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A potential role of chondroitin sulfate on bone in osteoarthritis: inhibition of prostaglandin E_2 and matrix metalloproteinases synthesis in interleukin-1β- stimulated osteoblasts

E. Pecchi †, S. Priam †, Z. Mladenovic †, M. Gosset †, A.-S. Saurel §, L. Aguilar §, F. Berenbaum †‡[a]*, C. Jacques †[a]

† UR 4, University Pierre & Marie Curie Paris VI, Paris Universitas, Aging, Stress and Inflammation Laboratory, 7 quai St-Bernard, 75252 Paris Cedex 5, France
‡ UFR Pierre et Marie Curie Paris VI, Department of Rheumatology, Saint-Antoine hospital, 75012 Paris, France
§ Pierre Fabre Laboratories, Castres, France

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SUMMARY

Objectives: To determine the effect of chondroitin sulfate (CS) on inflammatory mediators and proteolytic enzymes induced by interleukin-1β (IL-1β) and related to cartilage catabolism in murine osteoblasts.
Design: Osteoblasts were obtained by enzymatic digestion of calvaria from Swiss mice and cultured for 3 weeks as a primary culture. Cells were then stimulated with IL-1β (1 or 10 ng/ml). CS-treated osteoblasts were incubated with 100 μg/ml of CS during the last week of culture w/o IL-1β for the last 24 h. Expressions of cyclooxygenase-2 (COX-2), microsomal prostaglandin E synthase-1 (mPGES-1), 15-PG dehydrogenase (15-PGDH), matrix metalloproteinases-3 and -13 (MMP-3 and -13), osteoprotegerin (OPG) and receptor activator of nuclear factor-kappa B ligand (RANKL) were determined by real-time polymerase chain reaction (PCR). PGE_2, MMP-3 and MMP-13 release were assessed in the medium by enzyme-linked immunosorbent assay or western-blotting.
Results: IL-1β increased COX-2, mPGES-1, MMP-3, MMP-13, RANKL expressions, decreased 15-PGDH expression, and increased PGE_2, MMP-3 and MMP-13 release. Interestingly, 7 days of CS treatment significantly counteracted IL-1β-induced expression of COX-2 (−62%, $P < 0.001$), mPGES-1 (−63%, $P < 0.001$), MMP-3 (−39%, $P = 0.08$), MMP-13 (−60%, $P < 0.001$) and RANKL (−84%, $P < 0.001$). Accordingly, IL-1β-induced PGE_2, MMP-3 and MMP-13 releases were inhibited by 86% ($P < 0.001$), 58%($P < 0.001$) and 38% ($P < 0.01$) respectively.
Conclusions: In conclusion, our data demonstrate that, in an inflammatory context, CS inhibits the production of PGE_2 and MMPs. Since CS has previously been shown to counteract the production of these mediators in chondrocytes, we speculate that the beneficial effect of CS in Osteoarthritis (OA) could not only be due to its action on cartilage but also on subchondral bone.

Introduction

Osteoarthritis (OA) is not only characterized by articular cartilage destruction but also by an abnormal bone remodeling illustrated by subchondral bone sclerosis and varying degrees of osteophyte formation[1]. OA subchondral bone is the site of several dynamic morphological changes involving a remodeling process associated with an altered metabolism of osteoblasts[2]. A growing number of studies indicates that osteoblasts could partake in the disease process by releasing mediators involved in articular cartilage degradation[2]. Therefore, therapies that interfere with bone remodeling could possibly block or at least attenuate the progression of cartilage alterations.

Inflammation is a crucial component of the initiation and the evolution of OA[3]. Cartilage degradation in OA is due in part to an increased release of inflammatory mediators, such as interleukin-1 (IL-1β) and prostaglandin (PG) E_2[4]. PGs are also considered as critical local factors that modulate bone remodeling through their effects on both osteoblasts and osteoclasts and have been linked to many bone diseases[5]. PGE_2 is the most prominent eicosanoid in bone tissue, mainly produced by osteoblasts which release it upon different stimulations and also respond to external PGE_2[6].

* Address correspondence and reprint requests to: F. Berenbaum, UR 4, University Pierre & Marie Curie Paris VI, Paris Universitas, Aging, Stress and Inflammation Laboratory, 7 quai St-Bernard, 75252 Paris Cedex 5, France. Tel: 33-144-27-22-83; Fax: 33-144-27-51-40.
E-mail address: francis.berenbaum@sat.aphp.fr (F. Berenbaum).
[a] Dr. Berenbaum and Dr. Jacques contributed equally to this work.

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This prostanoid derived from arachidonic acid, and its production depends on the coordinated activities of cyclooxygenase-2 (COX-2) and microsomal PGE synthase 1 (mPGES-1). Collagen-induced arthritis is significantly less severe in mice lacking mPGES-1 than in control mice[7] indicating that PGE2 plays a pivotal role in inflammatory joint disease. The synthesis of mPGES-1 by chondrocytes from OA patients is also increased by IL-1β[8]. The coordinate induction of COX-2 and mPGES-1 is essential for PGE_2 production by osteoblasts treated by inflammatory stimulants. *In vivo*, PGE_2 is rapidly converted to an inactive metabolite by 15-hydroxy–PG dehydrogenase (15-PGDH)[9]. Synthesis of 15-PGDH is abnormal in various diseases[9]. We recently reported that 15-PGDH was synthesized by mouse chondrocytes and osteoblasts[10,11]. However its synthesis and regulation in bone tissue has not been characterized yet.

Matrix metalloproteinase (MMP) is a family of proteolytic enzymes involved in the degradation of the extracellular matrix of various tissues including bone. More than 20 different mammalian MMPs have been identified, and many physiological roles have been reported[12]. MMPs are involved in bone resorption and matrix degradation[13,14]. We have recently reported that murine osteoblasts produce MMP-3 and MMP-13[11].

Abnormal levels of two factors that play a major role in bone resorption, the receptor activator of nuclear factor-kappa B ligand (RANKL) and osteoprotegerin (OPG), have been found in human OA subchondral bone osteoblasts[15]. RANKL, a member of the tumor necrosis factor (TNF) family, is an essential cytokine for osteoclast differentiation and bone loss and acts as a survival factor for osteoclast precursors. OPG belongs to the family of TNF receptor[16] and is considered as a decoy receptor which inhibits the binding of RANKL to its membrane receptor RANK. OPG is able to inhibit the terminal stage of osteoclastic differentiation and to suppress its activation as well as inducing the apoptosis of mature osteoclasts[17]. This molecular triad OPG–RANKL–RANK is involved in the orchestration of pathophysiological bone remodeling.

Chondroitin sulfate (CS) is a major component of the extracellular matrix of many connective tissues. CS belongs to the family of glycosaminoglycans (GAG), long unbranched polysaccharides consisting of a repeating disaccharide unit which is often modified by sulfation, acetylation or epimerization[18]. CS is widely distributed in matrix where it forms an essential component of proteoglycans by covalent links with proteins[19]. Commonly referred as a "symptomatic slow-acting drug in OA" (SySADOA), CS is widely used in the management of OA patients[20]. *In vitro*, CS has been shown to have anti-inflammatory and anti-catabolic properties on chondrocytes (For review[21]) but little is known about its action on bone. In bone, CS and other GAG are actively synthesized by osteoblasts and are localized in the membrane of osteoblasts and in the extracellular matrix[22,23]. In osteoblasts, CS inhibits the osteoblast-mediated activation of osteoclasts by upregulating the expression ratio of OPG/RANKL[24,25]. However, the molecular mechanisms on how GAG mediates these effects in osteoblasts are not yet understood.

The aim of the present study was to investigate the effect of CS on inflammatory mediators and proteolytic enzymes induced by IL-1β and related to cartilage catabolism in murine osteoblasts. Altogether, our data suggest that CS inhibits the synthesis of proinflammatory and prodegradative mediators known to exert deleterious effect at the bone-cartilage interface.

Materials and methods

Materials

All reagents were purchased from Sigma–Aldrich (Lyon, France), unless stated otherwise. Fetal calf serum (FCS) was obtained from Invitrogen (Cergy-Pontoise, France). Collagenase A, trypsin, hyaluronidase, collagenase D and complete protease inhibitor mixture were from Roche Diagnostics (Meylan, France). The enhanced chemiluminescence (ECL) Western-blot analysis system was purchased from Amersham Pharmacia Biotech (Orsay, France). The Immunoblot polyvinylidene difluoride (PVDF) membranes for western-blotting and kaleidoscope prestained standards were obtained from Bio-Rad (Marnes-la-coquette, France). Recombinant human IL-1β was from PeproTech (Tebu-bio). CS (avian CS, average MW 18 kDa, less than 1.5% protein contaminant) was from Pierre Fabre Laboratories, France.

Primary calvaria mouse osteoblasts isolation and culture

Osteoblasts isolation and culture were performed as described previously[11]. On day 15, osteoblasts were incubated with 10% FCS-containing medium with CS (100 μg/ml) and medium changes were done every 2–3 days. On day 20, IL-1β (1 or 10 ng/ml) was added during 24 h. For acute phase stimulation, CS was not pre-incubated at day 15 but added on day 20 in the same time as IL-1β (same protocol using indomethacin at 10 μg/ml instead of CS). The osteoblasts were then isolated from the extracellular matrix by an enzymatic digestion with collagenase D (3 mg/ml, 30 min at 37°C under agitation). After centrifugation, osteoblasts were kept at −80°C until ribonucleic acid (RNA) extraction. Cell viability was assessed by trypan blue exclusion test. Osteoblast differentiation and mineralization were tested by staining of alkaline phosphatase and alizarin red respectively. These two parameters were not modified by CS (data not shown). All experiments were performed in duplicate or triplicate, 3 or 4 times with different calvaria osteoblasts populations.

RNA extraction, reverse transcription, and real-time quantitative reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted from chondrocytes using the RNeasy kit (Qiagen, Hilden, Germany), and concentrations determined spectrophotometrically. Reverse transcription was performed on 1 μg total RNA, using the OmniScript RT kit (Qiagen). Messenger RNA (mRNA) for HPRT, COX-2, mPGES-1, 15-PGDH, MMP-3, MMP-13, OPG and RANKL were quantified using the Light Cycler LC480 (Roche Diagnostics) as described[26]. Specific primers for complementary DNA (cDNA) were chosen with the LightCycler Probe Design 2 program based on mouse sequence information (Table I).

Proteins extraction and western-blotting

Protein from supernatant and immunoblot were prepared as previously described[8] with anti-human MMP-13 polyclonal Ab (from Santa Cruz Biotechnology, Tebu-Bio). Signals were detected by ECL and exposed to Fujifilm LAS-300 (Fujifilm Medical Systems, Stamford, CT). For densitometry analysis, we used Image-Gauge software (Science Lab 2004; Fujifilm).

Immunoassays for MMP-3 and PGE_2

MMP-3 and PGE_2 were directly measured in conditioned culture media by specific enzyme immunoassays (EIAs) (Biosource Europe and Cayman Chemical). The limit of detection of these immunoassays was 312 pg/ml for MMP-3 and 9 pg/ml for PGE_2. The MMP-3 and PGE_2 concentrations were analyzed at serial dilutions in duplicate and were read against standard curves.

Table I
Sequences of mouse primers for the quantitative RT-PCR experiments

mRNA gene	Sense (5′-3′)	Anti-sense (5′-3′)
HPRT	AGG-ACC-TCT-CGA-AGT-GT	ATT-CAA-ATC-CCT-GAA-GTA-CTC-AT
COX-2	GCA-CCA-ATC-TGA-TGT-TTG-CAT-TC-3	GGT-CCT-CGT-TCA-AAA-TCT-GTC-TTG
mPGES-1	CTG-CTG-GTC-ATC-AAG-ATG-TAC-G	CCC-AGG-TAG-GCC-ACG-GTG-TGT
15-PGDH	GCC-AAG-GTA-GCA-TTG-GTG-GAT	CTT-CCG-AAA-TGG-TCT-TGG-GGT-CT
MMP-3	TAC-GGG-TCT-CCC-CCA-GTT-TC	GGT-TCG-GGA-GGC-ACA-GAT-T
MMP-13	TTC-TTC-TGG-CGG-CTG-CAT	GGA-AGT-TCT-GGC-CCA-AAC-G
OPG	ATC-AGA-GCC-TCA-TCA-CCT-T	CTT-AGG-TCC-AAC-TAC-AGA-GGA-AC
RANKL	TTT-CGT-GCT-CCC-TCC-TTT	GCT-TCT-ATT-ACC-TGT-ACG-CCA

Statistical analysis

All data were expressed as means with 95% confidence interval (CI). All experiments were repeated at least three times. Statistical analyses were performed by Welch's corrected *t*-test to compare the mean values for two groups, and with one-way analysis of variance with Bonferroni *post hoc* test to compare mean values among >2 groups, using GraphPad Prism version (GraphPad Software, San Diego, California, USA). *P* values of 0.05 or less were considered significant.

Results

Effects of CS on the release of PGE_2 in IL-1β-stimulated mouse osteoblasts

Since PGE_2 is a pivotal catabolic mediator in OA, we tested the effect of CS on PGE_2 synthesis by mouse osteoblasts. We quantified PGE_2 production into the conditioned media after CS and/or IL-1β treatment (Fig. 1). CS had no effect on the basal PGE_2 release (Fig. 1). As expected, a 24 h incubation with IL-1β at 1 or 10 ng/ml increased the basal level of PGE_2 release by 23- and 184-fold, respectively [$P < 0.001$] [Fig. 1(A)]. When CS (100 μg/ml) was pre-incubated for 7 days before IL-1β treatment, the effect of IL-1β at 10 ng/ml on PGE_2 production was decreased by 86% of inhibition [$P < 0.001$] [Fig. 1(A)]. When the period of pretreatment was reduced from 7 days to 24 h and CS incubated for 24 h with IL-1β, the level of PGE_2 production was not modified compared to osteoblasts stimulated by IL-1β alone [Fig. 1(B)]. In order to compare the effect of CS with a nonsteroidal anti-inflammatory drug (NSAID) in that particular condition, we treated the mouse osteoblasts by indomethacin at 10 μg/ml for 24 h w/o IL-1β. As expected, the level of IL-1β-induced PGE_2 was completely abolished by indomethacin [$P < 0.001$] [Fig. 1(C)].

CS inhibited the expression of COX-2 and mPGES-1 mRNA and increased the expression of 15-PGDH in IL-1β-stimulated mouse osteoblasts

We subsequently focused our study on the inducible enzymes involved in PGE_2 synthesis, COX-2 and mPGES-1. In addition, we

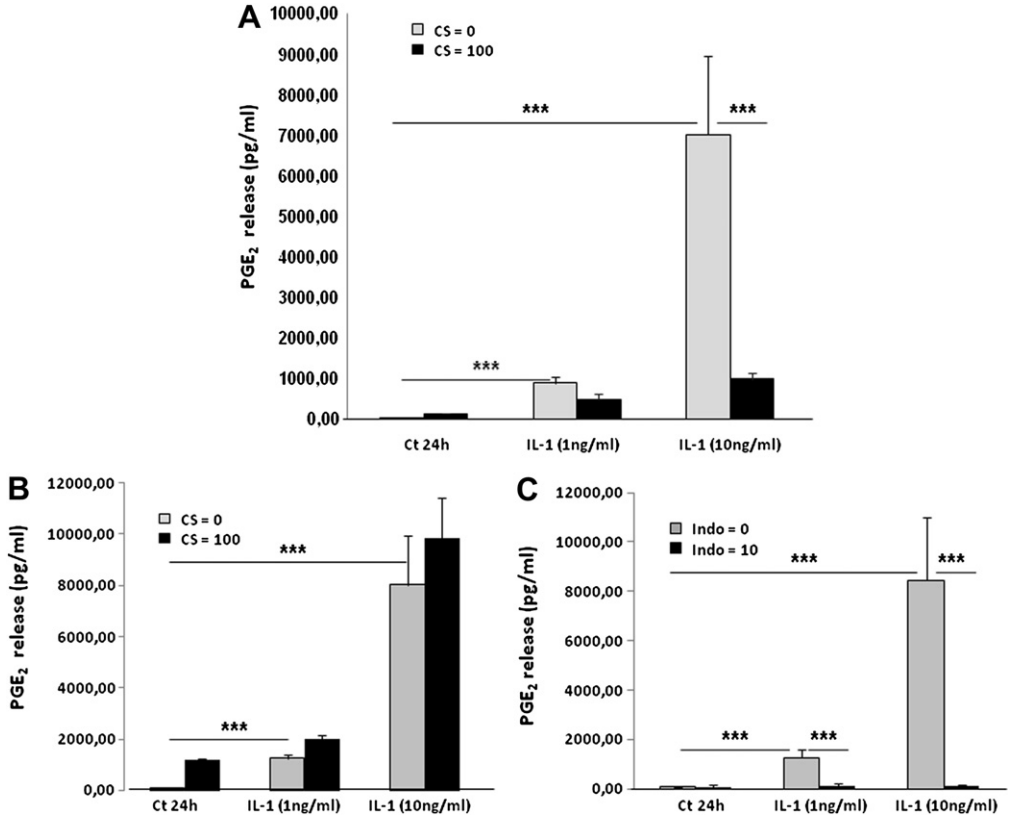

Fig. 1. Effect of CS on PGE2 release. Osteoblasts were treated for 7 days (A) or 24 h (B) in the presence or absence of CS (100 μg/ml) or for 24 h in the presence or absence of indomethacin (10 μg/ml) (C). IL-1β (1 or 10 ng/ml) was added for the latest 24 h. The amount of the PGE_2 release into the media (pg/ml) was measured by enzymatic immunoassay (EIA). PGE2 release are the mean with 95% CI of four independent experiments with two wells/condition, analyzed in duplicate (corrected *P*-values: $^{***}P < 0.001$).

studied 15-PGDH which is the key enzyme in the catabolism of PGE_2. Osteoblasts were treated for 7 days with CS at 100 μg/ml and with IL-1β for the latest 24 h. To determine the effect of CS on the steady-state mRNA levels of various enzymes involved in PGE_2 metabolism (COX-2, mPGES-1 and 15-PGDH), the cells were lysed and the RNA were extracted (Fig. 2). As expected, RT-PCR revealed that IL-1β at 1 and 10 ng/ml for 24 h increased synthesis of COX-2 mRNA by 4.7-fold [$P < 0.01$] and 10.8-fold, respectively [$P < 0.001$] [Fig. 2(A)] and mPGES-1 mRNA by 2.3-fold [$P = 0.09$] and 7.8-fold, respectively [$P < 0.001$] [Fig. 2(B)]. 15-PGDH mRNA expression was dramatically reduced by IL-1β at 1 and 10 ng/ml (70% and 90% of inhibition respectively; [all $P < 0.001$]) [Fig. 2(C)]. Then, we examined whether CS could modulate the expression of these three enzymes induced by IL-1β. CS did not significantly alter the mRNA basal levels of these three genes. However, the IL-1β-induced COX-2 and mPGES-1 mRNA amounts were decreased by a 7-days pretreatment with CS. Indeed, CS decreased the up-regulation of COX-2 and mPGES-1 mRNA levels induced by 24 h incubation with IL-1β at 10 ng/ml (−62% of inhibition for COX-2 [$P < 0.001$] [Fig. 2(A)] and −63% of inhibition for mPGES-1 [$P < 0.001$] [Fig. 2(B)]). 15-PGDH mRNA amounts induced by a 24-h incubation with IL-1β at 10 ng/ml were found to be not modulated by treatment of cells with CS [Fig. 2(C)].

CS decreased IL-1β stimulated MMP-3 gene expression and protein secretion in mouse osteoblasts

Since the matrix breakdown that occurs in OA is mainly due to MMPs (especially MMP-3 and MMP-13), we assessed the influence of CS on the synthesis of these enzymes by osteoblasts. After 7 days treatment with CS at 100 μg/ml and with IL-1β (1 or 10 ng/ml) for the latest 24 h, CS did not significantly alter the basal level of MMP-3 mRNA. Moreover, as expected, stimulation with IL-1β at 1 ng/ml or 10 ng/ml for 24 h, elevated this basal level of MMP-3 gene expression in mouse osteoblasts by 170- and 180-fold, respectively [$P < 0.001$] [Fig. 3(A)]. However, when CS was pre-incubated for 7 days before the IL-1β treatment at 10 ng/ml, IL-1β-induced-MMP-3 gene expression decreases by 39% of inhibition [$P = 0.08$] [Fig. 3(A)].

Using enzyme-linked immunosorbent assay (ELISA), we determined the effects of CS on MMP-3 release by measuring the protein concentration in the media from osteoblasts. IL-1β stimulation at 1 or 10 ng/ml during 24 h leads to an 88-fold and 101-fold increase of MMP-3 release in the media, respectively [$P < 0.001$] [Fig. 3(B)]. However, when CS was pre-incubated for 7 days before IL-1β treatment at 10 ng/ml, IL-1β stimulated MMP-3 production was decreased by 58% of inhibition [$P < 0.001$] [Fig. 3(B)].

CS inhibited MMP-13 gene expression and secretion following IL-1β stimulation in mouse osteoblasts

Osteoblasts were treated during 7 days with CS (100 μg/ml) and with IL-1β (1 or 10 ng/ml) for the latest 24 h. CS did not significantly alter the basal levels of MMP-13. Moreover, as expected, stimulation with IL-1β at 1 or 10 ng/ml for 24 h, elevated this basal level of MMP-13 gene expression by 9.32- and 17.46-fold, respectively [$P < 0.001$] [Fig. 4(A)]. However, pretreatment with CS for 7 days before the IL-1β treatment at 10 ng/ml, decreased the up-regulation

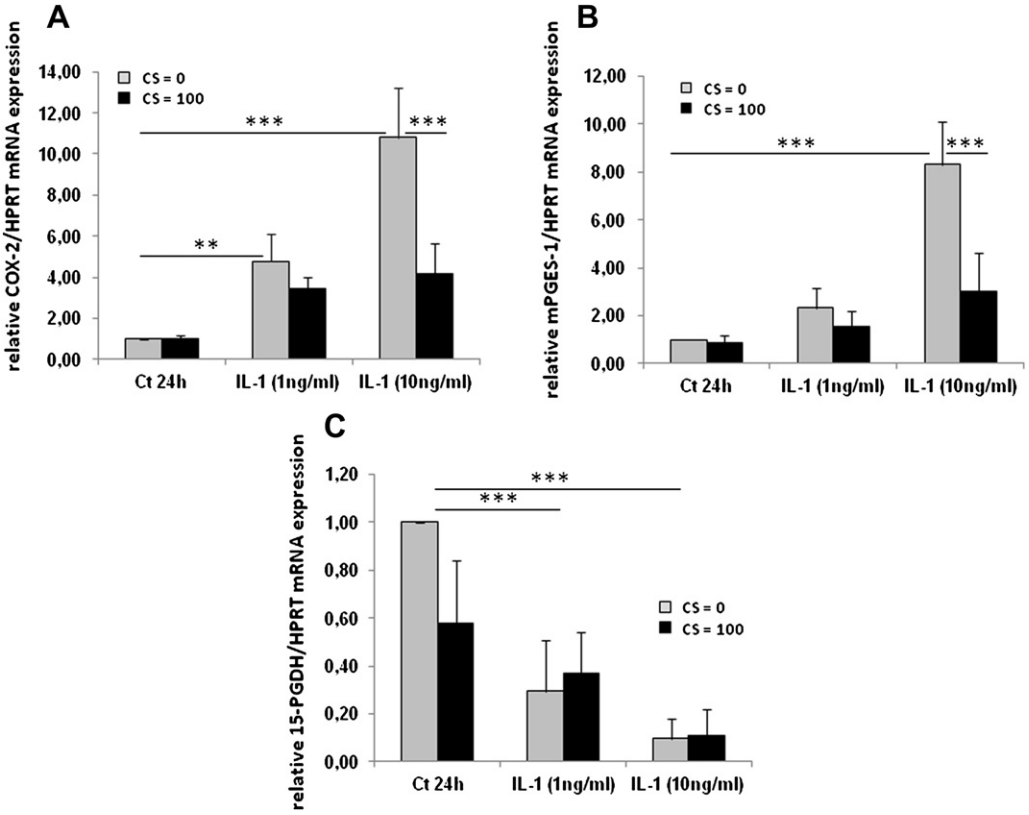

Fig. 2. Effect of CS on IL-1β-induced pro-inflammatory gene expression. Osteoblasts were treated for 7 days in the presence or absence of CS (100 μg/ml). IL-1β (1 or 10 ng/ml) was added for the latest 24 h. Total RNA was extracted and used in quantitative PCR to determine steady-state mRNA levels of COX-2 (A), mPGES-1 (B), and 15-PGDH (C). Standard curves for COX-2, mPGES-1, 15-PGDH and HPRT were generated by serial dilution of a cDNA mixture. The amount of COX-2, mPGES-1 and 15-PGDH mRNA was normalized against the amount of HPRT mRNA measured in the same cDNA. Values are the mean with 95% of four independent experiments with $n = 1$/group/experiment (corrected P-values: $^{**}P = 0.08$ and $^{***}P < 0.001$).

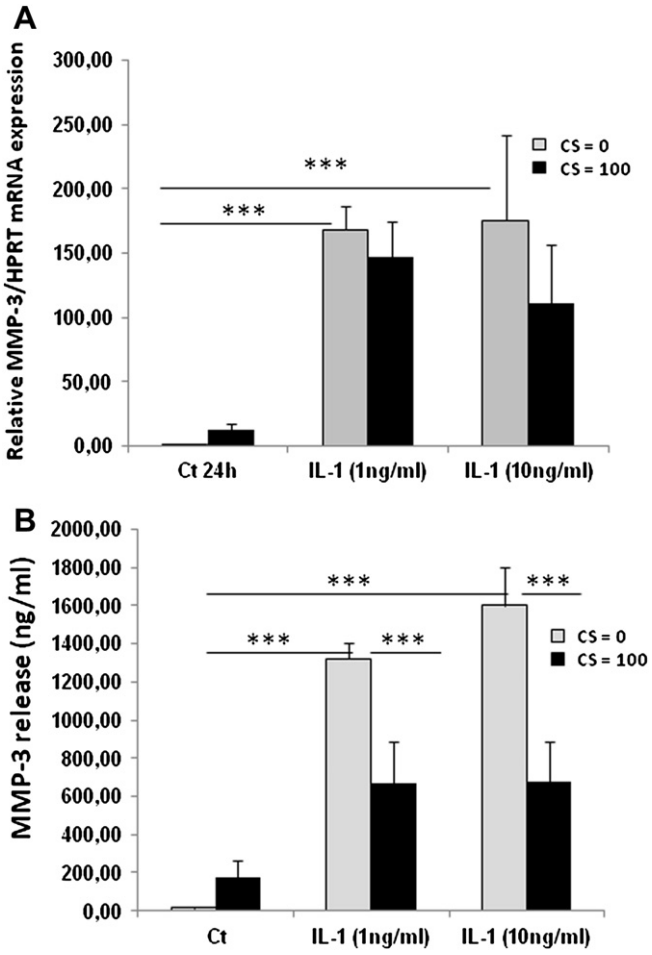

Fig. 3. Effect of CS on IL-1β-induced MMP-3 mRNA expression and protein release. Osteoblasts were treated for 7 days in the presence or absence of CS (100 μg/ml). IL-1β (1 or 10 ng/ml) was added for the latest 24 h. Total RNA was extracted and used in quantitative PCR to determine steady-state mRNA levels of MMP-3 (A). Media from cells incubated were assayed for analyzing MMP-3 release into the media (B). The amount of MMP-3 released into the medium (ng/ml) in response to IL-1β was measured by ELISA. Values are the mean with 95% CI of four independent experiments with $n = 1$/group/experiment, analyzed in duplicate (corrected P-values: $^{***}P < 0.001$).

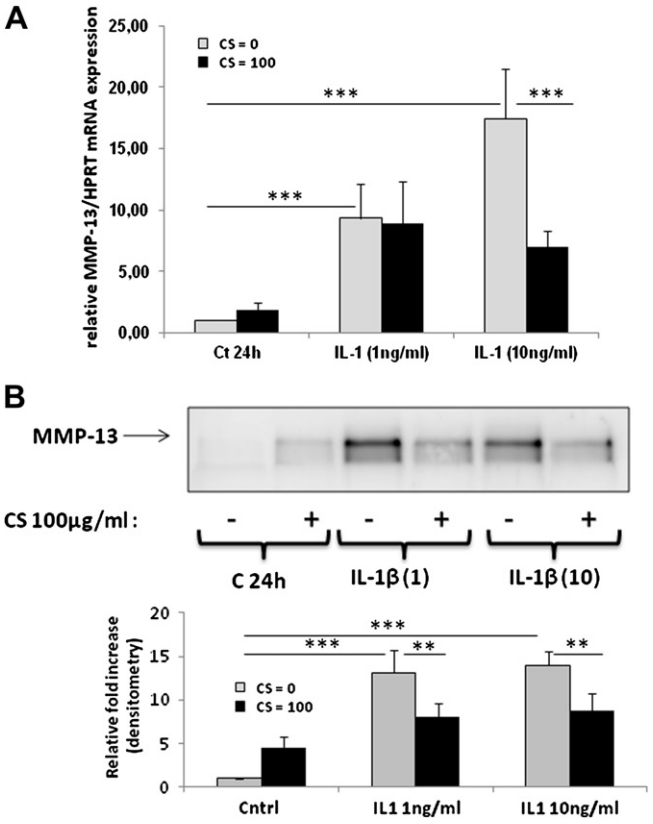

Fig. 4. Effect of CS on IL-1β-induced MMP-13 mRNA expression and protein release. Osteoblasts were treated for 7 days in the presence or absence of CS (100 μg/ml). IL-1β (1 or 10 ng/ml) was added for the latest 24 h. Total RNA was extracted and used in quantitative PCR to determine steady-state mRNA levels of MMP-13 (A). Media from cells incubated were assayed for analyzing MMP-13 release into the media (B). Values are the mean with 95% CI of four independent experiments with $n = 1$/group/experiment, analyzed in duplicate. MMP-13 protein secretion into the medium in response to IL-1β were assessed by immunoblotting. Blots in B are representative of three independent experiments. The densitometric analyses of western-blot is the mean with 95% CI of three independent experiments [corrected P-values: $^{**}P = 0.076$ for (IL-1 1 ng/ml), $^{**}P = 0.063$ for (IL-1 10 ng/ml), and $^{***}P < 0.001$].

of MMP-13 mRNA induced by a 24-h incubation with IL-1β by 60% of inhibition [$P < 0.001$] [Fig. 4(A)].

Using immunoblotting, we determined the effects of CS on MMP-13 release by osteoblasts by measuring the expression of this protein in the media. The analysis by densitometry of western-blot shows that IL-1β stimulation at 1 or 10 ng/ml for 24 h, elevated the level of MMP-13 release in the media by 13.12- and 13.94-fold, respectively [$P < 0.001$] [Fig. 4(B)]. However, upon addition of CS for 7 days before IL-1β treatment at 10 ng/ml, IL-1β stimulated MMP-13 production was decreased by 38% of inhibition [$P < 0.01$] [Fig. 4(B)].

CS increased OPG and inhibited RANKL gene expression following IL-1β stimulation in mouse osteoblasts

Osteoblasts were treated during 7 days with CS at 100 μg/ml and with IL-1β (1 or 10 ng/ml) for the latest 24 h. CS did not significantly alter the basal levels of OPG. Moreover, stimulation with IL-1β at 1 or 10 ng/ml for 24 h, not modified significantly the basal level of OPG gene expression. However, pretreatment with CS for 7 days before the IL-1β treatment at 1 or 10 ng/ml, increased significantly regulation of OPG mRNA induced by a 24-h incubation with IL-1β by 41% and 44% of increase respectively [$P < 0.001$] [Fig. 5(A)].

Stimulation with IL-1β at 1 or 10 ng/ml for 24 h, increased significantly the basal level of RANKL gene expression. Interestingly, pretreatment with CS for 7 days before the IL-1β treatment at 10 ng/ml, decreased significantly regulation of RANKL mRNA induced by a 24-h incubation with IL-1β by 84% of decrease [$P < 0.001$] [Fig. 5(B)].

Then, the OPG/RANKL ratio was determined and data showed [Fig. 5(C)] that, under basal condition, the expression ratio of OPG/RANKL was significantly increased by 35.7% when cells were incubated with CS for 7 days. Moreover, as expected, stimulation with IL-1β at 1 or 10 ng/ml for 24 h, decreased significantly the basal expression ratio of OPG/RANKL. Interestingly, pretreatment with CS for 7 days before the IL-1β treatment at 1 or 10 ng/ml, increased significantly expression ratio of OPG/RANKL induced by a 24-h incubation with IL-1β by 63% and 91% of increase [$P < 0.001$] [Fig. 5(C)].

Discussion

OA is now considered as a global organ failure involving all the tissues of the joint[1]. In the last 5 years, many studies have focused their attention on the role of the subchondral bone in the

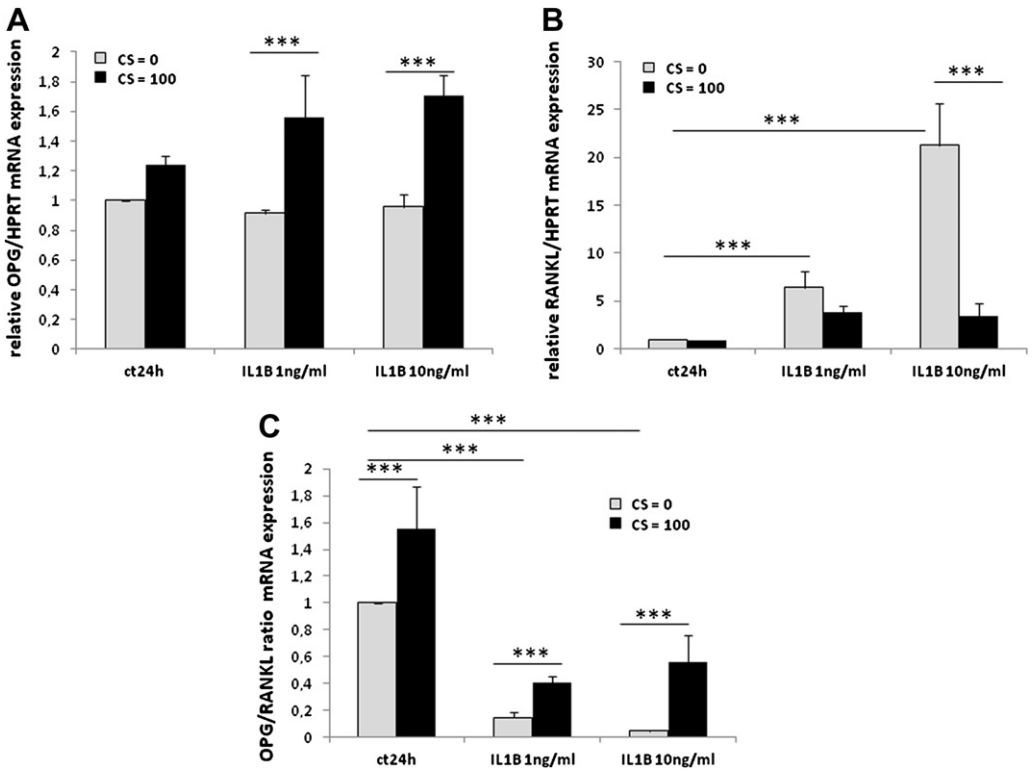

Fig. 5. Effect of CS on IL-1β-induced OPG, RANKL gene expression and OPG/RANKL ratio. Osteoblasts were treated for 7 days in the presence or absence of CS (100 μg/ml). IL-1β (1 or 10 ng/ml) was added for the latest 24 h. Total RNA was extracted and used in quantitative PCR to determine steady-state mRNA levels of OPG (A), RANKL (B), and OPG/RANKL ratio (C). Values are the mean with 95% CI of three independent experiments with $n = 1$/group/experiment (corrected P-values: $^{***}P < 0.001$).

pathophysiology of the disease. In OA, the subchondral bone demonstrates accelerated phases of bone resorption and bone formation[27–29]. These changes are associated with an altered metabolism of osteoblasts which lead to an abnormal production of soluble mediators. These mediators produced by bone cells can affect deep zone chondrocytes by going through the bone-cartilage interface[30]. Thus, it makes sense to target subchondral bone cells to treat OA in order to decrease cartilage degradation.

In order to assess the role of subchondral bone osteoblasts in the OA process we recently developed a new model of mouse osteoblasts in culture[14]. After 3 weeks of culture, cells formed a 3D membrane, showing a strong alkaline phosphatase activity and expressed genes characteristics of the osteoblastic phenotype [RUNX-2, COL1A1, Bone sialoprotein (BSP), osteopontin (OPN), osteocalcin (OC)][31]. This model is accurate to assess the role of subchondral osteoblasts in OA[14].

The aim of the present study was to investigate whether CS, a natural compound widely used in the treatment of OA symptoms, could counteract the production of mediators known to be deleterious for cartilage, by murine osteoblasts in an inflammatory context. We used 100 μg/ml of CS in our study. The choice of "physiological" dose was recently discussed[32]. A large range of CS concentrations have been used (i.e., 12.5–2000 μg/ml) in *in vitro* studies and most of them employed about 100–200 μg/ml. These CS concentrations are attainable in articular tissues *in vivo*. Pharmacokinetic studies performed on humans and experimental animals after oral administration of CS revealed that it can be absorbed orally[33–35], and a high content of labeled CS was found in the synovial fluid and cartilage[34,35]. Such concentrations for *in vitro* studies are required because the treatment with CS is characterized by a slow onset of action, with a maximal clinical effect being attained after several months (3–6 months) of treatment. CS was chosen not only because of its well-known anti-inflammatory and anti-degradative *in vitro* properties in cartilage but also because of recent studies demonstrating interesting effects on subchondral bone marrow lesions (BML)[36] and on the pro-resorptive properties of human osteoarthritis subchondral bone osteoblasts[26] underlining a critical role of CS in the crosstalk between cartilage and subchondral bone in OA.

Our data show that, when CS is pre-incubated for 7 days before IL-1β treatment, the effect of IL-1β on PGE_2 production is decreased by 86% of inhibition in mouse osteoblasts. The coordinate induction of COX-2 and mPGES-1 is essential for PGE2 production by osteoblasts treated by inflammatory stimulants. Indeed, our results show that CS inhibits the expression of COX-2 and mPGES-1 mRNA in IL-1β-stimulated mouse osteoblasts. PGs are considered as important local factors that modulate bone remodeling through their effects both on osteoblasts and osteoclasts and have been linked to many bone-resorptive diseases[37]. Among several PG produced, PGE2, a major mediator involved in the pathogenesis of OA, is the most prominent eicosanoid in bone tissue, mainly produced by osteoblasts[6,37]. PGE2 plays a multifaceted role in bone metabolism. This PG has indeed been shown to stimulate osteoclastogenesis leading to bone resorption but it also displays bone-forming activities including osteoblast formation and differentiation[37]. Therefore the role of PGE2 in bone remodeling during OA remains controversial. In OA patients, the production of high level of PGE2 by subchondral bone osteoblasts is mainly associated with an anabolic and anti-resorptive phenotype[15]. Accordingly, inhibition of COX in human OA osteoblasts restores the catabolic parathyroid hormone (PTH)-signaling pathway to the level of control osteoblasts[38]. Some anabolic processes occurring in bone during OA are responsible for the production of an abundant osteoid matrix with abnormal features,

which fails to mineralize normally and leads to bone sclerosis (For review[39]). Therefore, due to its anabolic properties in bone, the increase in PGE2 production could partake in this abnormal osteoblasts activity leading to bone sclerosis and then to a worsening of the OA progression. However, this potential role needs to be clarified. PGE2 is also significantly elevated in the synovial fluid of OA patients[40] and it enhances the degradative process of proteoglycans in cartilage[41]. Thus, we can speculate that the inhibition of PGE2 production by CS could decrease bone sclerosis and could also have an anti-degradative effect in the deep zone of OA cartilage.

PGE_2 is formed from a series of enzymatic reactions involving COXs and PGES. In an inflammatory context, COX-2 and mPGES-1 are induced and lead to an overproduction of PGE_2. The present findings in osteoblasts are comparable with what is seen in chondrocytes, that is an inhibition of both COX-2 mRNA levels and PGE_2 production in presence of CS in cultured cartilage explants[21]. Several transcription factors are reported to mediate the enhanced expression of COX-2 and mPGES-1 under inflammatory conditions such as nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1)[42,43]. Moreover, CS is involved in the formation of focal adhesions in osteoblasts[44] and earlier findings show that in osteocytes and osteoblasts, c-Fos, which dimerizes with C-jun to form the AP-1 transcription factor, is involved in the regulation of expression of COX-2 and consequently in a reorganization of the cytoskeleton and formation of focal adhesions[45]. Taken these data together, it can be postulated that the beneficial effects of CS might be due to a specific interaction of CS with the cytoskeleton of osteoblasts and a concomitant specific inhibition of the delayed PGE_2 release[5,21,30,46].

In the present study, we demonstrate that IL-1β induces MMP-3 and MMP-13 gene expression and synthesis, and that CS is able to significantly counteract this effect. One possible role for MMPs is to prepare recruitment sites for osteoclasts and its progenitors by degrading collagenous extracellular matrix covering the mineralized bone surface, and then to expose RGD (Arg-Gly-Asp) sequences which allow osteoclasts adhesion *via* alpha5/beta3 integrin receptor[47]. Further, degradative products of collagens may activate osteoclasts[48]. Thus, degradation of collagen on bone surface does not only allow osteoclasts attachment, but may also stimulate them to proceed to activation and resorption phases. Our results in osteoblasts strengthen the hypothesis that CS could have interesting properties by modulating osteoclast activation through the inhibition of MMP-3 expression. Moreover, by decreasing the production of MMP-3 and MMP-13 at the bone-cartilage interface by subchondral osteoblasts, we can speculate an anti-degradative effect of CS in the deep zone cartilage in OA[49]. Our results in osteoblasts are in agreement with previous data showing that CS down-regulated the expression of MMP-3 and MMP-13 in bovine cartilage explants[21].

Moreover, our data showing the effects of CS on the OPG and RANKL system highlight the ability of CS to modulate osteoclast activation. Indeed, our data revealed that CS can regulate the expression of these molecules. When CS was pre-incubated for 7 days before IL-1β treatment, the effect of IL-1β on OPG mRNA expression was increased by 44% and on RANKL mRNA expression was decreased by 84% thereby CS was able to increase the mRNA ratio of OPG/RANKL. A recent study about the use of chondroitin and glucosamine sulfate in combination in human OA subchondral bone osteoblasts showed the same effect of CS alone on OPG/RANKL ratio[26]. OPG contains a heparin-binding domain to which some GAGs were demonstrated to bind[50]. Nonetheless, it should not be excluded that CS may also act indirectly through the production of other factors that in turn modulate OPG/RANKL and/or resorption activity[51].

Interestingly, the effect of CS on PGE_2 production and MMP expression was present only when cells were pre-incubated with CS for a long period of time (7 days). We first decided to select a short period of time (24 h preincubation with CS) to test if the effect of CS on PGE_2 was due to a direct interaction between CS and IL-1β. Indeed, a study suggested that during the immune response, ILs tended to be retained at sites of secretion by interaction with GAG in the extracellular matrix[52]. Since our data showing that 24 h treatment of CS was not able to inhibit PGE_2 production, we considered that this hypothesis was invalidated. In addition, we have shown that indomethacin was effective even when the pre-incubation time was short. Such differences between an NSAID and CS may highlight differences in term of mechanism of action (fast vs long-acting effect) in OA. However, the reasons why these differences exist remain unknown yet. The mechanisms whereby CS binds to the cell membrane and elicits intracellular signaling to play these pleiotropic activities are quite unknown. CS is internalized in the cell *via* the hyaluronan receptor for endocytosis (HARE)[53], and other scavenger receptors such as CD36 and CD44[54]. The mechanism by which CS reduces p38 MAPK, ERK1/2 phosphorylation, and NF-κB nuclear translocation remains unclear but it has been reported that inhibition of hyaluronan binding to CD44 by CS diminishes ERK1/2 phosphorylation[55]. Since the activation of these signaling pathways is redox-sensitive, once internalized by HARE, the antioxidant activity of CS may have contributed to these effects[56].

In conclusion, our data demonstrate that, in an inflammatory context, CS inhibits the production of PGE_2 and MMPs. Since CS has previously been shown to counteract the production of these mediators in chondrocytes, we speculate that the beneficial effect of CS in OA could not only be due to its action on cartilage but also on subchondral bone.

Author contributions

Dr. Berenbaum had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Conception and Study design: Berenbaum, Aguilar, Saurel, Jacques and Pecchi.

Acquisition of data: Pecchi, Priam, Mladenovic.

Analysis and interpretation of data: Pecchi, Gosset, Jacques, Berenbaum, Aguilar.

Manuscript preparation: Berenbaum, Jacques.

Statistical analysis: Pecchi, Jacques.

Conflict of interest

We have no conflict of interest.

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References

1. Bijlsma J, Berenbaum F, Lafeber F. Osteoarthritis: an update with relevance for clinical practice. Lancet 2011;377(9783): 2115–26.
2. Martel-Pelletier J, Lajeunesse D, Reboul P, Pelletier JP. The role of subchondral bone in osteoarthritis. In: Sharma L, Berenbaum F, Eds. Osteoarthritis: A Companion to Rheumatology. Philadelphia: Mosby Elsevier; 2007:15–32.

3. Garstang SV, Stitik TP. Osteoarthritis: epidemiology, risk factors, and pathophysiology. Am J Phys Med Rehabil 2006;85: S2–11.
4. Goldring MB, Berenbaum F. The regulation of chondrocyte function by proinflammatory mediators: prostaglandins and nitric oxide. Clin Orthop Relat Res 2004;(427 Suppl):S37–46. Review.
5. Aigner T, Sachse A, Gebhard PM, Roach HI. Osteoarthritis: pathobiology-targets and ways for therapeutic intervention. Adv Drug Deliv Rev 2006;58(2):128–49.
6. Miyaura C, Inada M, Suzawa T, Sugimoto Y, Ushikubi F, Ichikawa A, *et al*. Impaired bone resorption to prostaglandin E2 in prostaglandin E receptor EP4-knockout mice. J Biol Chem 2000;275:19819–23.
7. Trebino CE, Stock JL, Gibbons CP, Naiman BM, Wachtmann TS, Umland JP, *et al*. Impaired inflammatory and pain responses in mice lacking an inducible prostaglandin E synthase. Proc Natl Acad Sci U S A 2003;100(15):9044–9.
8. Masuko-Hongo K, Berenbaum F, Humbert L, Salvat C, Goldring MB, Thirion S. Up-regulation of microsomal prostaglandin E synthase 1 in osteoarthritic human cartilage: critical roles of the ERK-1/2 and p38 signaling pathways. Arthritis Rheum 2004;50(9):2829–38.
9. Tai HH, Cho H, Tong M, Ding Y. NAD+-linked 15-hydroxyprostaglandin dehydrogenase: structure and biological functions. Curr Pharm Des 2006;12(8):955–62.
10. Gosset M, Berenbaum F, Levy A, Pigenet A, Thirion S, Saffar JL, *et al*. Prostaglandin E2 synthesis in cartilage explants under compression: mPGES-1 is a mechanosensitive gene. Arthritis Res Ther 2006;8(4):R135.
11. Sanchez C, Gabay O, Salvat C, Henrotin YE, Berenbaum F. Mechanical loading highly increases IL-6 production and decreases OPG expression by osteoblasts. Osteoarthritis Cartilage 2009;17(4):473–81.
12. Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases: role in arthritis. Front Biosci 2006;11:529–43.
13. Kusano K, Miyaura C, Inada M, Tamura T, Ito A, Nagase H, *et al*. Regulation of matrix metalloproteinases (MMP-2, -3, -9, and -13) by interleukin-1 and interleukin-6 in mouse calvaria: association of MMP induction with bone resorption. Endocrinology 2008;139:1338–45.
14. Inada M, Wang Y, Byrne MH, Rahman MU, Miyaura C, López-Otín C, *et al*. Critical roles for collagenase-3 (Mmp13) in development of growth plate cartilage and in endochondral ossification. Proc Natl Acad Sci U S A 2004;101:17192–7.
15. Kwan Tat S, Pelletier JP, Lajeunesse D, Fahmi H, Lavigne M, Martel-Pelletier J. The differential expression of osteoprotegerin (OPG) and receptor activator of nuclear factor kappaB ligand (RANKL) in human osteoarthritic subchondral bone osteoblasts is an indicator of the metabolic state of these disease cells. Clin Exp Rheumatol 2008;26(2):295–304.
16. The'oleyre T, Wittrant Y, Kwan Tat S, Fortun Y, Re'dini F, Heymann D. The molecular triad OPG/RANK/RANKL: involvement in the orchestration of pathophysiological bone remodeling. Cytokine Growth Factor Rev 2004;15:457–75.
17. Simonet WS, Lacey DL, Dunstan CR, Kelley M, Chang MS, Luthy R, *et al*. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. Cell 1997;89:309–19.
18. Taylor KR, Gallo RL. Glycosaminoglycans and their proteoglycans: host associated molecular patterns for initiation and modulation of inflammation. FASEB J 2006;20:9–22.
19. Dudhia J. Aggrecan, aging and assembly in articular cartilage. Cell Mol Life Sci 2005;62:2241–56.
20. Uebelhart D, Malaise M, Marcolongo R, DeVathaire F, Piperno M, Mailleux E, *et al*. Intermittent treatment of knee osteoarthritis with oral chondroitin sulfate: a one-year, randomized, double-blind, multicenter study versus placebo. Osteoarthritis Cartilage 2004;12:269–76.
21. Monfort J, Pelletier JP, Garcia-Giralt N, Martel-Pelletier J. Biochemical basis of the effect of chondroitin sulphate on osteoarthritis articular tissues. Ann Rheum Dis 2008;67:735–40.
22. Cool SM, Nurcombe V. The osteoblast-heparan axis: control of the bone cell lineage. Int J Biochem Cell Biol 2005;37:1739–45.
23. Bastow ER, Byers S, Golub SB, Clarkin CE, Pitsillides AA, Fosang AJ. Hyaluronan synthesis and degradation in cartilage and bone. Cell Mol Life Sci 2008;65:395–413.
24. Iovu M, Dumais G, du Souich P. Anti-inflammatory activity of chondroitin sulfate. Osteoarthritis Cartilage 2008;16:14–8.
25. Tat SK, Pelletier JP, Vergés J, Lajeunesse D, Montell E, Fahmi H, *et al*. Chondroitin and glucosamine sulfate in combination decrease the pro-resorptive properties of human osteoarthritis subchondral bone osteoblasts: a basic science study. Arthritis Res Ther 2007;9:R117.
26. Blaise R, Mahjoub M, Salvat C, Barbe U, Brou C, Corvol MT, *et al*. Arthritis Rheum 2009;60(2):428–39.
27. Seibel MJ, Duncan A, Robins SP. Urinary hydroxy-pyridinium crosslinks provide indices of cartilage and bone involvement in arthritic diseases. J Rheumatol 1989;16:964–70.
28. Sowers M, Zobel D, Weissfeld L, Hawthorne VM, Carman W. Progression of osteoarthritis of the hand and metacarpal bone loss. A twenty-year followup of incident cases. Arthritis Rheum 1991;34:36–42.
29. Li B, Marshall D, Roe M, Aspden RM. The electron microscope appearance of the subchondral bone plate in the human femoral head in osteoarthritis and osteoporosis. J Anat 1999;195:101–10.
30. Lories RJ, Luyten FP. The bone–cartilage unit in osteoarthritis. Nat Rev Rheumatol 2010;7:43–9.
31. Lamour V, Detry C, Sanchez C, Henrotin Y, Castronovo V, Bellahcene A. Runx2- and histone deacetylase 3-mediated repression is relieved in differentiating human osteoblast cells to allow high bone sialoprotein expression. J Biol Chem 2007;282:36240–9.
32. Martel-Pelletier J, Kwan Tat S, Pelletier JP. Effects of chondroitin sulfate in the pathophysiology of osteoarthritic joint: a narrative review. Osteoarthritis Cartilage 2010;18:S7–11.
33. Conte A, de Bernardi M, Palmieri L, Lualdi P, Mautone G, Ronca G. Metabolic fate of exogenous chondroitin sulfate in man. Arzneimittelforschung 1991;41:768–72.
34. Conte A, Palmieri L, Segnini D, Ronca G. Metabolic fate of partially depolymerized chondroitin sulfate administered to the rat. Drugs Exp Clin Res 1991;17:27–33.
35. Palmieri L, Conte A, Giovannini L, Lualdi P, Ronca G. Metabolic fate of exogenous chondroitin sulfate in the experimental animal. Arzneimittelforschung 1990;40:319–23.
36. Wildi LM, Raynauld JP, Martel-Pelletier J, Beaulieu A, Bessette L, Morin F, *et al*. Chondroitin sulphate reduces both cartilage volume loss and bone marrow lesions in knee osteoarthritis patients starting as early as 6 months after initiation of therapy: a randomised, double-blind, placebo-controlled pilot study using MRI. Ann Rheum Dis 2011;70(6):982–9.
37. Hikiji H, Takato T, Shimizu T, Ishii S. The roles of prostanoids, leukotrienes, and platelet-activating factor in bone metabolism and disease. Prog Lipid Res 2008;47:107–26.
38. 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(4):713–21.
39. Lajeunesse D. The role of bone in the treatment of osteoarthritis. Osteoarthritis Cartilage 2004;12(Suppl A):S34–8. Review.

40. Kirker-Head CA, Chandna VK, Agarwal RK, Morris EA, Tidewell A, O'Callaghan MW, *et al*. Concentrations of substance P and prostaglandin E2 in synovial fluid of normal and abnormal joints of horses. Am J Vet Res 2000;61:714–8.
41. Yamada H, Kikuchi T, Nemoto O, Obata K, Sato H, Seiki M, *et al*. Effects of indomethacin on the production of matrix metalloproteinase-3 and tissue inhibitor of metalloproteinases-1 by human articular chondrocytes. J Rheumatol 1996;23:1739–43.
42. Park JY, Pillinger MH, Abramson SB. Prostaglandin E_2 synthesis and secretion: the role of PGE2 synthases. Clin Immunol 2006;119:229–40.
43. Kunze R, Hempel U, Dieter P. Differential effect of chondroitin-4-sulfate on the immediate and delayed prostaglandin E2 release from osteoblasts. Prostaglandins Other Lipid Mediat 2010;92(1–4):8–12.
44. Douglas T, Heinemann S, Mietrach C, Hempel U, Bierbaum S, Scharnweber D, *et al*. Interactions of collagen types I and II with chondroitin sulfates A–C and their effect on osteoblast adhesion. Biomacromolecules 2007;8:1085–92.
45. Ponik SM, Pavalko FM. Formation of focal adhesions on fibronectin promotes fluid shear stress induction of COX-2 and PGE2 release in MC3T3-E1 osteoblasts. J Appl Phys 2004;97:135–42.
46. Legendre F, Baugé C, Roche R, Saurel AS, Pujol JP. Chondroitin sulfate modulation of matrix and inflammatory gene expression in IL-1beta-stimulated chondrocytes—study in hypoxic alginate bead cultures. Osteoarthritis Cartilage 2008;16:105–14.
47. Helfrich MH, Nesbitt SA, Lakkakorpi PT, Barnes MJ, Bodary SC, Shankar G, *et al*. Beta 1 integrins and osteoclast function: involvement in collagen recognition and bone resorption. Bone 1996;19:317–28.
48. Holliday LS, Welgus HG, Fliszar CJ, Veith GM, Jeffrey JJ, Gluck SL. Initiation of osteoclast bone resorption by interstitial collagenase. J Biol Chem 1997;272:22053–8.
49. Shibakawa A, Yudoh K, Masuko-Hongo K, Kato T, Nishioka K, Nakamura H. The role of subchondral bone resorption pits in osteoarthritis: MMP production by cells derived from bone marrow. Osteoarthritis Cartilage 2005;13(8):679–87.
50. Theoleyre S, Kwan Tat S, Vusio P, Blanchard F, Gallagher J, Ricard-Blum S, *et al*. Characterization of osteoprotegerin binding to glycosaminoglycans by surface plasmon resonance: role in the interactions with receptor activator of nuclear factor kappaB ligand (RANKL) and RANK. Biochem Biophys Res Commun 2006;347:460–7.
51. Itonaga I, Sabokbar A, Sun SG, Kudo O, Danks L, Ferguson D, *et al*. Transforming growth factor-beta induces osteoclast formation in the absence of RANKL. Bone 2004;34:57–64.
52. Ramsden L, Rider CC. Selective and differential binding of interleukin (IL-)-1a, IL1β, IL-2 and IL-6 to glycosaminoglycans. Eur J Immunol 1992;22:3027–31.
53. Sobal G, Sinzinger H. Binding of [99mTc]chondroitin sulfate to scavenger receptors on human chondrocytes as compared to binding of oxidized [125I]LDL on human macrophages. J Recept Signal Transduct Res 2002;22:459–70.
54. Kawashima H, Hirose M, Hirose J, Nagakubo D, Plaas AH, Miyasaka M. Binding of a large chondroitin sulfate/dermatan sulfate proteoglycan, versican, to L-selectin, P-selectin, and CD44. J Biol Chem 2000;275:35448–56.
55. Suwan K, Choocheep K, Hatano S, Kongtawelert P, Kimata K, Watanabe H. Versican/PG-M assembles hyaluronan into extracellular matrix and inhibits CD44-mediated signaling toward premature senescence in embryonic fibroblasts. J Biol Chem 2009;284:8596–604.
56. Ajisaka K, Agawa S, Nagumo S, Kurato K, Yokoyama T, Arai K, *et al*. Evaluation and comparison of the antioxidative potency of various carbohydrates using different methods. J Agric Food Chem 2009;57:3102–7.