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OSTEOARTHRITIS and CARTILAGE

Effects of chondroitin sulfate and interleukin-1β on human articular chondrocytes cultivated in clusters

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Summary

Objective: To test the effects of chondroitin sulfate (ACS, a glycosaminoglycan of cartilage) with and without interleukin-1β (IL-1β) on human articular chondrocytes cultivated in clusters and in long-term (0–16 days or 16–32 days).

Design: Chondrocyte productions of proteoglycans (PGs), type II collagen (coll-II) and prostaglandin E_2 (PGE_2) were assayed by specific radioimmunoassays applied to conditioned culture media and to clusters.

Results: During the two culture periods (0–16 days or 16–32 days), ACS (100–1000 μg/ml) increased total PG production and had no effect on the production of coll-II by chondrocytes. During the first 16 days, ACS (500–1000 μg/ml) decreased total PGE_2 synthesis. IL-1β decreased PG and coll-II productions and increased PGE_2 synthesis. During the first period (0–16 days), while the cluster is forming, ACS counteracted the IL-1β-induced effects on PG (500–1000 μg ACS/ml), coll-II (100–1000 μg ACS/ml) and PGE_2 (500–1000 μg ACS/ml) productions. During the second period (16–32 days), when the cluster is already formed, ACS counteracted the IL-1β-induced effects on total PG (100–1000 μg ACS/ml), coll-II (1000 μg ACS/ml) and PGE_2 (1000 μg ACS/ml) productions.

Conclusions: These *in vitro* studies suggest that ACS is able to increase matrix component production by human chondrocytes and to inhibit the negative effects of IL-1β.

Key words: Osteoarthritis, Cartilage, Culture, Glycosaminoglycan.

Introduction

Articular cartilage is a connective tissue composed of cells (chondrocytes) located inside a matrix essentially constituted by proteoglycans (PGs) and type II collagen (coll-II) [1]. Cartilage is subject to anabolic processes (chondroformation) and catabolic processes (chondroresorption). Chondrocytes are able to maintain a dynamic equilibrium between these processes. In articular diseases, such as in osteoarthritis (OA), catabolic processes exceed anabolic processes, leading to a net decrease of matrix material [2]. Anabolic and catabolic processes are influenced by agents such as cytokines, hormones, growth factors and precursors of matrix elements. Interleukin-1 (IL-1) is a cytokine that is involved in the cartilage degradation [3]. IL-1 is produced by various cell types (monocytes, macrophages, chondrocytes, etc.) [4] and has been detected in synovial fluid of patients with OA [5]. IL-1 influences chondrocyte metabolism: it induces prostaglandin E_2 (PGE_2) production [6] and protease activation and production (e.g., metalloproteases such as collagenase and stromelysin) [7].

We have previously described a system of three-dimensional culture of chondrocytes [8]. This culture model allows enzymatically isolated human chondrocytes to aggregate and form a cluster. In the cluster, chondrocytes are morphologically and biosynthetically differentiated (cells with a round shape located inside a newly synthesized matrix composed of coll-II and cartilage PGs [8–12]). In this culture model there are two phases: the cluster formation (a large production of matrix components taking place during the first 2 weeks),

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then a decrease in matrix component production when cells are surrounded by the matrix.

In this work, we have studied the *in vitro* effects of chondroitin sulfate (ACS) on human articular chondrocytes. These cells were extracted from osteoarthritic femoral head cartilage and cultivated in clusters in the presence or in the absence of IL-1β, during the first 16 days of culture or from day 16 to day 32. By use of specific and sensitive radioimmunoassays, we have studied the effects of ACS ± IL-1β on two anabolic processes (PG and coll-II production in conditioned culture media and inside clusters) and PGE_2 production.

Materials and Methods

THREE-DIMENSIONAL CULTURE OF HUMAN CHONDROCYTES

The effects of ACS ± IL-1β were assessed in human chondrocytes in culture, according to a previously defined methodology [8]. Human chondrocytes were cultivated in Dulbecco's modified of Eagle's medium, supplemented with 1% Ultroser G (Gibco, Gent, Belgium) and 50 μg/ml ascorbate. Macroscopically healthy cartilage was taken from three osteoarthritic human femoral heads, immediately after surgery for total hip prostheses (mean age of the patients: 65 years). Histological analyses of cartilage from these samples showed some typical osteoarthritic modifications such as clusters of chondrocytes, loss of metachromasy and fibrillation. Cartilage was digested by 1 mg/ml clostridial collagenase (Boehringer Mannheim GmbH, Germany) in buffer (NaCl 120 mmol/l, KH_2PO_4 5 mmol/l, $NaHCO_3$ 25 mmol/l, glucose 3 mmol/l and Hepes 30 mmol/l, pH 7.4) for 24 h. After six successive washings and centrifugations, the cells were suspended in glass flasks (Sovirel; 10^6 cells/2 ml culture medium) and placed on a gyrotary shaker (100 rpm). Cultures were maintained at 37°C in a 95% air/5% CO_2 atmosphere.

Chondroitin sulfate (ACS, Structum®, 24 kDa; Pierre Fabre Médicament, France) was extracted from bovine cartilage. The product, chondroitin sulfate, has been characterized, using various analytical methods (high performance liquid chromatography, electrophoreses). These analyses clearly showed a high level of purity (>98%). No other glycosaminoglycan (GAG) has been detected in the preparation. No proteins were detected either by gel electrophoresis or by Lowry and Bradford methods in the final ACS preparation. Assays of amino acids showed levels <0.1% for five amino acids. The extraction process including acidic and basic phases can definitely exclude the presence of agents such as growth factors or cytokines. The ACS-4:ACS-6 ratio is approximatively 1.5. ACS (0, 100, 500, 1000 μg/ml) with or without human recombinant IL-1β (Boehringer Mannheim GmbH, Germany; 2 IU/ml) was added to and maintained in the culture medium throughout the whole culture duration (0–16 days) or from day 16 to day 32. Culture media were renewed every 4 days. For each concentration of the drug and for corresponding controls, four flasks were used; each flask contained one chondrocyte cluster obtained from $\pm 10^6$ isolated chondrocytes.

PGs, coll-II and PGE_2 were radioimmunologically assayed in conditioned culture media and in clusters. At the end of the culture, the chondrocyte clusters were prepared for further fluorimetric DNA assay and for radioimmunoassays, as follows: chondrocyte clusters were washed three times with phosphate-buffered saline (PBS: PO_4 0.05 mol/l, NaCl 0.15 mol/l, pH 7.4), then homogenized in PBS in the presence of protease inhibitors [13], sodium azide 6.7×10^{-3} mol/l and superoxide dismutase (200 U/ml) by ultrasonic dissociation for two 30-s intervals at 4°C (power: 200 W/cm^2). The protease inhibitors were 6-amino-hexanoic acid 0.1 mol/l, EDTA 0.01 mol/l, benzamidine chlorhydrate 0.05 mol/l, trypsin inhibitor 5×10^{-8} mol/l.

PG ASSAY

PGs released into culture media and chondrocyte clusters contents were radioimmunologically assayed according to a previously described method [14]. Briefly, human cartilage PG were extracted according to the methods of Roughley *et al*. [15] and Bayliss and Venn [16]. Antiserum against human cartilage PG was raised in rabbits according to the method of Vaitukaitis *et al*. [17]. The radioimmunoassay was performed in a 0.4 ml incubation volume consisting of 0.1 ml tracer solution (15 000–20 000 cpm [^{125}I]-PG labeled by the chloramine-T method) [18], 0.1 ml anti-PG antiserum diluted 1:5000 in PBS, 0.15 mol/l NaCl, pH 7.4) containing 5 g/l bovine serum albumin (incubation buffer) and 0.2 ml of serial dilutions of conditioned culture media, cluster extracts or of unlabeled antigen ranging from 0.1 to 500 ng/tube (reference curve). After 4 days at 4°C, the labeled PG-antibody complexes were separated by double precipitation [19]. The analytical sensitivity of the radioimmunoassay was 0.6 ng/tube. The intra- and inter-assay coefficients of variation were less than 10 and 20%,

Table I
Binding measures performed

Receptor	Cell culture	Reference molecule	Bibliographic reference
IL-1β	Balb/c 3T3	IL-1β	Bird *et al*. [25]
IL-1α	CHO cells	IL-1α	Horuk [26]

respectively, along the linear part of the curve. As proven by digestion experiments and the absence of cross-reactivity of GAGs, the antibodies were solely directed against the antigenic determinants of the PG core protein. In addition, there were no interference of coll-II, fibronectin, ACS or hyaluronic acid with the assay [14]. ACS (up to 10 mg/mL) did not cross-react in the assay.

COLL-II ASSAY

Type II collagen was assayed radioimmunologically according to a previously described methodology [20]. Briefly, coll-II was extracted from human articular cartilage [21] and used as immunogen, tracer and reference preparation. A polyclonal antiserum was obtained in the guinea-pig. The radioimmunoassay was performed in a sequential manner. First, 0.1 ml incubation buffer (PBS; 0.3 mol/l NaCl, pH 7.4), 0.1 ml of coll-II reference solution (1–500 ng), culture media or cluster extracts and 0.1 ml guinea-pig antiserum (1:5000) were mixed. Second, 0.1 ml [^{125}I]-labeled coll-II (using the Iodogen method [22] and diluted to obtain 20 000 cpm) was added. After 18 h at 4°C, the labeled PG-antibody complexes were separated by double precipitation [19]. Cartilage PG, fibronectin, laminin, type I or type III collagen, GAGs did not interfere in the assay. The detection limit of the assay was 20 ng/ml. Intra- and inter-assay coefficients of variation were 8 and 15% respectively.

DNA ASSAY

Cluster DNA content was assayed according to the fluorimetric method of Labarca and Paigen [23]. This method is based on the principle of a fluorescent emission when the reactive (fluorochrome-bis-benzimidazol, Calbiochem-Behring, La Jolla, CA, U.S.A.; Hoechst dye 33258) is bound to DNA.

PROSTAGLANDIN E_2 ASSAY

Prostaglandin E_2 was assayed in conditioned culture media according to a radioimmunoassay previously described [24]. Briefly, a polyclonal antiserum was obtained in the rabbit. The [^{3}H]-PGE_2 tracer came from Amersham. This tracer was diluted in the incubation buffer in order to obtain 100 000 cpm/ml. The PGE_2 standard came from Sigma Chemical Corporation (Saint Louis, U.S.A.). The incubation buffer was composed of Tris-HCl 0.01 mol/l, NaCl 0.15 mol/l, gelatin 1 g/l, pH 7.4. To 0.1 ml of the sample to be assayed or to the cold reference antigen (0–2500 pg/ml) were added successively 0.1 ml buffer, 0.1 ml [^{3}H]-PGE_2 (100 000 cpm/ml) and 0.1 ml antiserum (diluted at 1:4000). After 48 h at 4°C, the free antigen was separated from the antigen bound to antibodies by charcoal (5 g/l) and dextran T70 (500 mg/l) precipitation. There was no cross-reaction between this antiserum anti-PGE_2 and other prostanoids, nor with other fatty acids. The detection limit of the assay was 20 pg/ml. Intra- and inter-assay coefficients of variation were 6 and 10% respectively.

MEASURES OF ACS BINDING TO IL-1β

The binding measures were performed as in Table I. Experimental conditions are summarized as in Table II. After the incubation, monolayer cells were washed with cold buffer. Fixated radioactivity was measured with a liquid scintillation counter (Packard) using scintillant liquid (Microscint 0) [25, 26].

Table II
Summary of experimental conditions

Receptor	Ligand	Concentration	Nonspecific	Incubation
IL1-β	[^{125}I]-IL-1β	0.1 nM	IL-1β (10 nM)	240 min/4°C
IL-1α	[^{125}I]-IL-1α	60 pM	IL-1α (4.4 nM)	195 min/37°C

CALCULATION AND STATISTICAL ANALYSIS

The mean ± S.D of each variable (released proteoglycans, coll-II, PGE_2 in culture media and cluster contents) was calculated. Comparison of mean values was performed using the analysis of variance (ANOVA) test. The results were expressed as amounts measured in culture media per microgram of DNA, and as cluster contents per microgram DNA.

Results

PROTEOGLYCAN AND TYPE II COLLAGEN PRODUCTIONS

Results concerning proteoglycan and type II collagen assayed in culture media and clusters for cultures incubated with ACS ± IL-1β at each time point are presented in Figs 1, 2, 3, 4.

Three-dimensional culture during the first 16 days of culture PG production

When chondrocytes are cultivated in the presence of ACS (100, 500, 1000 μg/ml), ACS significantly increases ($P < 0.05$) total PG production in media (0–16 days) and inside chondrocyte clusters (at day 16) (Fig. 1).

IL-1β (2 IU/ml) induces a significant decrease of total PG production in culture media and inside chondrocyte clusters (IL-1β vs controls: $P < 0.05$) (Fig. 1).

When chondrocytes are cultivated in the presence of ACS and IL-1β, ACS (500–1000 μg/ml)

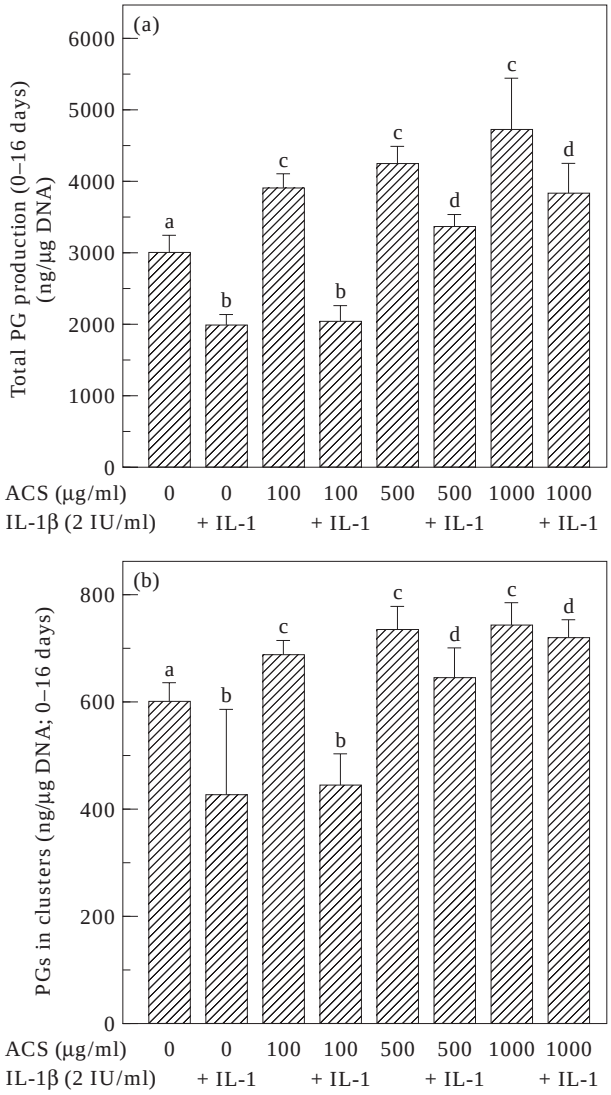

FIG. 1. PG production [(a) in culture medium; (b) in clusters] by human chondrocytes cultivated in clusters in the presence of ACS ± IL-1β during the first 16 days of culture. b and c vs a; b vs c; d vs b: $P < 0.05$.

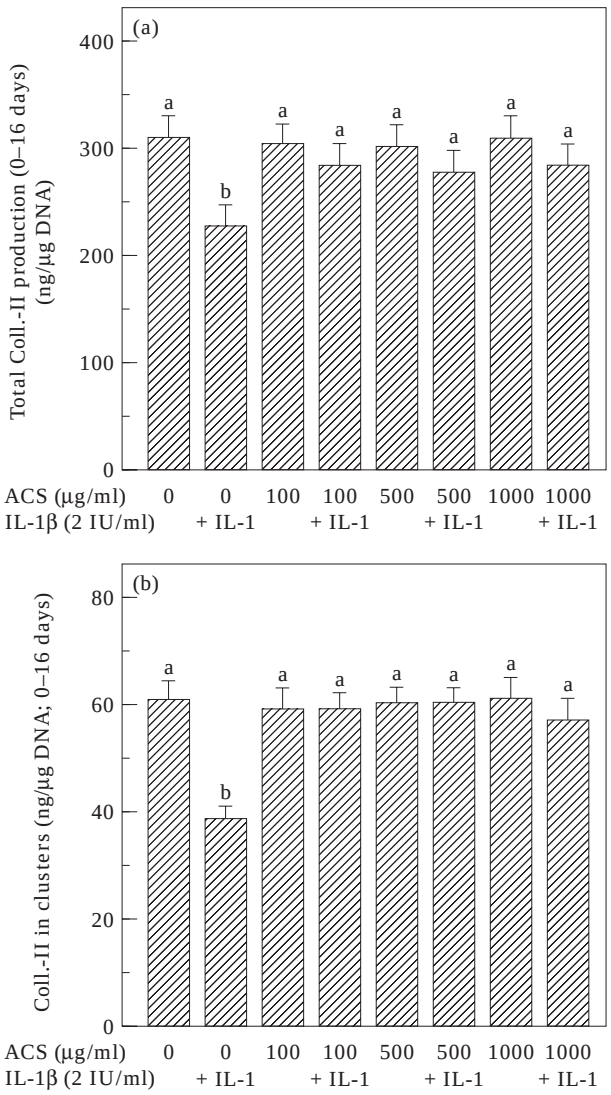

FIG. 2. Coll-II production [(a) in culture medium; (b) in clusters] by human chondrocytes cultivated in clusters in the presence of ACS ± IL-1β during the first 16 days of culture. a vs b: $P < 0.05$.

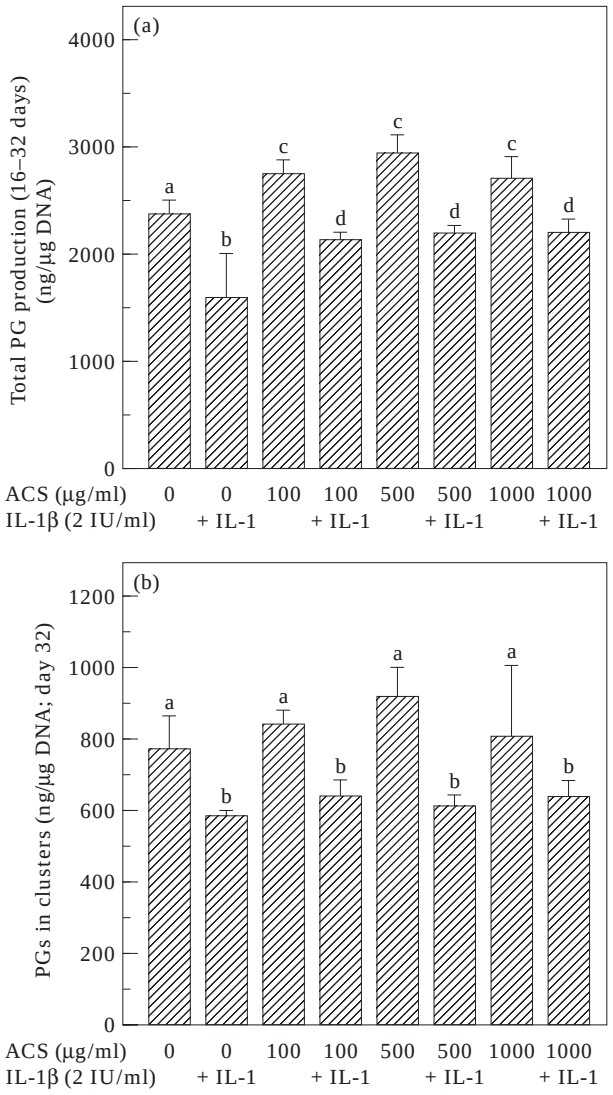

FIG. 3. PG production [(a) in culture medium; (b) in clusters] by human chondrocytes cultivated in clusters in the presence of ACS ± IL-1β, from day 16 to day 32. b and c vs a; b vs c; d vs b and c: $P < 0.05$.

counteracts the IL-1β-induced effects, in culture media and in the clusters (IL-1β vs IL-1β + ACS 500 or vs IL-1β + ACS 1000: $P < 0.05$) (Fig. 1).

Coll-II production

When human chondrocytes are cultivated in the presence of ACS (100, 500 or 1000 µg/ml), ACS does not affect total coll-II production in culture media and inside clusters (Fig. 2).

IL-1β (2 IU/ml) induces a significant decrease of total coll-II production in culture media (0–16 days) and inside chondrocyte clusters (at day 16) (IL-1β vs controls: $P < 0.05$) (Fig. 2).

Total coll-II production in media (from day 0 to day 16) and coll-II inside clusters are decreased by IL-1β ($P < 0.05$). ACS (100, 500, 1000 µg/ml) inhibits the IL-1β-induced effects (IL-1β vs ACS 100 + IL-1β or vs ACS 500 + IL-1β or vs ACS 1000 + IL-1β: $P < 0.05$) (Fig. 2).

Three-dimensional culture from day 16 to day 32

PG production

When chondrocytes are cultivated in the presence of ACS (100, 500, 1000 µg/ml), ACS significantly increases ($P < 0.05$) total PG production in media ($P < 0.05$) (Fig. 3).

IL-1β (2 IU/ml) induces a significant decrease of PG production in culture media (IL-1β vs controls: $P < 0.05$) and inside chondrocyte clusters at day 32 (IL-1β vs controls: $P < 0.05$) (Fig. 3).

As compared to controls, total PG production in culture media (from day 16 to day 32) is decreased by IL-1β ($P < 0.05$), is increased by ACS (100, 500 or 1000 µg/ml). ACS (at 100, 500 or 1000 µg/ml) has no effect on the PG amount in clusters. ACS (at 100, 500 or 1000 µg/ml) counteracts the IL-1β-induced effects IL-1β vs IL-1β + ACS 100 µg/ml or vs IL-1β + ACS 500 µg/ml or vs IL-1β + ACS 1000 µg/ml: $P < 0.05$) (Fig. 3).

Coll-II production

Coll-II production in culture media after 16 days of culture is not detectable by our radioimmunoassay (sensitivity: 20 ng/ml).

When human chondrocytes are cultivated in the presence of ACS (100, 500 or 1000 µg/ml), ACS does not affect coll-II production inside clusters (Fig. 4).

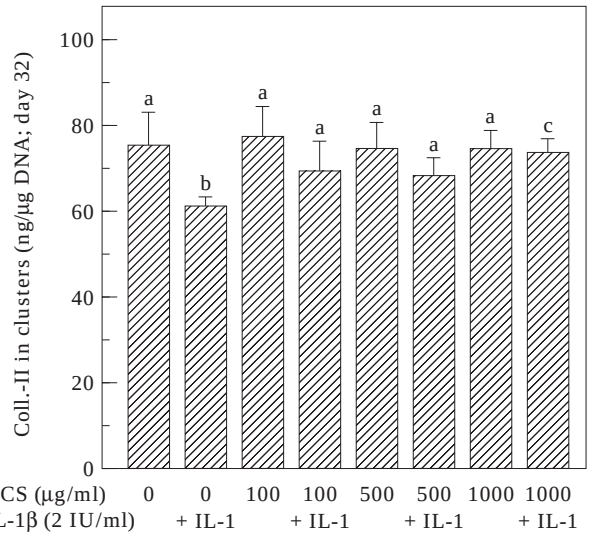

FIG. 4. Coll-II inside human chondrocyte clusters cultivated in the presence of ACS ± IL-1β from day 16 to day 32. a vs b; b vs c: $P < 0.05$.

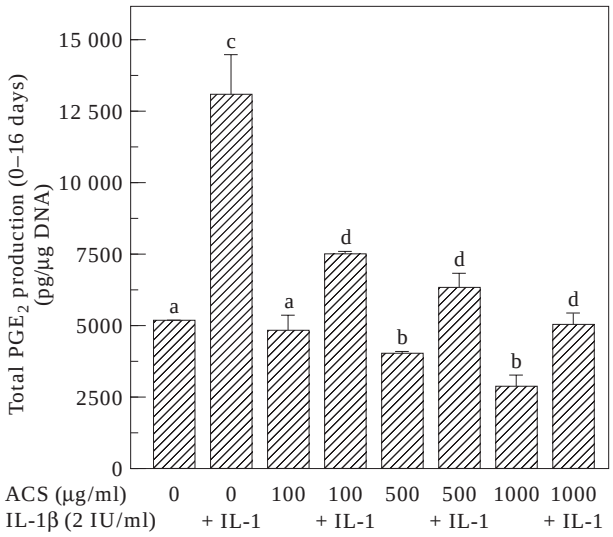

FIG. 5. Total PGE_2 production in culture medium by human chondrocytes cultivated in clusters in the presence of ACS ± IL-1β during the first 16 days of culture. b and c vs a; c and d v b; c v d: $P < 0.05$.

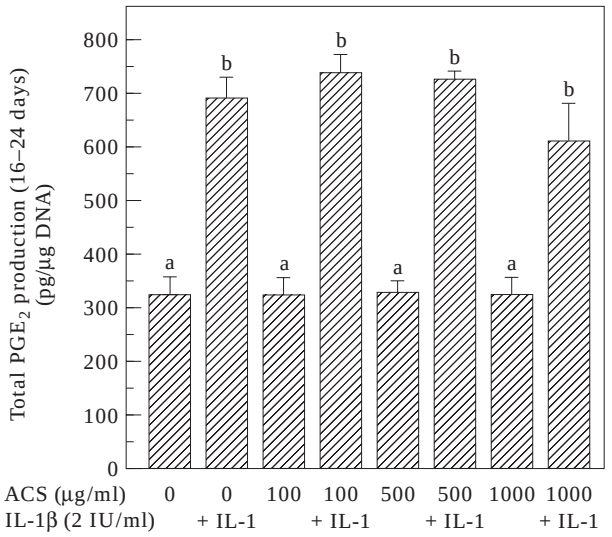

FIG. 6. Total PGE_2 production in culture medium by human chondrocytes cultivated in clusters in the presence of ACS ± IL-1β from day 16 to day 24. a vs b: $P < 0.05$.

IL-1β (2 IU/ml) induces a significant decrease of coll-II content inside chondrocyte clusters (at day 32) (IL-1β vs controls: $P < 0.05$) (Fig. 4).

When chondrocytes are cultivated in the presence of ACS and IL-1β, ACS (1000 µg/ml) counteracts the IL-1β-induced effects ($P < 0.05$) in chondrocyte clusters (Fig. 4).

PGE_2 PRODUCTION

Three-dimensional culture during the first 16 days of culture

IL-1β (2 IU/ml) induces a significant increase in total PGE_2 production in culture media (IL-1β vs controls: $P < 0.05$) (Fig. 5).

When compared with untreated controls, total PGE_2 production in media (from day 0 to day 16) is increased by IL-1β ($P < 0.05$) and decreased by ACS (500 or 1000 µg/ml) ($P < 0.05$) (Fig. 5). When chondrocytes are cultivated in the presence of ACS and IL-1β, ACS (100, 500 or 1000 µg/ml) significantly counteracts the IL-1β-induced effects (IL-1β vs ACS 100 µg/ml + IL-1β, or vs ACS 500 µg/ml + IL-1β or vs ACS 1000 µg/ml + IL-1β: $P < 0.05$) (Fig. 5).

Three-dimensional culture from day 16 to day 32

After 24 days of culture, PGE_2 production in culture media is not detectable by our radioimmunoassay (<20 pg/ml). Fig. 6 represents the total PGE_2 production in culture media from day 16 to day 24.

When chondrocytes are cultivated in the presence of ACS (100, 500 or 1000 µg/ml), ACS does not affect total PGE_2 production in culture media (Fig. 6) IL-1β (2 IU/ml) induces a significant increase of total PGE_2 production in culture media (IL-1β vs controls: $P < 0.05$) (Fig. 6).

When chondrocytes are cultivated in the presence of ACS and IL-1β, ACS (100, 500 or 1000 µg/ml) does not counteract the IL-1β-induced effects.

ACS BINDING TO IL-1α OR IL-1β

There is no binding of ACS to IL-1α or IL-1β (Table III).

Discussion

In our study, the long-term cluster culture model [8, 27–29] was used to test the effects of a cartilage

Table III
Binding of ACS to IL-1α and IL-1β

ACS concentration	IL-1α binding (%)	IL-1β binding (%)
100	102	109
300	110	104
1000	89	118

GAG, chondroitin sulfate, in the presence or absence of IL-1β, on human chondrocyte metabolism *in vitro*. These experiments confirm our previous results [11] and many studies conducted in various experimental models [30–33]: IL-1β induces a decrease in cartilage matrix element production (i.e.: coll-II and PGs) and an increase in PGE_2 synthesis. These effects of IL-1β are stronger at the beginning of the culture, during the first 2 weeks, than thereafter, from day 16 to day 32. During the first 2 weeks of culture, the chondrocyte cluster is forming and flaky, then it condenses while its weight and its PG and coll-II contents increase with culture duration [8]. It is possible that the access and fixation of IL-1β to its receptors is facilitated when the cells are surrounded by a flaky matrix. When the cluster is constituted, the IL-1β effects are weaker. Indeed, some works have shown that cartilage matrix is composed of small PGs (fibromodulin, decorin, biglycan) which are able to bind cytokines [34] and may decrease the effects of IL-1β.

Here, we show that ACS increases PG production by human chondrocytes in culture media and in clusters. Moreover, the ACS effects are stronger during the first 2 weeks of culture than from day 16 to day 32 when the cluster is constituted: ACS is more active when chondrocytes produce large amounts of matrix elements and organize their matrix. ACS does not affect basal coll-II production by chondrocytes. This is not surprising, since ACS is a cartilage GAG and might be used as substrate for PG synthesis. Other works (in other experimental models) have shown that ACS increases GAG synthesis by chondrocytes *in vitro* [35] and hyaluronic acid synthesis by synoviocytes *in vitro* [36].

When ACS is added to the culture medium during the first days, ACS counteracts the IL-1β-induced effects. This effect of ACS is stronger on PG production than on coll-II production. When ACS is added to culture medium from day 16 to day 32, when the cluster is constituted, ACS counteracts the IL-1β effects in a less potent way than when ACS is added at the beginning of culture. Concerning PG cluster content, there is a slow increase with culture duration (for example, in the presence of 100 µg ACS/ml: PG cluster content increases from 686.7 ± 25.2 ng/µg DNA at day 16 to 840.0 ± 35.2 ng/µg DNA at day 32). This slow PG accumulation in cluster may be due to an effect of ACS on PG synthesis processes and/or to the integration of ACS in aggrecan.

Concerning PGE_2 production, ACS (at 500 or 1000 µg/ml) decreases total PGE_2 production during the first 16 days of culture. Moreover, during this culture period, ACS (100–1000 µg/ml) inhibits the stimulating IL-1β effect on PGE_2 production. The large PGE_2 release observed at the beginning of culture and under the effect of IL-1β is significantly reduced by ACS. Mitrovic *et al*. [37] have shown that PGE_2 inhibit matrix element production by chondrocytes *in vitro*. In our experimental model, we have previously shown that shark ACS (10–1000 µg/ml) increases PG content in clusters and decreases collagenolytic activity measured in culture media [38]. Baici *et al*. [39–41] have shown that ACS partially inhibits elastase activity (elastase is a potent mediator of cartilage degradation). Moreover, according to the latter authors, interaction between elastase and GAGs seems achieved through electrostatic bonds between negatively charged sulfate groups and the enzyme positive charges. However, this interaction does not influence the enzyme active site, but induces an indirect loss of its catalytic efficacy. Kato *et al*. [42] have shown that another GAG, hyaluronic acid, forms a viscous barrier protecting cartilage, inhibiting IL-1-induced PGE_2 production by synoviocytes and decreasing the loss of cartilage PGs [43–44]. Another hypothesis is that the electronegative charges of ACS could bind cytokines responsible for matrix degradation [45]. However, studies measuring specific binding using IL-1 and ACS (100 to 1000 µg/ml) showed no binding of ACS to IL-1α or IL-1β. Further experiments are needed to determine if ACS can bind other cytokines such as TNFα or IL-6.

Transposition of these *in vitro* results to *in vivo* situation must consider *in vivo* ACS concentrations, particularly the synovial fluid concentration. Actually, total sulfated GAG concentrations in humans vary according to pathologies [46–47]: from 80–330 µg/ml in synovial fluid. ACS-4 and ACS-6 disaccharide assays seem to indicate 40–100 µg/ml. The ACS (Structum®) administration to patients would increase ACS and total GAG concentrations up to 100 µg/ml. At this concentration, ACS might inhibit IL-1β-induced effects *in vivo*; this hypothesis has to be tested in *in vivo* experimental models.

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