

Avocado/Soybean Unsaponifiables Prevent the Inhibitory Effect of Osteoarthritic Subchondral Osteoblasts on Aggrecan and Type II Collagen Synthesis by Chondrocytes

YVES E. HENROTIN, MICHELLE A. DEBERG, JEAN-MICHEL CRIELAARD, NATHALIE PICCARDI, PHILIPPE MSIKA, and CHRISTELLE SANCHEZ

ABSTRACT. *Objective.* To determine the effects of avocado/soybean unsaponifiables (ASU) on osteoblast-induced dysregulation of chondrocyte metabolism.

Methods. Human chondrocytes were isolated from osteoarthritis (OA) cartilage and cultured in alginate beads for 4 or 10 days in the absence or presence of osteoblasts isolated from nonsclerotic (NSC) or sclerotic (SC) zones of OA subchondral bone plate in monolayer. Before co-culture, osteoblasts were incubated or not with 10 μ g/ml ASU for 72 hours. Aggrecan, type II collagen, matrix metalloproteinase-3 (MMP-3) and MMP-13, tissue inhibitor of metalloproteinase (TIMP-1), transforming growth factor- β 1 (TGF- β 1) and TGF- β 3, inducible NO synthase (iNOS), and cyclooxygenase-2 (COX-2) mRNA levels in chondrocytes were quantified by RT-PCR. Aggrecan, osteocalcin, TGF- β 1, interleukin 1 β (IL-1 β), and IL-6 production were assayed by immunoassays.

Results. In co-culture, SC osteoblasts induced a significant inhibition of matrix protein production and a significant increase of MMP synthesis by chondrocytes. In contrast, SC osteoblasts did not modify TIMP-1, TGF- β 1 and TGF- β 3, iNOS, or COX-2 mRNA levels in chondrocytes. The pretreatment of SC osteoblasts with ASU fully prevented the inhibitory effects of SC osteoblasts on matrix component production, and even significantly increased type II collagen mRNA level over the control (chondrocytes alone) value. In contrast, pretreatment of SC osteoblasts with ASU did not significantly modify the expression of MMP, TIMP-1, TGF- β 1, TGF- β 3, iNOS, or COX-2 gene by chondrocytes.

Conclusion. ASU prevent the osteoarthritic osteoblast-induced inhibition of matrix molecule production, suggesting that this compound may promote OA cartilage repair by acting on subchondral bone osteoblasts. This finding constitutes a new mechanism of action for this compound, known for its beneficial effects on cartilage. (First Release July 1 2006; J Rheumatol 2006;33:1668–78)

Key Indexing Terms:

OSTEOARTHRITIS

CARTILAGE

BONE

AVOCADO

SOYBEAN

There is consensus that osteoarthritis (OA) is characterized by subchondral bone thickening, accompanied by increased osteoid volume and low mineralization¹. These histomorphological modifications indicate that dysregulation of bone

remodelling may be part of OA. Indeed, osteoblasts isolated from subchondral OA bone demonstrate an altered phenotype². In comparison to normal osteoblasts, they produce more alkaline phosphatase (ALP), osteocalcin (OC), transforming growth factor- β 1 (TGF- β 1), insulin-like growth factor-1 (IGF-1), and urokinase plasminogen activator (uPA), while IGF binding protein, plasminogen activator inhibitor-1, and interleukin 1 β (IL-1 β) levels remain unchanged^{3–5}. The imbalance between urokinase and its inhibitor may favor the hydrolysis of IGF binding protein, resulting in the freeing of IGF-1 locally, which may then act via an autocrine/paracrine pathway to enhance bone matrix formation². Further, OA osteoblasts are resistant to parathyroid hormone (PTH) stimulation, a situation that can also contribute to abnormal bone remodelling and bone sclerosis in OA³. Because microcracks, vascular channels, and neovascularization provide a link between subchondral bone tissue and cartilage, it was hypothesized that IL-6, TGF- β , and probably other unexplored factors produced by osteoblasts may also contribute to the abnormal remodelling of OA cartilage^{6–8}. Westacott, *et al* showed

From the Bone and Cartilage Metabolism Research Unit, Institute of Pathology B23; Centre for Oxygen Research and Development, CHU Sart-Tilman; and Centre for Immunology, CHU Sart-Tilman, University of Liège, Liège, Belgium; and Laboratoires Expanscience, Epéron, France.

Supported in part by Expanscience and Belgian FNRS grants. C. Sanchez was supported as a pre-doctoral fellow by Belgian FNRS.

Y.E. Henrotin, PhD, Professor, Bone and Cartilage Metabolism Research Unit, Institute of Pathology B23; Centre for Oxygen Research and Development, CHU Sart-Tilman; and Centre for Immunology, CHU Sart-Tilman, University of Liège; M.A. Deberg, PhD; J.-M. Crielaard, MD, PhD, Professor, Bone and Cartilage Metabolism Research Unit, Institute of Pathology B23, University of Liège; N. Piccardi, PhD; P. Msika, PhD, Laboratoires Expanscience; C. Sanchez, PhD, Bone and Cartilage Metabolism Research Unit, Institute of Pathology B23, University of Liège.

Address reprint requests to Dr. Y.E. Henrotin, Bone and Cartilage Research Unit, Institute of Pathology, CHU Sart-Tilman, 4000 Liège, Belgium. E-mail: yhenrotin@ulg.ac.be.

Accepted for publication March 15, 2006.

Personal non-commercial use only. The Journal of Rheumatology Copyright © 2006. All rights reserved.

that OA osteoblasts in monolayer increased glycosaminoglycan release from cartilage explants, whereas normal cells did not⁹. However, no single effector responsible for osteoblast-induced cartilage degradation has been identified. That study was designed to determine the effects of avocado/soybean unsaponifiables (ASU) on the subchondral osteoblast/chondrocyte pathophysiological axis. To this end, we developed an original co-culture model, in which OA subchondral osteoblasts in monolayer are cultured with OA chondrocytes in alginate beads. Using this co-culture model, we previously showed that OA subchondral osteoblasts from the sclerotic (SC) zone modulate chondrocyte metabolism. The presence of SC subchondral osteoblasts induced a decrease of aggrecan, type II collagen, SOX-9, and parathyroid hormone related protein (PTHrP) mRNA levels in chondrocytes, but an increase of levels of osteoblast-stimulating factor-1 (OSF-1), matrix metalloproteinase-3 (MMP-3), and MMP-13^{5,10}.

In Europe, the unsaponifiable elements of avocado (A) and soybean (S) oils mixed in a ratio 1:2 (ASU, Piasclédine, Laboratoires Expanscience, Courbevoie, France) are commonly used in humans to treat OA. Clinical studies have shown the beneficial effects of ASU on symptoms of hip and knee OA^{11,12}. Further, a 2-year placebo controlled trial reported evidence of reduced joint space narrowing (JSN) in OA of the hip in a population treated with ASU, but only in the subgroup of patients with severe JSN (under the median value of joint space width) at baseline¹³. *In vitro*, ASU stimulates matrix component synthesis, and partially counteracts the inhibitory effect of IL-1, possibly via the production of TGF- β , an important family of growth factors in terms of cartilage homeostasis^{14,15}. ASU is also a potent inhibitor of the production by human OA chondrocytes of proinflammatory mediators, including IL-6, IL-8, macrophage inflammatory protein-1 β , nitric oxide (NO), and prostaglandin E₂¹⁵.

We demonstrate that ASU prevent the inhibition of matrix molecule production by sclerotic subchondral osteoblasts, suggesting a new mechanism of action for this drug.

MATERIALS AND METHODS

Subchondral osteoarthritic osteoblasts in monolayer culture. Tibial and femoral subchondral bone plates were from the knees of cadavers (3 men, ages 48, 57, and 65 yrs) with idiopathic OA obtained immediately after death. All tissues used in this study were obtained after University of Liège, Medicine Faculty Ethics Committee approval (reference number 2005/8). After careful elimination of trabecular bone and articular cartilage, OA subchondral bone was dissected under a microscope to separate nonsclerotic (NSC) from SC zones. NSC and SC zones were characterized by a marked difference in their thickness. We considered as SC bone only the subchondral bone zones with a thickness > 2 mm and either denuded or overlaid by fibrillated cartilage. Also, we considered as NSC bone only the subchondral bone zones with a maximal thickness of 1 mm. Intermediate zones of the subchondral bone plate were discarded. Osteoblasts from SC or NSC subchondral bone were then obtained by outgrowth from explants as described^{5,10}. At confluence, cells were collected by trypsinization, seeded (50,000 cells/cm²) in 12-well plates (12-well companion plates, Falcon, BD Biosciences, Erembodegem, Belgium), and grown for 3 days in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U/ml

penicillin, 100 μ g/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 μ g/ml streptomycin, 10 mM HEPES, 2% Ultrosor G, a serum substitute, 10⁻⁸ M 1,25(OH)₂ vitamin D₃ (Sigma-Aldrich, Bornem, Belgium), 50 μ g/ml ascorbic acid (Sigma-Aldrich), and 20 μ g/ml proline (Invitrogen, Merelbeke, Belgium). At the end of this differentiation period, cells showed an osteoblastic phenotype characterized by the production of OC and ALP. After washings, cells were cultured for 72 h in the absence or presence of ASU (one part avocado unsaponifiable and 2 parts soybean unsaponifiable) at the recommended and currently used *in vitro* concentration of 10 μ g/ml^{14,15}. ASU composition has been described¹⁴. ASU was first dissolved in ethanol and then diluted in incubation medium to the required final concentration. The final concentration of ethanol was 0.1% in all culture conditions. 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 μ g/ml streptomycin, 50 μ g/ml ascorbic acid (Sigma-Aldrich), 20 μ g/ml proline (Invitrogen). ITS+ is a premixed cell growth system containing in 1 ml: 0.625 mg insulin, 0.625 mg transferrin, 0.625 μ g selenious acid, 0.125 g bovine serum albumin, and 0.535 mg linoleic acid.

Chondrocyte culture in alginate beads. Cartilage was from the knees of cadavers (3 men ages 50, 52, and 63 yrs) with idiopathic OA obtained immediately 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 our scale¹⁶. The severity of pitting was recorded for each donor. Four different grades were considered: 0, normal white cartilage in all areas examined; I, presence of a yellow-grey 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 specimens showing OA cartilage lesions of grade II or III. Cartilage was cut into small fragments and then subjected to enzymatic digestion sequentially with hyaluronidase, pronase, and collagenase (3 g cartilage per 10 ml of enzyme solution) as described¹⁷. The cells were filtered through a nylon mesh with a pore diameter of 70 μ m, and then washed 3 times with sterile water 0.9% NaCl. Cell viability was estimated by trypan blue exclusion test and in all cases was superior to 95%. Chondrocytes were suspended in 1.2% low viscosity alginate (Sigma-Aldrich) in 0.9% NaCl solution at a density of 4 \times 10⁶ cells/ml, which was slowly passed through a 25 gauge needle in a dropwise fashion into 102 mM CaCl₂ solution (Sigma-Aldrich). After instantaneous gelation, the beads were allowed to polymerize further for 10 min in the CaCl₂ solution. Ten alginate beads containing OA chondrocytes were placed in porous inserts (with 1 μ m pore size; Falcon, BD Biosciences). The chondrocytes in alginate beads were maintained 2 days in DMEM supplemented with 2% Ultrosor G, 10 mM HEPES, 100 U/ml penicillin, and 100 μ g/ml streptomycin, as a precaution to avoid contamination with drugs that donors might have taken before death. After this washout period, culture medium was changed and chondrocytes were placed in co-culture with NSC or SC subchondral osteoblasts or with normal skin fibroblasts in monolayer. Normal skin fibroblasts obtained by outgrowth from explants as described¹⁸ were provided by Lambert Ch. (Liège, Belgium).

OA osteoblast/chondrocyte co-culture. The inserts (with a pore size of 1 μ m; BD Biosciences) containing 10 alginate beads were co-cultured for 4 days with osteoblasts in monolayer preincubated or not with ASU. Before co-culture, osteoblasts were extensively washed with phosphate buffered saline (PBS). Co-culture medium was DMEM supplemented with 1% ITS+, 10 mM HEPES, 100 U/ml penicillin, 100 μ g/ml streptomycin, 50 μ g/ml ascorbic acid, 20 μ g/ml proline. Six wells of a 12-well plate were used for each culture condition. The experiment was repeated 3 times, each time using subchondral osteoblasts coming from a different donor. Conversely, the OA chondrocytes selected for each experiment were not from the same donor as the osteoblasts. As controls, OA chondrocytes in alginate beads were cultured alone (mono-

culture) or together with normal skin fibroblasts in monolayer. At the end of the co-culture period, cell conditioned culture medium (S) was carefully collected and kept at -20°C until analysis. The alginate beads were dissolved in 1 ml 0.1 M citrate for 10 min. The resulting suspension was centrifuged at 1200 rpm for 10 min. By this method, 2 fractions were collected: the supernatant, so-called further-removed matrix (FRM), containing the majority of the matrix macromolecules, 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). The cell pellets of chondrocytes or osteoblasts were washed 3 times with PBS and then either homogenized in 1 ml PBS by ultrasonic dissociation for DNA and aggrecan 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 Paigen¹⁹.

Immunoassays for aggrecan, OC, IL-1 β , IL-6, and TGF- β 1. OC, IL-1 β , IL-6, and TGF- β 1 were directly measured in osteoblast-conditioned culture supernatant by specific EASIA (enzyme amplified sensitivity immunoassays; Biosource Europe, Fleurus, Belgium). Aggrecan was measured in the different compartments of alginate bead and in culture supernatants by a specific EASIA. The limit of detection of these immunoassays was 5.5 ng/ml for aggrecan, 1.9 ng/ml for OC, 10 pg/ml for IL-1 β , 8 pg/ml for IL-6, and 30 pg/ml for TGF- β 1. The intra- and interassay coefficients of variation were less than 5% for all immunoassays.

Alkaline phosphatase assay. ALP activity was quantified in the cellular fraction of the osteoblast culture. Fifty microliters of cell extract were incubated with 100 μl of p-nitrophenylphosphate (liquid p-NPP, ready to use, KEM-EN-TEC, Copenhagen, Denmark). In the presence of ALP, p-NPP is transformed to p-nitrophenol and inorganic phosphate; p-nitrophenol absorbance is measured at 405 nm. 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.

Immunoassay for PTHrP. PTHrP was directly measured in osteoblast-conditioned culture supernatant by a specific IRMA kit (immunoradiometric assay; Diagnostic Systems Laboratories, Oxon, UK). In this assay, a first antibody is coated and the second is ^{125}I labelled. Sample incubation was performed overnight at ambient temperature under constant agitation (180 rpm), rinsed with demineralized water, and counted. The limit of detection of this immunoassay was 3 pg/ml.

Quantitative real-time reverse transcription-polymerase chain reaction. RNA from 3×10^6 cells was isolated by SV total RNA isolation system (Promega) and polymerase chain reaction (PCR) was performed using the Light Cycler-FastStart DNA Master Sybr Green I (Roche Diagnostics, Brussels, Belgium) as described⁵. The PCR template source was either 3 ng first-strand cDNA or purified DNA standard. Primer sequences used to amplify the desired cDNA were as shown in Table 1. To standardize mRNA levels, we amplified glycer-

aldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, as an internal control. Gene expression was normalized by calculating the ratio between the number of copies of GAPDH and of the following: cDNA copies of aggrecan, type II collagen (COL2A1), MMP-3, MMP-13, tissue inhibitor of metalloproteinase (TIMP-1), TGF- β 1 and TGF- β 3, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2).

Calculation and statistical analysis. The results (mean \pm SEM) were expressed as GAPDH-normalized gene expression or as the concentration of aggrecan per μg DNA. Total aggrecan production corresponded to the sum of aggrecan found in the supernatant, CM and FRM. A nonparametric Mann-Whitney U test was performed on all the experiments.

RESULTS

In comparison with NSC, SC subchondral osteoblasts produced higher amounts of ALP (+ 95%), OC (+ 98%), IL-6 (+ 134%), and TGF- β 1 (+ 41%), whereas PTHrP was decreased (-35%), and IL-1 β concentration was similar (Table 2). These findings indicate that SC osteoblasts had an altered phenotype. Aside from inhibition of IL-1 β production ($p < 0.001$), ASU (10 $\mu\text{g}/\text{ml}$) had no effect on NSC osteoblasts. In contrast, ASU significantly decreased ALP, OC, and TGF- β 1 synthesis by SC osteoblasts ($p < 0.01$), but had no effect on PTHrP, IL-1 β , or IL-6 production.

We then cultured NSC 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, $3.5 \pm 0.8 \mu\text{g}$ of DNA were measured in alginate beads, osteoblasts, and fibroblast 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 numbers remained stable over the co-culture period (data not shown).

When they were cultured alone (monoculture), human OA chondrocytes in alginate beads spontaneously produced 8.5 μg of aggrecan per μg of DNA after 4 days, and 14.2 μg after 10 days. As previously described, newly synthesized aggrecan was essentially accumulated in alginate bead²⁰. 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-

Table 1. Sequences of primers for the quantitative PCR experiments.

mRNA	Forward (5'-3')	Reverse (5'-3')
GAPDH	TTG GTA TCG TGG AAG GAC TCA	TGT CAT CAT ATT TGG CAG GTT T
Aggrecan	GCA CCA TGC CTT CTG CTT CCG AG	CTC CAC TGC CTG TGA AGT CAC CAC
COL2A1	TGC TGC CCA GAT GGC TGG AGG A	TGC CTT GAA ATC CTT GAG GCC C
MMP-3	ATG AGG TAC GAG CTG G	TCA CGC TCA AGT TCC C
MMP-13	CAA CGG ACC CAT ACA G	ACA GAC CAT GTG TCC C
TIMP-1	TTC CGA CCT CGT CAT CAG	TGA GAA ACT CCT CGC T
TGF- β 1	ATC TAT GAC AAG TTC AAG CAG AGT	ACC ACT GCC GCA CAA CTC CGG TGA
TGF- β 3	AAG TGG GTC CAT GAA CCT AA	GCT ACA TTT ACA AGA CTT CAC
iNOS	CCA TGG AAC ATC CCA AAT AC	TCT GCA TGT ACT TCA TGA AGG
COX-2	TTC AAA TGA GAT TGT GGG AAA A	AGA TCA TCT CTG CCT GAG TAT CTT

Table 2. Phenotypic characteristics of subchondral osteoblasts. Production of alkaline phosphatase (ALP), osteocalcin (OC), parathyroid hormone related protein (PTHrP), interleukin 1 β (IL-1 β), IL-6, and transforming growth factor (TGF)- β 1 by nonsclerotic (NSC) or sclerotic (SC) subchondral osteoblasts cultured 72 h with or without 10 μ g/ml ASU. Values are means \pm SEM (n = 9) of 3 independent cultures with subchondral osteoblasts from 3 different donors. Statistical significance between NSC and SC subchondral osteoblasts *** p < 0.001; between ASU treated group and controls ## p < 0.01 and ### p < 0.001.

	Control	ASU (10 μ g/ml)
ALP, nmol/ μ g DNA* min		
NSC	9.1 \pm 0.6	9.2 \pm 1.1
SC	17.8 \pm 0.6***	13.1 \pm 0.3##
OC, pg/ μ g DNA		
NSC	6,808 \pm 302	6,013 \pm 854
SC	13,507 \pm 411***	9,154 \pm 724##
PTHrP, pg/ μ g DNA		
NSC	31.2 \pm 2.1	33.5 \pm 2.4
SC	20.3 \pm 1.6***	22.2 \pm 1.8
IL-1 β , pg/ μ g DNA		
NSC	4.4 \pm 0.6	2.7 \pm 0.1###
SC	4.5 \pm 0.7	4.2 \pm 0.5
IL-6, pg/ μ g DNA		
NSC	595 \pm 35	533 \pm 19
SC	1397 \pm 114***	1339 \pm 170
TGF- β 1, pg/ μ g DNA		
NSC	37.5 \pm 0.5	34.5 \pm 2.8
SC	52.9 \pm 3.4***	39.1 \pm 3.3##

cultured with SC osteoblasts, aggrecan content in alginate bead was decreased by 27% \pm 7% and by 31% \pm 5% compared to monoculture after 4 or 10 days, respectively (p < 0.001), whereas NSC osteoblasts or normal skin fibroblasts did not significantly modify aggrecan content (Figure 1). The decrease of aggrecan content was significantly more marked in the CM compartment of alginate beads than in FRM. After 4 days, the aggrecan content was decreased by 45% \pm 8% in CM (p < 0.01), but only by 20% \pm 7% in FRM (p < 0.05). In co-culture, ASU (10 μ g/ml) completely reversed osteoblast-induced inhibition of aggrecan production (Figure 1).

After 4 days of culture in alginate beads (monoculture), OA chondrocytes in alginate beads spontaneously expressed 22 copies of aggrecan and 1.8 copies of type II collagen (COL2A1) per 1000 copies of GAPDH. After 10 days, the expression of these genes was strongly decreased, and chondrocytes expressed only 10.1 aggrecan copies and 0.45 COL2A1 copy per 1000 GAPDH copies. When they were co-cultured with osteoblasts, aggrecan mRNA levels in chondrocytes were significantly reduced by SC (p < 0.001) but not by NSC osteoblasts (Figure 2). On the other hand, NSC and SC osteoblasts inhibited COL2A1 gene expression by chondrocytes (p < 0.001; Figure 3), but the effect was significantly greater with SC osteoblasts (p < 0.001; Figure 3). After 4 days of co-culture, ASU (10 μ g/ml) fully prevented osteoblast-induced inhibition of aggrecan and COL2A1 gene expression, and even raised type II collagen expression level over the

monoculture level (Figures 2A and 3A). After 10 days of incubation, ASU prevented osteoblast-induced inhibition of aggrecan core protein production, but not aggrecan gene expression (Figures 1B and 2B).

After 4 days of culture, OA chondrocytes in alginate beads spontaneously expressed 1400 copies of MMP-3 and 5 copies of MMP-13 per 1000 copies of GAPDH. After 10 days in alginate beads, MMP-3 and MMP-13 gene expressions fell to 822 copies and 4 copies per 1000 copies of GAPDH, respectively. After 4 days of co-culture with SC osteoblasts, MMP-3 and MMP-13 mRNA levels in chondrocytes were significantly increased by 1.65 and 2 times, respectively (p < 0.001). In comparison, NSC osteoblasts or skin fibroblasts did not modify the level of MMP-3 mRNA, while NSC osteoblasts decreased the level of MMP-13 mRNA (p < 0.01). In co-culture, pretreatment of NSC and SC osteoblasts with ASU did not significantly modify MMP-3 or MMP-13 mRNA levels in chondrocytes (Figures 4 and 5).

After 4 days in monoculture, OA chondrocytes in alginate beads spontaneously expressed 417 copies of TIMP-1, 1.8 copies of TGF- β 1, 0.7 copy of TGF- β 3, 2.8 copies of COX-2, and 90 copies of iNOS per 1000 copies of GAPDH. After 10 days, chondrocytes expressed 256 copies of TIMP-1, 1.6 copies of TGF- β 1, 1.2 copies of TGF- β 3, 3.1 copies of COX-2, and 34 copies of iNOS per 1000 copies of GAPDH. The expression of these genes was not modified in co-culture with SC or NSC osteoblasts. In co-culture, the pretreatment of osteoblasts with ASU did not influence the expression of these genes (data not shown).

DISCUSSION

Although OA has long been considered primarily a cartilage disorder associated with focal articular cartilage degradation, this disease is accompanied by well defined changes in intra- and periarticular tissues, including subchondral bone sclerosis. The abnormal remodelling of the subchondral bone plate that is exposed to excessive nonphysiologic mechanical loads results in stiffer bone that is no longer an effective shock absorber. This increases mechanical strain in the overlying cartilage and can accelerate its damage over time. Further, epidemiologic studies have clearly documented increased subchondral bone with disease progression²¹. Recently, it was reported that alendronate, a potent inhibitor of bone resorption, had a potential structure-modifying effect as observed in a study performed on rats, protecting cartilage deterioration and preventing osteophyte formation²². These observations suggest a role for subchondral bone changes in OA, supporting the concept that intervention that reduces bone sclerosis might slow progressive cartilage degradation. Since it has been demonstrated that osteoblasts from sclerotic subchondral bone (SC osteoblasts) showed an altered phenotype⁵, they constitute a potential therapeutic target for drugs used in the treatment of OA. We observed that SC osteoblasts secreted more ALP, OC, IL-6, and TGF- β 1 and less PTHrP than NSC

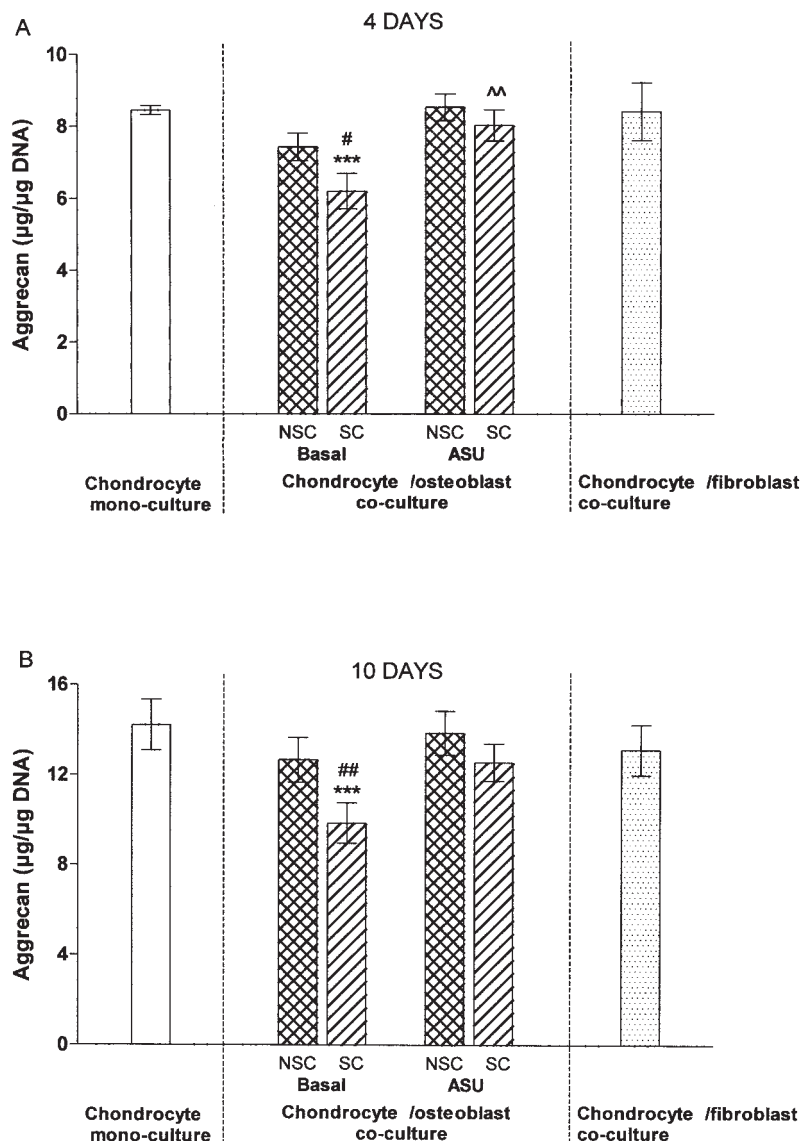


Figure 1. Aggrecan content in alginate beads after 4 days (A) or 10 days (B) of culture in the absence (monoculture) or presence (co-culture) of osteoblasts from nonsclerotic (NSC) or sclerotic (SC) subchondral bone zones. Before co-culture, osteoblasts were incubated or not (basal) for 72 h with ASU (10 µg/ml). As a control, OA chondrocytes in alginate beads were also co-cultured with normal human skin fibroblasts. Results are expressed as mean ± SEM of 3 experiments performed with cells from 3 different donors. Comparison of mean values of total aggrecan production was by Mann-Whitney U test. Chondrocyte/osteoblast co-culture compared to monoculture ***p < 0.001; pretreated osteoblasts compared to controls ^^p < 0.01; SC compared to NSC osteoblasts #p < 0.05 and ##p < 0.01.

cells⁵, corroborating results of previous work^{3,4,23}. This finding indicates that SC subchondral osteoblasts used in this study show an altered phenotype compared to NSC cells and that they secrete high levels of mediators such as TGF-β1 and IL-6 involved in subchondral bone sclerosis^{5,22,24,25}. For the first time, we also demonstrate that ASU, used in Europe to treat OA, inhibit ALP and OC synthesis by SC osteoblasts, 2 markers of new bone formation, and decrease the synthesis of TGF-β1 by osteoblasts, a growth factor postulated to be one of

the key regulators of local bone formation²⁶. TGF-β1 is synthesized locally in the bone tissue and presumed to act by autocrine/paracrine stimulation of proliferation of the osteoblastic precursors and production of bone matrix molecules²⁷⁻²⁹. Altogether, these observations indicate that ASU might be effective on SC subchondral osteoblasts and favor a return to bone homeostasis. These results suggest that ASU could have beneficial effects on subchondral bone sclerosis.

In previous work¹⁵, we reported that ASU stimulates TGF-

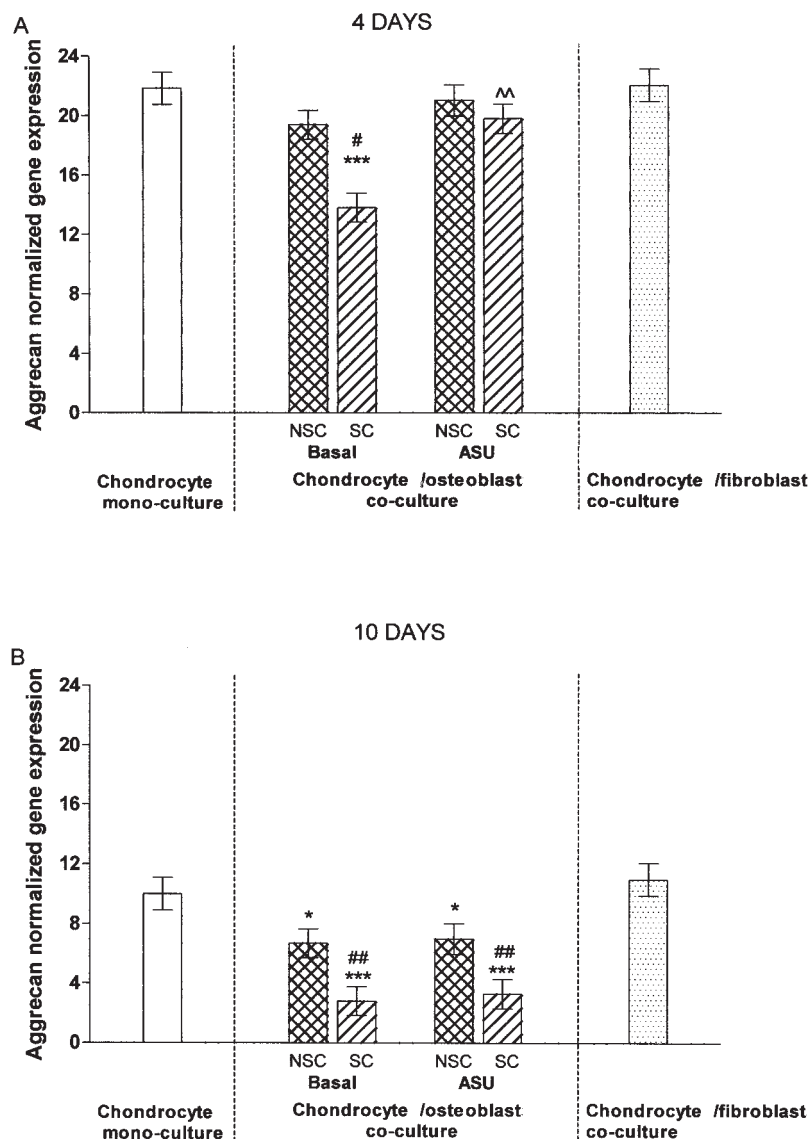


Figure 2. Aggrecan gene expression by human OA chondrocytes in alginate beads after 4 days (A) or 10 days (B) of culture in the absence (monoculture) or presence (co-culture) of osteoblasts from nonsclerotic (NSC) or sclerotic (SC) subchondral bone zones. Before co-culture, osteoblasts were incubated or not (basal) for 72 h with ASU (10 μ g/ml). As a control, OA chondrocytes in alginate beads were also co-cultured with normal human skin fibroblasts. The mRNA copy numbers were normalized against the corresponding copy number of GAPDH mRNA. Comparison of mean values was by Mann-Whitney U test. Chondrocyte/osteoblast co-culture compared to monoculture * $p < 0.05$ and *** $p < 0.001$; pretreated osteoblasts compared to controls ^ $p < 0.01$; SC compared to NSC osteoblasts # $p < 0.05$ and ## $p < 0.01$.

β 1 production by human OA chondrocytes. In our present study we have demonstrated that ASU inhibit TGF- β 1 synthesis by human SC osteoblasts. As ASU contain multiple active compounds that target multiple pathways, we can speculate that the spectrum of compounds active on chondrocytes is different from that active on osteoblasts. Therefore, we can hypothesize that different regulatory pathways of TGF- β 1 synthesis are activated in chondrocytes and in osteoblasts,

leading to different effects of ASU on chondrocytes and osteoblasts.

Since the presence of tidemark microcracks that appear early in OA⁶ have been identified in OA cartilage, we can speculate that soluble mediators produced by SC osteoblasts may modulate chondrocyte metabolism and contribute to cartilage degradation. To verify this hypothesis, we developed an original model of culture in which SC osteoblasts and OA

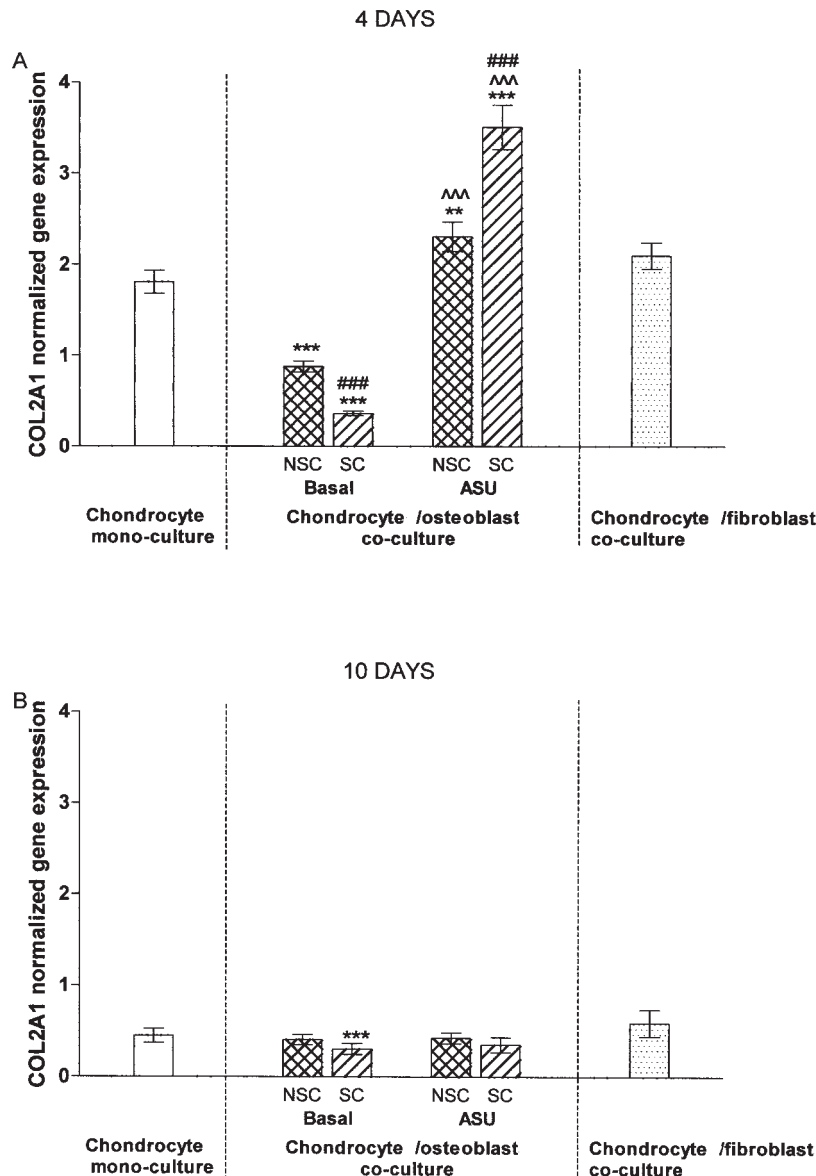


Figure 3. Type II collagen (COL2A1) gene expression by human OA chondrocytes in alginate beads after 4 days (A) or 10 days (B) of culture in the absence (monoculture) or presence (co-culture) of osteoblasts from nonsclerotic (NSC) or sclerotic (SC) subchondral bone zones. Before co-culture, osteoblasts were incubated or not (basal) for 72 h with ASU (10 μ g/ml). As a control, OA chondrocytes in alginate beads were also co-cultured with normal human skin fibroblasts. The mRNA copy numbers were normalized against the corresponding copy number of GAPDH mRNA. Comparison of mean values was by Mann-Whitney U test. Chondrocyte/osteoblast co-culture compared to monoculture ** $p < 0.01$ and *** $p < 0.001$; pretreated osteoblasts compared to controls ^^^ $p < 0.001$; SC compared to NSC osteoblasts ### $p < 0.001$.

chondrocytes are cultured in the same environment, but do not come into contact. The originality of our model lies in the fact that we use osteoblasts from the sclerotic subchondral bone, and OA chondrocytes are cultured in alginate beads. Westacott, *et al* demonstrated that OA trabecular osteoblasts in monolayer degraded cartilage explants in about half of the cases⁹. A key element in cartilage degradation is an increase in MMP activity. Interestingly, we observed that SC

osteoblasts induced a strong elevation of MMP-3 and MMP-13 synthesis by chondrocytes, whereas NSC osteoblasts or normal skin fibroblasts had no effect⁵. This observation indicates that osteoblast-induced cartilage degradation is related to its particular SC phenotype. In addition to this stimulating effect of SC osteoblasts on MMP synthesis, we also observed that aggrecan content in alginate beads decreased when chondrocytes were co-cultured with SC osteoblasts⁵. Again, this

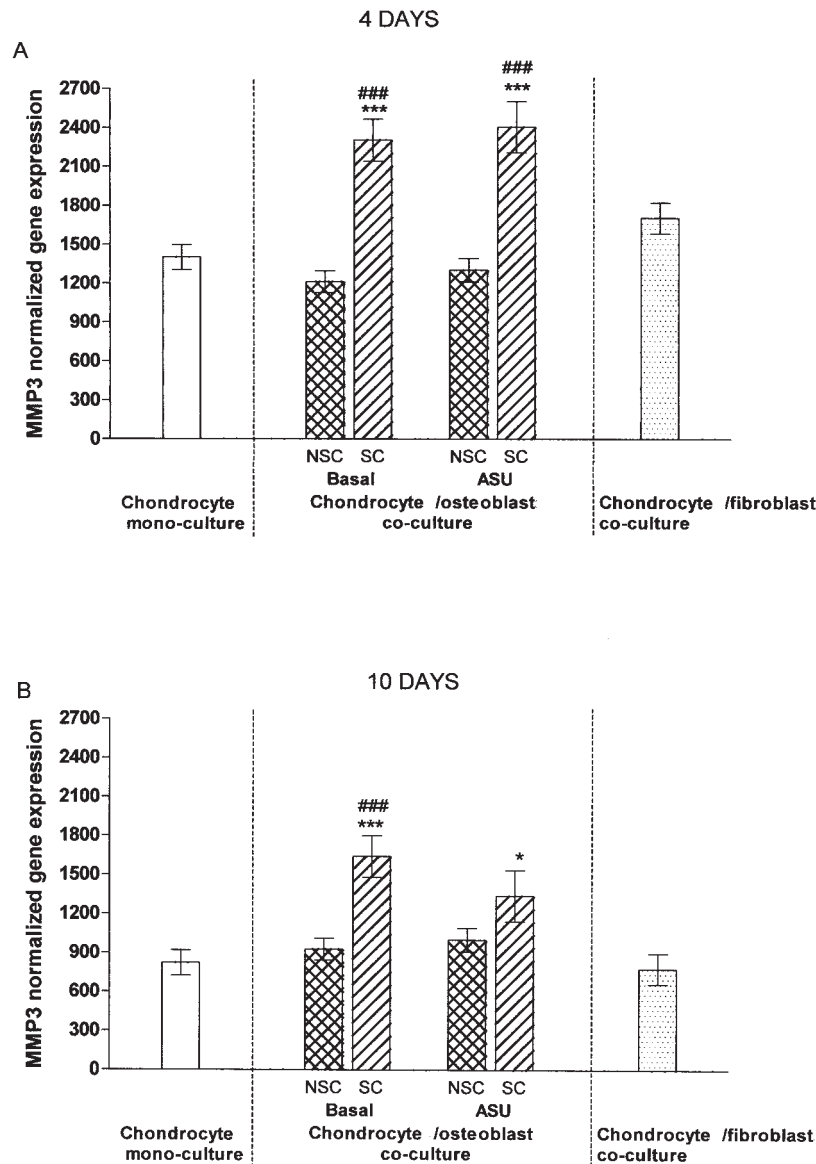


Figure 4. MMP-3 gene expression by human OA chondrocytes in alginate beads after 4 days (A) or 10 days (B) of culture in the absence (monoculture) or presence (co-culture) of osteoblasts from nonsclerotic (NSC) or sclerotic (SC) subchondral bone zones. Before co-culture, osteoblasts were incubated or not (basal) for 72 h with ASU (10 μ g/ml). As a control, OA chondrocytes in alginate beads were also co-cultured with normal human skin fibroblasts. The mRNA copy numbers were normalized against the corresponding copy number of GAPDH mRNA. Comparison of mean values was by Mann-Whitney U test. Chondrocyte/osteoblast co-culture compared to monoculture * $p < 0.05$ and *** $p < 0.001$; SC compared to NSC osteoblasts ### $p < 0.001$.

effect seems to be related to SC phenotype of osteoblasts as suggested by the absence of effect of NSC osteoblasts and fibroblasts. SC osteoblasts also induce a decrease of aggrecan mRNA levels in chondrocytes, suggesting that the decrease of aggrecan content results from a decrease of aggrecan synthesis. In parallel, SC osteoblasts decrease type II collagen (A1) gene expression by OA chondrocytes¹⁰. Together, these findings indicate that SC phenotype of osteoblasts induces a marked dysregulation of chondrocyte metabolism, character-

ized by a decrease of matrix component synthesis and an increase of MMP production. This imbalance between anabolic and catabolic factors could lead to cartilage matrix depletion.

SC subchondral bone is now recognized to play a major role in OA development and is considered a potential therapeutic target. Previously, we reported that ASU stimulated aggrecan synthesis and decreased MMP-3 production by OA human chondrocytes in alginate beads¹⁵. This effect was sus-

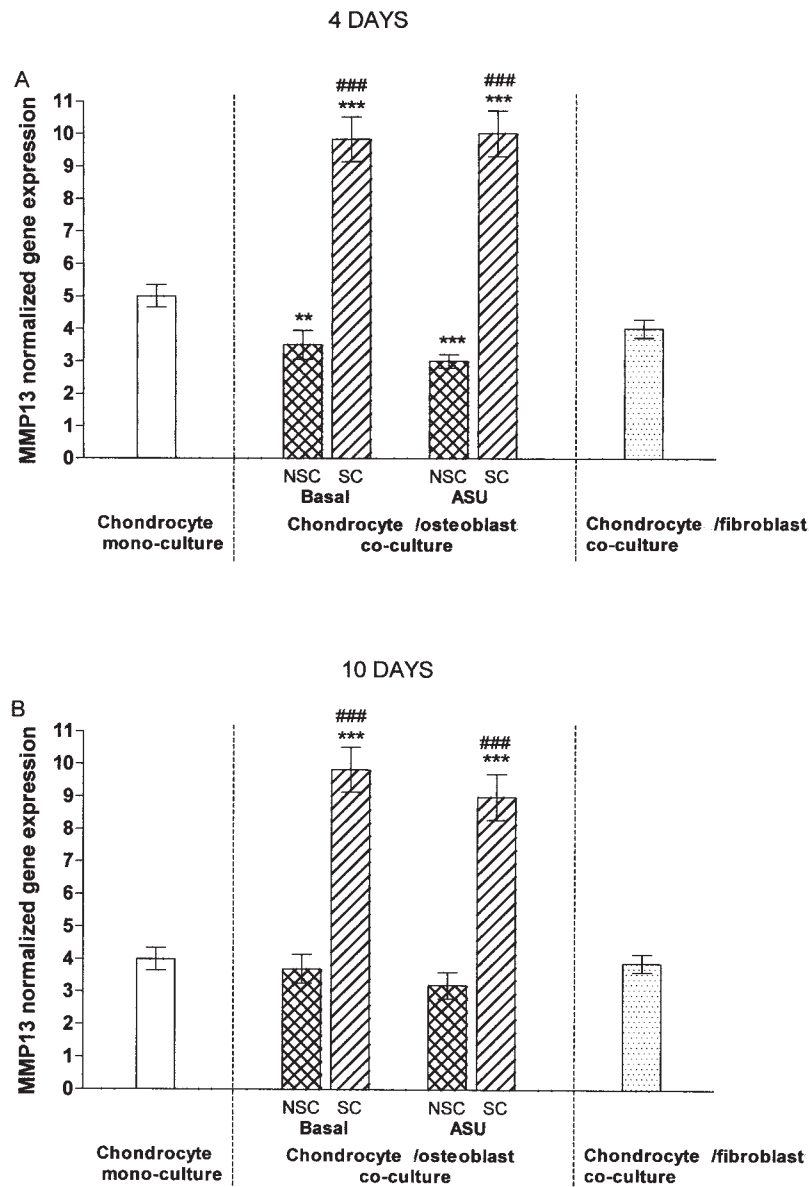


Figure 5. MMP-13 gene expression by human OA chondrocytes in alginate beads after 4 days (A) or 10 days (B) of culture in the absence (monoculture) or presence (co-culture) of osteoblasts from nonsclerotic (NSC) or sclerotic (SC) subchondral bone zones. Before co-culture, osteoblasts were incubated or not (basal) for 72 h with ASU (10 μ g/ml). As a control, OA chondrocytes in alginate beads were also co-cultured with normal human skin fibroblasts. The mRNA copy numbers were normalized against the corresponding copy number of GAPDH mRNA. Comparison of mean values was by Mann-Whitney U test. Chondrocyte/osteoblast co-culture compared to monoculture *** $p < 0.001$; SC compared to NSC osteoblasts ### $p < 0.001$.

pected to be secondary to TGF- β secretion, since TGF- β 1 and TGF- β 2 expression have been reported to be upregulated by ASU in bovine chondrocyte cultured in monolayer and human chondrocytes in alginate beads^{14,15}. In that study, we observed that ASU prevented the inhibitory effect of SC osteoblasts on aggrecan synthesis but had no significant effect on MMP, TIMP-1, COX-2, or iNOS expressions. This finding demonstrates that ASU may protect cartilage by acting at the sub-

chondral bone level and suggests a new mechanism of action for this potential structure-modifying drug. As ASU decrease TGF- β 1 production by SC osteoblasts, we can exclude that this factor is involved in the ASU effect. Indeed, TGF- β 1 is largely described as a stimulating factor for aggrecan synthesis in chondrocyte culture³⁰. Nevertheless, other mechanisms are not excluded. Besides the effect on TGF- β production, ASU could also upregulate other TGF- β or other TGF- β

superfamily members as bone morphogenetic protein (BMP). Indeed, many BMP (BMP-2, -4, -6, -7, -9) have been shown to increase aggrecan and type II collagen production by human chondrocytes³¹⁻³³. Further investigations need to be conducted to clarify the mechanism of action of ASU.

In summary, our study shows that SC subchondral osteoblasts show an altered phenotype characterized by an increased production of OC, ALP, TGF- β 1, and IL-6. ASU, a drug commonly used in Europe to treat symptomatic OA, downregulates the production of ALP, OC, and TGF- β 1 by human SC osteoblasts, suggesting that this drug could promote the return to subchondral bone homeostasis. Further, we have demonstrated that osteoblasts from OA sclerotic subchondral bone induce a decrease of aggrecan synthesis and an increase of metalloproteinase synthesis by human OA chondrocytes, a mechanism that may contribute to cartilage degradation in OA. This OA osteoblast-induced imbalance between anabolic and catabolic processes in cartilage can be prevented by ASU. These effects of ASU on osteoblasts suggest a new mechanism of action for this drug, known for its action on cartilage, and may explain its therapeutic effect, although we are aware that the effects of ASU on osteoblasts cannot be responsible for the full spectrum of beneficial effects observed in clinical studies.

ACKNOWLEDGMENT

The authors extend their appreciation to Elisabeth Kaut, Marianne Mathy-Hartert, Paul Simonis, and Murielle Lemestre for their skilful technical assistance.

REFERENCES

- Grynpas MD, Alpert B, Katz I, Lieberman I, Pritzker KP. Subchondral bone in osteoarthritis. *Calcif Tissue Int* 1991;49:20-6.
- Hilal G, Martel-Pelletier J, Pelletier JP, Ranger P, Lajeunesse D. Osteoblast-like cells from human subchondral osteoarthritic bone demonstrate an altered phenotype in vitro: possible role in subchondral bone sclerosis. *Arthritis Rheum* 1998;41:891-9.
- Hilal G, Massicotte F, Martel-Pelletier J, Fernandes JC, Pelletier JP, Lajeunesse D. Endogenous prostaglandin E2 and insulin-like growth factor 1 can modulate the levels of parathyroid hormone receptor in human osteoarthritic osteoblasts. *J Bone Miner Res* 2001;16:713-21.
- Hilal G, Martel-Pelletier J, Pelletier JP, Duval N, Lajeunesse D. Abnormal regulation of urokinase plasminogen activator by insulin-like growth factor 1 in human osteoarthritic subchondral osteoblasts. *Arthritis Rheum* 1999;42:2112-22.
- Sanchez C, Deberg MA, Piccardi N, Msika P, Reginster JY, Henrotin Y. Osteoblasts from the sclerotic subchondral bone downregulate aggrecan but upregulate metalloproteinases expression by chondrocytes. This effect is mimicked by interleukin-6, -1b and oncostatin M pre-treated non sclerotic osteoblasts. *Osteoarthritis Cartilage* 2005;13:979-87.
- Sokoloff L. Microcracks in the calcified layer of articular cartilage. *Arch Pathol Lab Med* 1993;117:191-5.
- Imhof H, Breitenscher M, Kainberger F, Rand T, Trattnig S. Importance of subchondral bone to articular cartilage in health and disease. *Top Magn Reson Imaging* 1999;10:180-92.
- Malinin T, Ouellette EA. Articular cartilage nutrition is mediated by subchondral bone: a long-term autograft study in baboons. *Osteoarthritis Cartilage* 2000;8:483-91.
- Westacott CI, Webb GR, Warnock MG, Sims JV, Elson CJ. Alteration of cartilage metabolism by cells from osteoarthritic bone. *Arthritis Rheum* 1997;40:1282-91.
- Sanchez C, Deberg MA, Piccardi N, Msika P, Reginster JY, Henrotin Y. Subchondral bone osteoblasts induce phenotypic changes in human osteoarthritic chondrocytes. *Osteoarthritis Cartilage* 2005;13:988-97.
- Maheu E, Mazieres B, Valat JP, et al. Symptomatic efficacy of avocado/soybean unsaponifiables in the treatment of osteoarthritis of the knee and hip: a prospective, randomized, double-blind, placebo-controlled, multicenter clinical trial with a six-month treatment period and a two-month followup demonstrating a persistent effect. *Arthritis Rheum* 1998;41:81-91.
- Appelboom T, Schuermans J, Verbruggen G, Henrotin Y, Reginster JY. Symptoms modifying effect of avocado/soybean unsaponifiables (ASU) in knee osteoarthritis. A double blind, prospective, placebo-controlled study. *Scand J Rheumatol* 2001;30:242-7.
- Lequesne M, Maheu E, Cadet C, Dreiser RL. Structural effect of avocado/soybean unsaponifiables on joint space loss in osteoarthritis of the hip. *Arthritis Rheum* 2002;47:50-8.
- Boumediene K, Felisaz N, Bogdanowicz P, Galera P, Guillou GB, Pujol JP. Avocado/soya unsaponifiables enhance the expression of transforming growth factor beta 1 and beta 2 in cultured articular chondrocytes. *Arthritis Rheum* 1999;42:148-56.
- Henrotin YE, Sanchez C, Deberg MA, et al. Avocado/soybean unsaponifiables increase aggrecan synthesis and reduce catabolic and proinflammatory mediator production by human osteoarthritic chondrocytes. *J Rheumatol* 2003;30:1825-34.
- Henrotin Y, Labasse A, Zheng SX, et al. Strontium ranelate increases cartilage matrix formation. *J Bone Miner Res* 2001;16:299-308.
- Sanchez C, Mateus MM, Defresne MP, Crielaard JM, Reginster JY, Henrotin YE. Metabolism of human articular chondrocytes cultured in alginate beads. Longterm effects of interleukin 1 beta and nonsteroidal antiinflammatory drugs. *J Rheumatol* 2002;29:772-82.
- Delvoe P, Wiliquet P, Leveque JL, Nussgens BV, Lapiere CM. Measurement of mechanical forces generated by skin fibroblasts embedded in a three-dimensional collagen gel. *J Invest Dermatol* 1991;97:898-902.
- Labarca C, Paigen K. A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* 1980;102:344-52.
- Sanchez C, Mathy-Hartert M, Deberg MA, Fichoux H, Reginster JY, Henrotin YE. Effects of rhein on human articular chondrocytes in alginate beads. *Biochem Pharmacol* 2003;65:377-88.
- Lane NE, Nevitt MC. Osteoarthritis, bone mass, and fractures: how are they related? *Arthritis Rheum* 2002;46:1-4.
- Hayami T, Pickarski M, Wesolowski GA, et al. The role of subchondral bone remodeling in osteoarthritis: reduction of cartilage degeneration and prevention of osteophyte formation by alendronate in the rat anterior cruciate ligament transection model. *Arthritis Rheum* 2004;50:1193-206.
- Massicotte F, Lajeunesse D, Benderdour M, et al. Can altered production of interleukin-1 beta, interleukin-6, transforming growth factor-beta and prostaglandin E(2) by isolated human subchondral osteoblasts identify two subgroups of osteoarthritic patients. *Osteoarthritis Cartilage* 2002;10:491-500.
- van Beuningen HM, van der Kraan PM, Arntz OJ, van den Berg WB. Transforming growth factor-beta 1 stimulates articular chondrocyte proteoglycan synthesis and induces osteophyte formation in the murine knee joint. *Lab Invest* 1994;71:279-90.
- Tardif G, Reboul P, Dupuis M, et al. Transforming growth factor-beta induced collagenase-3 production in human osteoarthritic chondrocytes is triggered by Smad proteins: cooperation between activator protein-1 and PEA-3 binding sites. *J Rheumatol* 2001;28:1631-9.

26. Kassem M, Kveiborg M, Eriksen EF. Production and action of transforming growth factor-beta in human osteoblast cultures: dependence on cell differentiation and modulation by calcitriol. *Eur J Clin Invest* 2000;30:429-37.
27. Rickard DJ, Gowen M, MacDonald BR. Proliferative responses to estradiol, IL-1 alpha and TGF beta by cells expressing alkaline phosphatase in human osteoblast-like cell cultures. *Calcif Tissue Int* 1993;52:227-33.
28. Robey PG, Termine JD. Human bone cells in vitro. *Calcif Tissue Int* 1985;37:453-60.
29. Ong JL, Carnes DL, Sogal A. Effect of transforming growth factor-beta on osteoblast cells cultured on 3 different hydroxyapatite surfaces. *Int J Oral Maxillofac Implants* 1999;14:217-25.
30. Wang L, Almqvist KF, Veys EM, Verbruggen G. Control of extracellular matrix homeostasis of normal cartilage by a TGF beta autocrine pathway. Validation of flow cytometry as a tool to study chondrocyte metabolism in vitro. *Osteoarthritis Cartilage* 2002;10:188-98.
31. Majumdar MK, Wang E, Morris EA. BMP-2 and BMP-9 promotes chondrogenic differentiation of human multipotential mesenchymal cells and overcomes the inhibitory effect of IL-1. *J Cell Physiol* 2001;189:275-84.
32. Grunder T, Gaissmaier C, Fritz J, et al. Bone morphogenetic protein (BMP)-2 enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads. *Osteoarthritis Cartilage* 2004;12:559-67.
33. Fan Z, Chubinskaya S, Rueger DC, Bau B, Haag J, Aigner T. Regulation of anabolic and catabolic gene expression in normal and osteoarthritic adult human articular chondrocytes by osteogenic protein-1. *Clin Exp Rheumatol* 2004;22:103-6.