.4 | 3.5 ± 0.3 |
| ALP ((nmol/μg DNA)/min) | N | 9.1 ± 0.6 | 12.2 ± 0.4** | 10.9 ± 1.5 | 14.3 ± 0.6*** |
| | SC | 17.8 ± 0.6### | 23.5 ± 1.2*** | 17.7 ± 1.2 | 27.6 ± 4.3*** |
| OC (ng/μg DNA) | N | 6.8 ± 0.3 | 1.9 ± 0.1*** | 3.3 ± 0.1*** | 2.6 ± 0.7*** |
| | SC | 13.5 ± 0.4### | 3.4 ± 1.0*** | 6.5 ± 0.8*** | 5.3 ± 0.5*** |
| IL-1β (pg/μg DNA) | N | 4.4 ± 0.6 | N.D. | 5.1 ± 0.3 | 5.1 ± 1.1 |
| | SC | 4.5 ± 0.7 | N.D. | 6.3 ± 0.7 | 5.1 ± 1.0 |
| IL-6 (pg/μg DNA) | N | 595 ± 35 | 3745 ± 80*** | N.D. | 2946 ± 28*** |
| | SC | 1397 ± 114### | 4620 ± 73*** | N.D. | 3690 ± 104*** |
| TGF-β1 (pg/μg DNA) | N | 37.5 ± 0.5 | 29.6 ± 2.5*** | 62.6 ± 5.5*** | 46.0 ± 2.1** |
| | SC | 52.9 ± 3.4### | 38.4 ± 3.3*** | 71.9 ± 5.2** | 60.7 ± 3.2 |

Values are means ± S.E.M. ($n = 9$) of three independent cultures performed with subchondral osteoblasts coming from three different donors. Subchondral osteoblasts were cultured in the presence of 1.7 ng/ml IL-1β, 100 ng/ml IL-6, 50 ng/ml IL-6sR or 10 ng/ml OSM during 72 h. Statistical significance between N and SC subchondral osteoblasts: ###$P < 0.001$ or between controls and cytokine-stimulated osteoblasts: **$P < 0.01$ and ***$P < 0.001$.

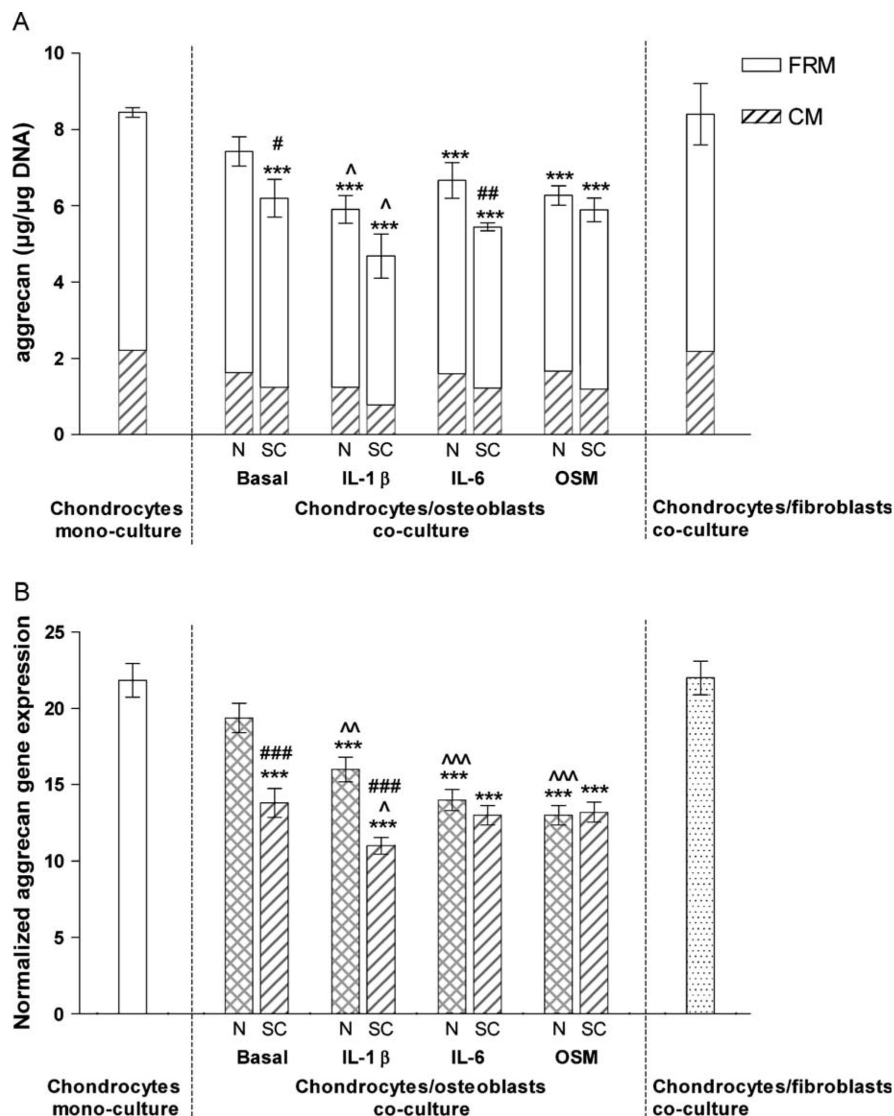
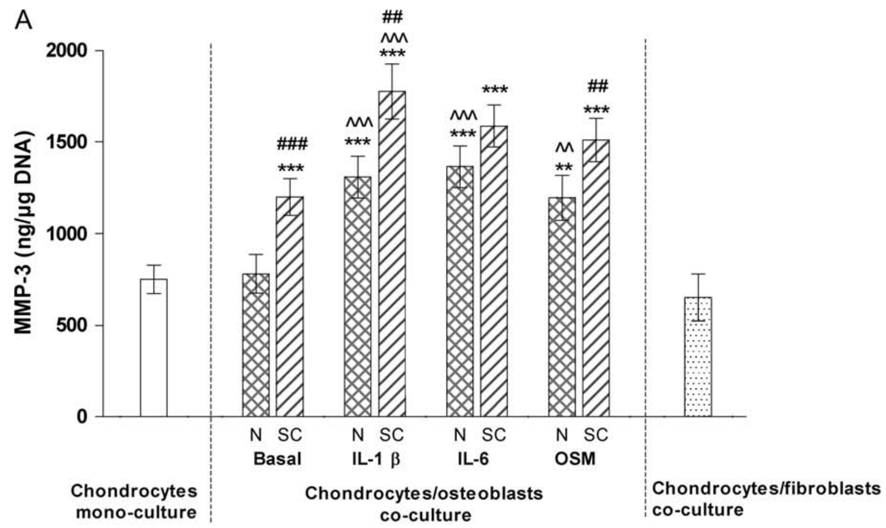



Fig. 2. AGG content in alginate beads (A) and AGG gene expression by chondrocytes (B) after 4 days of culture in the absence (mono-culture) or in the presence (co-culture) of osteoblasts isolated from SC or N zones of subchondral bone. Before co-culture, human osteoblasts were incubated or not (control) for 72 h with IL-1β (1.7 ng/ml), IL-6 (100 ng/ml in combination with 50 ng/ml of IL-6sR), or OSM (10 ng/ml). As a control, human OA chondrocytes in alginate beads were also co-cultured with normal human skin fibroblasts. The results are expressed as the mean ± S.E.M. of three experiments performed with cells coming from three different donors. Comparison of mean values of total AGG production (A) or GAPDH mRNA normalized AGG mRNA copies (numbers of mRNA gene copies per 1000 GAPDH mRNA copies, B) was performed by repeated measures ANOVA analysis. Statistical significances: chondrocytes/osteoblasts co-culture compared to mono-culture ***$P < 0.001$; pre-treated osteoblasts compared to the controls ^$P < 0.05$, ^^$P < 0.01$, ^^^$P < 0.001$; SC osteoblasts compared to N osteoblasts #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$.

cultured together with chondrocytes in alginate beads. Whilst N osteoblasts had no effect on AGG synthesis, IL-1β-treated N osteoblasts decreased AGG content in alginate beads and AGG gene expression in similar order to that of SC osteoblasts ($P < 0.01$, Fig. 2). Pre-treatment of SC osteoblasts with IL-1β significantly accentuated their inhibitory effect on AGG synthesis by chondrocytes ($P < 0.05$, Fig. 2). In comparison with untreated N osteoblasts, IL-1β-treated N osteoblasts induced a significant elevation of MMP-3 and MMP-13 mRNA levels in chondrocytes ($P < 0.001$, Fig. 3). IL-1β treatment also enhanced the stimulating effect of SC osteoblasts on MMPs gene expression (Fig. 3).

EFFECTS OF PRE-TREATMENT OF OSTEOBLASTS WITH IL-6/IL-6SR ON HUMAN CHONDROCYTE METABOLISM

In both N and SC osteoblasts monolayer culture, IL-6 (100 ng/ml) in the presence of its soluble receptor (IL-6sR) significantly decreased OC and increased TGF-β1 ($P < 0.001$) production, but did not significantly modify ALP and IL-1β synthesis (Table I). IL-6/IL-6sR-treated osteoblasts were then cultured together with chondrocytes in alginate beads. Whilst N osteoblasts had no effect on AGG synthesis, IL-6/IL-6sR-treated N osteoblasts decreased AGG content in alginate beads and AGG gene expression in the same order of magnitude as OA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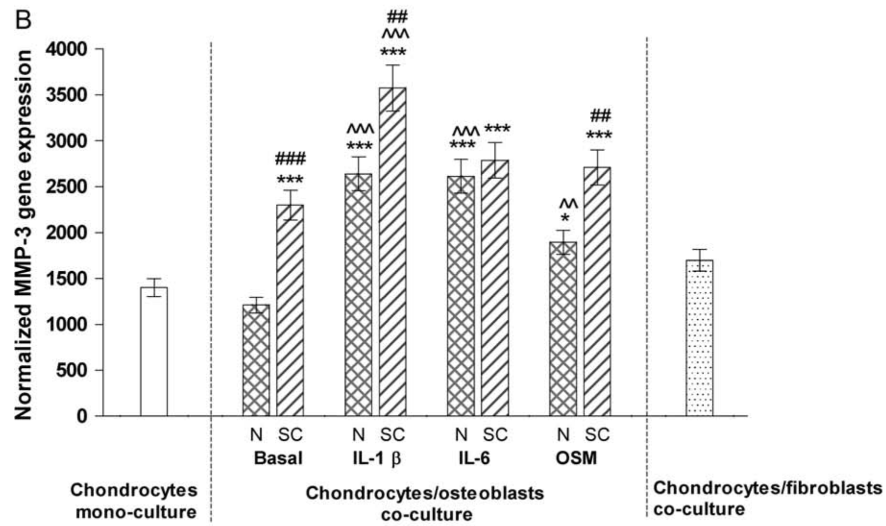
Chondrocytes/fibroblasts co-culture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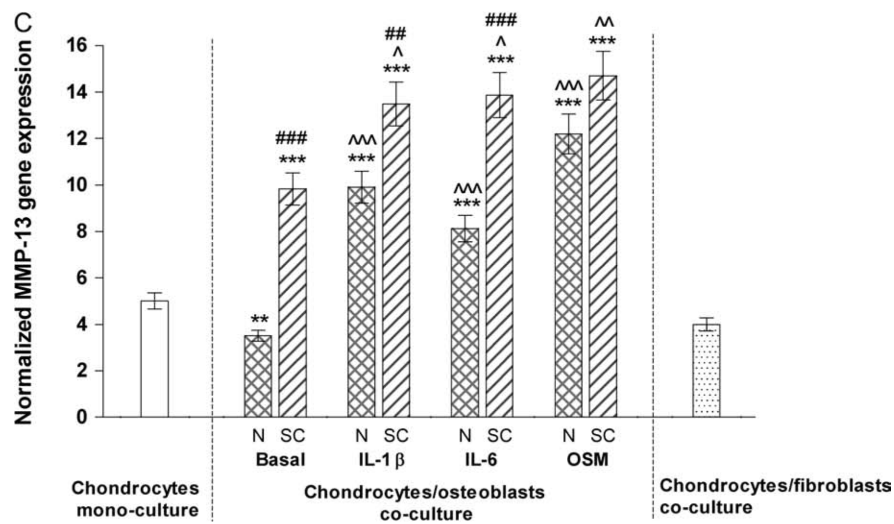
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Basal IL-1 β IL-6 OSM
Chondrocytes mono-culture
Chondrocytes/osteoblasts co-culture
Chondrocytes/fibroblasts co-culture

osteoblasts ($P < 0.001$, Fig. 2). Pre-treatment of SC osteoblasts with IL-6/IL-6sR did not increase their inhibitory effect on AGG synthesis by chondrocytes (Fig. 2). In comparison with untreated N osteoblasts, IL-6-treated N osteoblasts induced a significant elevation of MMP-3 and MMP-13 mRNA levels in chondrocytes ($P < 0.001$, Fig. 3). IL-6 treatment significantly enhanced the stimulating effect of SC osteoblasts on MMP-13 synthesis ($P < 0.05$) but did not modify the stimulating effect of SC osteoblasts on MMP-3 (Fig. 3).

### EFFECTS OF PRE-TREATMENT OF OSTEOBLASTS WITH OSM ON HUMAN CHONDROCYTE METABOLISM

In both N and SC osteoblasts in monolayer, OSM (10 ng/ml) significantly inhibited OC but stimulated ALP and IL-6 ($P < 0.001$) production. OSM did not significantly modify IL-1β production. It also increased TGF-β1 synthesis but only in N osteoblasts culture ($P < 0.01$) (Table I). OSM-treated osteoblasts were then co-cultured for 4 days with chondrocytes in alginate beads. Whilst N osteoblasts had no effect on AGG synthesis, OSM-treated N osteoblasts decreased AGG content in alginate beads and AGG gene expression in the same order of magnitude as SC osteoblasts ($P < 0.001$, Fig. 2). Pre-treatment of SC osteoblasts with OSM did not accentuate their inhibitory effect on AGG synthesis by chondrocytes (Fig. 2). By comparison with untreated N osteoblasts, OSM-treated N osteoblasts induced a significant elevation of MMP-3 and MMP-13 mRNA levels in chondrocytes ($P < 0.05$ and $P < 0.001$, respectively, Fig. 3). OSM treatment also enhanced the stimulating effect of SC osteoblasts on MMP-13 gene expression (Fig. 3), but had no effect on MMP-3.

## Discussion

Recent evidences suggest a key role for subchondral bone tissue in the pathogenesis of OA, possibly via the production of cytokines and growth factors. Using bone explants from OA patients, number of investigators have previously shown that these explants contain more TGF-β and IGF compared with normal bone tissues[27,28]. Herein, we have observed that SC osteoblasts secreted more ALP, OC, IL-6 and TGF-β1 than cells isolated from N zones of subchondral bone, corroborating the Lajeunesse's group results[2,5]. This finding indicates that OA subchondral osteoblasts used in this study show an altered phenotype compared to N cells and that they secrete mediators, such as TGF-β1 and IL-6, involved in the structural changes of OA cartilage[29–32]. As previous pathological studies have shown the presence of micro-cracks in the tidemark that appear early in OA[6], we have speculated that soluble mediators produced by SC osteoblasts could modulate chondrocyte metabolism and contribute to cartilage degradation. To verify this hypothesis, we have developed an original model of culture in which human osteoblasts from the SC subchondral bone and OA chondrocytes are cultured in the same environment, but without any contact. In parallel, we have tested the effects of three cytokines on the osteoblasts phenotype. These three cytokines have been chosen because there is a body of evidence suggesting that these mediators are involved in the communication between osteoclasts, bone marrow cells and osteoblasts. Further, these cytokines have been shown to influence bone remodeling and therefore, could be involved in subchondral bone sclerosis.

The originality of our model lies in the fact that we use osteoblasts from the SC subchondral bone and that OA chondrocytes are cultured in alginate beads. In a previous work, Westacott *et al.*[10] have demonstrated that OA trabecular osteoblasts in monolayer degraded cartilage explants in about half of the cases. A key element in cartilage degradation is an increase in metalloproteinase activities. MMP-3 and MMP-13 are two MMPs of particular importance in the degradation of cartilage in OA, because these MMPs are highly expressed by OA chondrocytes compared to normal cells. MMP-3 cleaves a wide variety of matrix macromolecules, e.g., proteoglycan, fibronectin and several collagens whereas MMP-13 is the most active collagenase to degrade type II collagen network. It has been shown to be 5–10 folds more active on type II collagen than MMP-1 and MMP-8[33]. Interestingly, we demonstrate that OA osteoblasts induced a strong elevation of MMP-3 and MMP-13 gene expression and MMP-3 secretion whereas N osteoblasts or normal skin fibroblasts have no effect. This observation indicates that osteoblast-induced MMPs production is related to its particular OA phenotype. These data suggest that SC osteoblasts could potentially contribute to cartilage degradation if activation were to occur *in vivo*. Indeed, we have not detected MMP activity estimated by casein–resorufin and 3H-collagen degradation tests in co-culture supernatants, suggesting that newly-synthesized proMMPs were not processed in active MMPs in our model. This data must be paralleled with those reported by Westacott *et al.*, showing that OA osteoblasts-induced cartilage explants degradation. A potential candidate responsible for this MMPs induction could be the hepatocyte growth factor (HGF), found in the deep layer of OA cartilage and recently found to be produced by OA subchondral osteoblasts and not by chondrocytes themselves[9]. HGF is a growth factor known to induce the expression of MMP-13 *in vitro* by OA chondrocytes[34]. Beside this stimulating effect of SC OA osteoblasts on MMPs synthesis, we have also observed that AGG content in alginate beads decreases when chondrocytes are co-cultured with SC osteoblasts. Again, this effect seems to be related to SC phenotype of

Fig. 3. MMP-3 production in the culture supernatant (A) and MMP-3 (B) and -13 (C) gene expressions by human OA chondrocytes in alginate beads after 4 days of culture in the absence (mono-culture) or in the presence (co-culture) of osteoblasts isolated from SC or N zones of subchondral bone. Before co-culture, human osteoblasts were either incubated or not (control) for 72 h with IL-1β (1.7 ng/ml), IL-6 (100 ng/ml in combination with 50 ng/ml of IL-6sR), or OSM (10 ng/ml). As a control, human OA chondrocytes in alginate beads were also co-cultured with normal human skin fibroblasts. The results are expressed as the mean ± S.E.M. of three experiments performed with cells coming from three different donors. The mRNA copy numbers were normalized against the corresponding copy number of GAPDH mRNA and expressed in numbers of mRNA gene copies per 1000 GAPDH mRNA copies. Comparison of mean values was performed by repeated measures ANOVA analysis. Statistical significances: chondrocytes/osteoblasts co-culture compared to mono-culture $^{**}P < 0.01$, $^{***}P < 0.001$; pre-treated osteoblasts compared to the controls $^{\wedge}P < 0.05$, $^{\wedge\wedge\wedge}P < 0.001$; SC osteoblasts compared to N osteoblasts $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$.

osteoblasts as suggested by the absence of effect of N osteoblasts and fibroblasts. SC osteoblasts also induce a decrease of AGG mRNA level in chondrocytes, indicating that the decrease of AGG content results in a decrease of AGG synthesis. Among the factors stimulating MMP synthesis and inhibiting AGG synthesis, IL-1β is probably one of the most powerful. Nevertheless, in our experimental conditions, SC and N osteoblasts produce similar amounts of IL-1β, indicating that the overproduction of MMPs by chondrocytes does not result in an increase of IL-1β concentration. Also, the IL-1β quantities found in the 4 days co-culture supernatant were below the detection limit of our EASIA assay (10 pg/ml, data not shown), even in the co-culture with IL-1β-stimulated osteoblasts. Nevertheless, we cannot exclude other mechanisms including an increase of OA chondrocytes sensitivity to IL-1β, as a consequence of an overexpression of IL-1 receptors at its membrane level, and/or an increase of IL-1β bioactivity secondary to a decrease of the synthesis of the natural receptor antagonist (IL-1Ra). Another potential candidate could be IL-6 that is overproduced by OA osteoblasts. In a previous study, we have reported that IL-6 stimulated MMP-3 but inhibited AGG production by human OA chondrocytes in alginate beads[32]. In addition, Flannery *et al.*[35] have observed that IL-6 and/or IL-6sR increased AGG catabolism above that seen in the presence of IL-1α alone, suggesting that IL-6 acts in synergy with IL-1 to degrade cartilage. Nevertheless, we are aware that our study fails to clearly identify the mediator(s) responsible for the OA osteoblasts-induced dysregulation of chondrocyte metabolism. Future investigations have been planed in which neutralizing antibodies against cytokines, inhibitors of cyclooxygenases or nitric oxide synthase will be used.

Another hypothesis is that IL-1β, IL-6 and OSM which are overproduced by OA chondrocytes could modulate subchondral osteoblasts creating a physiopathological loop between cartilage and subchondral bone. Of interest is the observation that IL-1β, IL-6 and OSM modulate N osteoblasts phenotype and induce an increase of ALP and a decrease of OC. Interestingly, the cytokine-modified osteoblasts phenotype is different to that of SC osteoblasts. In comparison to N osteoblasts, SC osteoblasts produce more ALP and OC while cytokine-treated osteoblasts produce more ALP but less OC. This observation suggests that osteoblasts acquired SC phenotype does not only result from IL-1β, IL-6 or OSM activities and that probably other mechanical and/or biochemical factors (e.g., tumor necrosis factor alpha, bone morphogenetic proteins) are involved[36,37]. However, cytokine-treated N osteoblasts decrease AGG synthesis and increase MMPs production by chondrocytes, in the same order of magnitude as SC osteoblasts. This finding indicates that SC osteoblasts effects on cartilage can be mimicked by stimulating N osteoblasts by one of these cytokines. However, IL-1β, IL-6 and OSM have different effects on N osteoblasts. One of the major difference concerns the production of TGF-β1. Whilst IL-1β decreases TGF-β production by N osteoblasts, IL-6 and OSM increase it. This observation suggests that probably TGF-β1 does not play a major role in osteoblasts-induced cartilage metabolic dysfunction.

In summary, our work demonstrated that the SC phenotype of osteoblasts induces a marked dysregulation of chondrocyte metabolism, characterized by a decrease of AGG synthesis and an increase of proMMPs synthesis. These findings suggest that osteoblasts from the SC subchondral bone, besides synoviocytes, may contribute to OA physiopathology.

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