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OsteoArthritis and Cartilage (2006) **14**, 631–640
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doi:10.1016/j.joca.2006.01.008

Osteoarthritis and Cartilage

International Cartilage Repair Society

Activation by IL-1 of bovine articular chondrocytes in culture within a 3D collagen-based scaffold. An *in vitro* model to address the effect of compounds with therapeutic potential in osteoarthritis

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Summary

Objective: To determine the best protocol for the preparation of a tissue-engineered cartilage to investigate the potential anti-arthritic and/or anti-osteoarthritic effects of drugs.

Methods: Calf articular chondrocytes, seeded in collagen sponges were grown in culture for up to 1 month. At day 14 cultures received interleukin (IL)-1β (ranging from 0.1 to 20 ng/ml) for 1 to 3 days. Analyses of gene expression for extracellular matrix proteins, collagen-binding integrins, matrix metalloproteinases (MMPs), aggrecanases, TIMPs, IL-1Ra and Iκ-Bα were carried out using real-time polymerase chain reaction (PCR). Metalloproteinase activities were analysed in the culture medium using both zymography and fluorogenic peptide substrates.

Results: We selected a culture for 15 or 17 days with collagen sponges seeded with 10^7 chondrocytes showing a minimal cell proliferation, a maximal sulphated glycosaminoglycan (sGAG) deposition and a high expression of COL2A1, aggrecan and the α10 integrin sub-unit and low expression of COL1A2 and the α11 integrin sub-unit. In the presence of 1 ng/ml IL-1β, we observed at day 15 up-regulations of 450-fold for MMP-1, 60-fold for MMP-13, 54-fold for ADAMTS-4 and MMP-3 and 10-fold for ADAMTS-5 and IL-1Ra. Down-regulations of 2.5-fold for COL2A1 and aggrecan were observed only at day 17. At the protein level a dose-dependent increase of total MMP-1 and MMP-13 was noted with less than 15% in the active form.

Conclusions: This *in vitro* model of chondrocyte culture in three dimensional (3D) seems well adapted to investigate the responses of these cells to inflammatory cytokines and to evaluate the potential anti-inflammatory effects of drugs.
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Key words: Cartilage construct, Bovine chondrocyte, Cytokine (IL-1), Osteoarthritis.

Introduction

Several studies have demonstrated the important role played by inflammatory cytokines such as interleukin-1 (IL-1α and IL-1β) or tumour necrosis factor α (TNF-α) in mediating the cartilage degradation observed in rheumatoid arthritis (RA) and osteoarthritis (OA)[1–3]. The *in vitro* activation of cultured chondrocytes by IL-1 is associated with dramatic changes in the expression of a large number of genes that contribute to cartilage degradation. For example IL-1 stimulates the synthesis of several matrix metalloproteinases (MMPs) and other inflammatory cytokines and inhibits the production of key extracellular matrix proteins such as type II collagen and aggrecan[4,5].

In order to investigate the potential anti-arthritic and/or anti-osteoarthritic effects of drugs and to obtain information on their mechanism of action, it is important to culture chondrocytes within microenvironments as close as possible to *in vivo* conditions and without major changes in their cartilage phenotype. Stimulated by the first clinical results of cartilage defect repair by the transplantation of human autologous chondrocytes, reported by Brittberg *et al.*[6], several tissue-engineered cartilages have been prepared with isolated chondrocytes grown within various three-dimensional (3D) scaffolds. Synthetic (e.g. poly[DL-lactic-co-glycolic acid]) or natural (agarose, alginate, hyaluronan, chitosan and collagen) polymers have been seeded with differentiated or dedifferentiated chondrocytes or with mesenchymal stem cells (MSCs), under different culture conditions in order to select the best protocol for the clinical repair of partial- or full-thickness articular cartilage defects[7–13].

In our laboratory, we chose to seed freshly isolated bovine articular chondrocytes into collagen-based 3D matrices in the form of sponges[14–16] or gels[17]. We compared the influence of different culture parameters (static, stirred or perfusion conditions; presence of 10% foetal calf serum (FCS) or serum-free media supplemented with ITS (insulin, transferrin, selenium) and cysteine[16]; absence of fresh ascorbic acid in the culture medium[15]) on the composition and properties of reconstituted cartilage under long-term culture (up to 3 months). In order to characterise the mechanism of action of a symptomatic acting drug in OA (Avocado/Soybean

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Received 27 April 2005; revision accepted 13 January 2006.

Unsaponifiables, ASU)[18,19] we have developed a model of *in vitro* reconstituted articular cartilage using bovine chondrocytes activated or not by an inflammatory cytokine. In this first paper we describe the best protocol selected to test the activation of chondrocytes by IL-1 and their response at the gene and protein level. Articular chondrocytes from young calves, easily available in large numbers, were seeded at two initial cell densities into collagen sponges and cultured for 15 or 30 days. Under these conditions, matrix deposition and density remain low, thereby permitting easy access of the active molecules to cells even in the centre of the construct[20]. To investigate the effects of IL-1 (0.1–20 ng/ml from day 14 for 1 or 3 days) on chondrocyte behaviour, we determined gene expression by using real-time reverse transcriptase polymerase chain reaction (RT-PCR) for several bovine genes coding for (1) matrix proteins (type I and II collagens, aggrecan), (2) collagen receptor integrin sub-units (α1, α2, α10, α11), (3), MMP-1, -2, -3, -13 and aggrecanases (ADAMTS-4, -5), and (4) TIMP-1, -2 (tissue inhibitor metalloproteinase) and proteins involved in the IL-1 signalling pathway (IL-1Ra, Iκ-Bα). At the protein level we measured the active and inactive forms of collagenases 1 and 3 (MMP-1 and MMP-13), using specific fluorogenic substrates and gelatinases A and B (MMP-2 and MMP-9) using zymography.

Materials and methods

CELL CULTURE

Collagen sponges, manufactured by Coletica (Lyon, France), were composed of collagen extracted from the skin of young calves containing 90–95% (dry weight) of native type I collagen and 4–9% type III collagen. In this study, 6 mm thick sponges were cross-linked using the carbodiimide method[21] to increase their stability. In brief, collagen sponges (9 × 8 cm) were incubated for 30 min in a 2-morpholinoethane sulphonic acid buffer (MES, Fluka, St Quentin Fallavier, France) in ethanol/water (40% v/v) (50 mM, pH 5.5) (400 ml/g). Cross-linking was carried out in the same buffer containing 14 mM *N*-[3-dimethylaminopropyl]-*N′*-ethylcarbodiimide hydrochloride (EDC) and 8 mM *N*-hydroxy-succinimide (NHS) (Accros, Noissy Le Grand, France). The reaction was performed at room temperature for 4 h. After removal of the cross-linking solution, samples were incubated for 2 h in sodium phosphate (0.1 M, pH 8.8). Finally the matrices were washed successively with 1 M NaCl and 2 M NaCl for 2 h (with five changes of washing solution), respectively, followed by washing with distilled water for 1 day and lyophilisation. Individual discs were cut out with a 10 mm diameter punch before being sterilised with 15 kGy β radiation. Such sponges, after sterilisation, exhibited thermal transition temperatures of 39°C (onset) and 48°C (peak) as measured by differential scanning calorimetry (Symatèse biomatériaux, Chaponost, France).

Freshly isolated chondrocytes (day 0) were obtained by enzymatic digestion of cartilage from the metacarpophalangeal joints of 6-month-old calves and 10^6 and 10^7 cells were seeded onto each sponge placed in a 24-well culture plate, as previously described[14,15]. Two hours after seeding, 2 ml of culture medium [RPMI/NCTC (v/v) (Sigma) containing 10% FCS (Biochrom KG, Berlin, Germany), 50 U/ml penicillin, 50 μg/ml streptomycin and 1 ng/ml amphotericin] were added to each well and the plates were further incubated at 37°C and 5% CO_2. Two days later, the sponges were cultured in 12.5 cm^2 culture flasks (three sponges in 15 ml of culture medium) and placed on a Polymax 1040 orbital shaker (30 rpm). As the drug to be tested in this model is soluble in ethanol, we checked the influence of this solvent added in the culture medium at a concentration of 0.2%. At day 14, some sponges were grown in serum-free medium containing rHu IL-1β (PromoKine, Heidelberg, Germany) at 0.1, 0.25, 0.5, 1, 5 and 20 ng/ml for 1 or 3 days. The medium was replaced every day during the whole culture.

CELL PROLIFERATION AND GAG CONTENT

The amount of DNA was measured using Hoechst 33258 dye and cell numbers were calculated based on the known DNA content per chondrocyte of 7.8 pg[14]. The sulphated glycosaminoglycan (sGAG) content in the sponges was determined spectrophotometrically using a modified dimethylmethylene blue method and shark chondroitin sulphate as a standard[14]. In all cases, three samples were analysed per condition in three independent experiments. The sGAG content was also measured in the culture medium of one experiment. In brief, the media of treated cultures were collected and pooled every 3 days for up to 15 or 30 days of treatment. Each sample (45 ml) was freeze-dried and re-suspended with 5 ml 10 mM Tris HCl buffer, pH 8 before papain (Sigma) digestion (6 mg/ml in 10 mM Tris HCl, 1 mM $CaCl_2$) for 2 h at 60°C. Then, the samples were dialysed against water for 2 days at 4°C before measurement of the sGAGs.

GENE EXPRESSION ANALYSIS

Total RNA was isolated from the chondrocyte-seeded collagen sponges with the RNeasy kit (Qiagen, Courtaboeuf, France) and digested with DNase to remove any contaminating genomic DNA. Reverse transcription of 0.5 μg total RNA was carried out under conditions previously described[15].

For real-time PCR, the reaction contained 10 μl of IQ SYBR green supermix (Biorad, Marnes la Coquette, France), 4 μl of 1/3 diluted RT in sterilised water, 1 μl of each primer at 0.3 μM and 4 μl water. Following an initial step of 1 min at 95°C and a denaturation step of 2 min at 95°C, fragments were amplified with n cycles of denaturation (95°C, 15 s), annealing/elongation for 30 s using primers specifically designed for this application (Table I). Amplification was performed in an iCycler IQ™ (Biorad). The PCR efficiencies of amplification (at threshold level = 200) for the studied genes ranged from 90 to 110%. For each cDNA sample the C_t value of the reference gene L30[22] was subtracted from the C_t value of the target sequence to obtain the ΔC_t. The level of expression was then calculated as $2^{-\Delta C_t}$ and expressed in relative quantity/L30 (RQ/L30) as the mean ± SD of three or six samples in three experiments. In this work relative changes equal to or greater than 2-fold were considered as biologically significant. All the PCR amplified products were purified for identification by sequence analysis (Genome express, Meylan, France). L30 was used to verify that equal amounts of RNA were added to the reaction. Differences in gene expression were analysed just after collection of the chondrocytes (day 0) and at 14 + 1 and 14 + 3 days of culture.

ANALYSIS OF MMP-1, -2, -9 AND -13 IN CULTURE MEDIUM OF CHONDROCYTES TREATED OR NOT WITH IL-1β

To assay metalloproteinase activities as described[23,24], 50 μl of conditioned media, collected at day 15 or 17 was incubated for 1 h in 440 μl Tris HCl buffer containing

Table I

Genes	Forward and reverse primers (5′−3′)	Product size (bp)	Annealing temperature (°C)	References
MMP-2	GGGGAGATTCCCACTTTGAT TGCAGCTGGTGTACTCCTTG	138	57	NM174745
MMP-3	TGGACCTGGAAAAGTTTTGG AGCTTCACGTTCGGTTGAGT	178	59	AF135232
ADAMTS-4	CTCCATGACAACTCGAAGCA CTAGGAGACAGTGCCCGAAG	169	59	NM181667
ADAMTS-5	CACCTCAGCCACCATCACAG AGTACTCTGGCCCGAAGGTC	152	59	AF192771
IL-1Ra	CTCCAGAGATGCCTGTCCAC CCAGGGCTCTTGTACCTCCT	106	59	AB005148
Iκ-Bα	TACTCCCCGTACCAGCTCAC GCTCTCCTCATCCTCGCTCT	114	59	AJ420928
TIMP-1	CACCCACAGACGGCCTTCT CTGGTATAAGGCAGTTTCATTGACTT	90	60	AF144763
TIMP-2	TGGGTCACGGAGAAGAACATC GAGCCGTCGCTTCTCTTGAT	71	60	AF144764

200 mM NaCl and 5 mM $CaCl_2$. The buffers were prepared at 125 mM and 100 mM for MMP-1 and MMP-13, respectively. To measure total MMP (active MMP + proMMP) 5 μg/ml trypsin (Sigma) was added for the activation of proMMP. The active MMP level was measured in the sample not incubated with trypsin. To stop the reaction, 10 μl of trypsin inhibitor (50 μg/ml) (Sigma) was added to the samples and incubated for 15 min at RT. Finally the specific fluorogenic substrates [(DNP-Pro-Cha-Abu-Cys(Me)-His-Ala-Lys(N-Me-Abz)-NH_2] for MMP-1 at 5 μM and [MCA-Pro-Cha-Gly-Nva-His-Ala-Dpa-NH_2] for MMP-13 at 8 μM (Calbiochem, Strasbourg, France) were added to the reaction vial and 2 aliquots of 200 μl were added to individual wells in a 96-well plate. The plate was further incubated for 4 h at 37°C and the fluorescence read in an FLX-800 Bio-Tek Instrument at Ex/Em 365 nm/450 nm for MMP-1 and 325 nm/393 nm for MMP-13. A control corresponding to the medium collected from non-seeded sponges was also analysed.

Gelatinase activity was measured by gelatin-substrate zymography as previously described[15] using the culture medium from chondrocytes seeded in collagen sponges and grown with 1, 5 or 20 ng/ml IL-1β for 1 or 3 days in medium without serum. Thirty microlitre samples of medium collected after 24 or 72 h of treatment were mixed with 4× Laemmli's non-reducing loading buffer and subjected to electrophoresis in 10% polyacrylamide gels containing 0.1% gelatin. After electrophoresis, the gels were treated as previously published[15].

STATISTICAL ANALYSIS

The statistical significance of quantitative parameters was assessed using a Student's *t*-test.

Results

CALF CHONDROCYTES CULTURED IN COLLAGEN SPONGES: CELL PROLIFERATION AND sGAG DEPOSITION

First, in order to determine the best experimental conditions to check the effect of IL-1β on chondrocytes, we compared the cell proliferation and sGAG deposition of calf articular chondrocytes seeded in collagen sponges at two initial cell densities (10^6 and 10^7 cells/sponge) and cultured for 15 and 30 days. As shown in Fig. 1(A, B), a 6−7-fold increase in cell numbers after 15 and 30 days of culture was observed in sponges initially seeded with 10^6 cells (A), whereas cell numbers remained unchanged, in sponges seeded with 10^7 cells (B) after 15 and 30 days (11×10^6 and 9×10^6 cells, respectively). In all cases no significant differences in cell content were observed in culture media with or without 0.2% ethanol.

As shown in Fig. 1(C, D), the sGAG content of sponges seeded with 10^6 (C) and 10^7 (D) cells were 106 and 170 μg/sponge, respectively, after 15 days in culture and 150 and 180 μg/sponge, respectively, after 30 days. In one set of experiments (10^7 cells/sponge) we measured (not shown) the total sGAG secreted in the medium. After 15 and 30 days in culture the values obtained were 750 and 1100 μg/sponge, respectively. If compared to the sGAG content present in the sponges at the same time (around 180 μg/sponge) it appears that after 15 days more than 82% of the sGAG are secreted in the medium and more than 86% after 30 days.

In all cases no significant differences were observed in sGAG content in sponge or in culture media with or without 0.2% ethanol. A slight contraction (15% of their initial diameter) of the sponges seeded with 10^6 or 10^7 cells was observed after 15 days, without modification after 30 days.

From these data, we selected for the rest of the study a culture for 15 days of sponges initially seeded with 10^7 calf chondrocytes. Under these conditions cell multiplication was minimal and sGAG deposition was maximal whereas the addition of 0.2% ethanol in the medium was without effect on these parameters.

GENE EXPRESSION OF CALF CHONDROCYTES, IN 3D CULTURE, TREATED OR NOT BY IL-1β

Real-time PCR (Figs. 2−5) was used to quantify and compare the expression of the genes coding for collagen types I (α2 chain) and II (α1 chain), aggrecan, integrin sub-units α1, α2, α10 and α11, MMP-1, -2, -3 and -13, ADAMTS-4 and -5, TIMP-1 and -2 and for two molecules involved in the IL-1β signalling pathway, IL-1Ra and Iκ-Bα. The following conditions were analysed: freshly isolated cells (day 0) and after 15 and 17 days in culture without IL-1β (open bars in Figs. 2−5) and after IL-1β treatment at day 14 for 1 and 3 days (grey bars in Figs. 2−5). For all the genes studied the addition of 0.2% ethanol in the medium did not modify their expression at day 15 (as shown by comparison of the open bars noted 0 and 0/+ in Figs. 2−5).

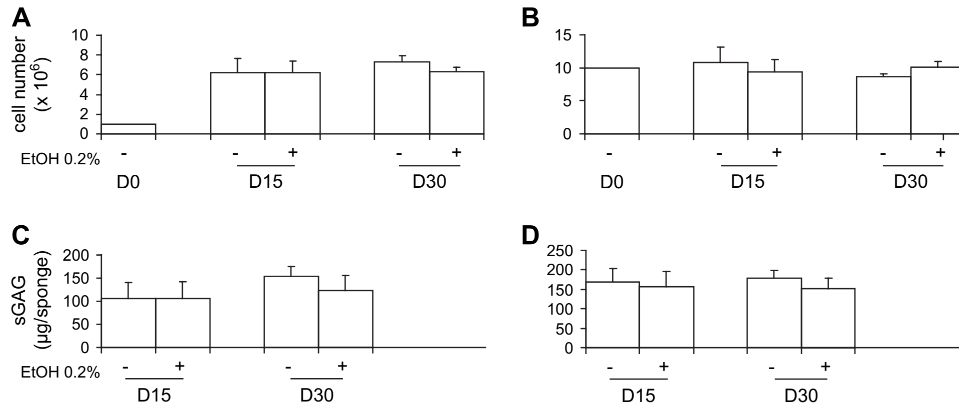

Fig. 1. Cell number and sGAG content of bovine chondrocytes seeded in collagen sponges. One (A and C) or ten million (B and D) cells were initially seeded in sponges and grown throughout the culture in serum supplemented medium (10% FCS) containing or not 0.2% ethanol. Cell numbers were quantified by measuring the DNA content of the sponges after 15 and 30 days (A and B) of culture. sGAG content was measured using a colorimetric method with dimethylmethylene blue after 15 and 30 days (C and D). Data are presented as the mean ± S.E.M. of triplicate samples obtained from three independent experiments.

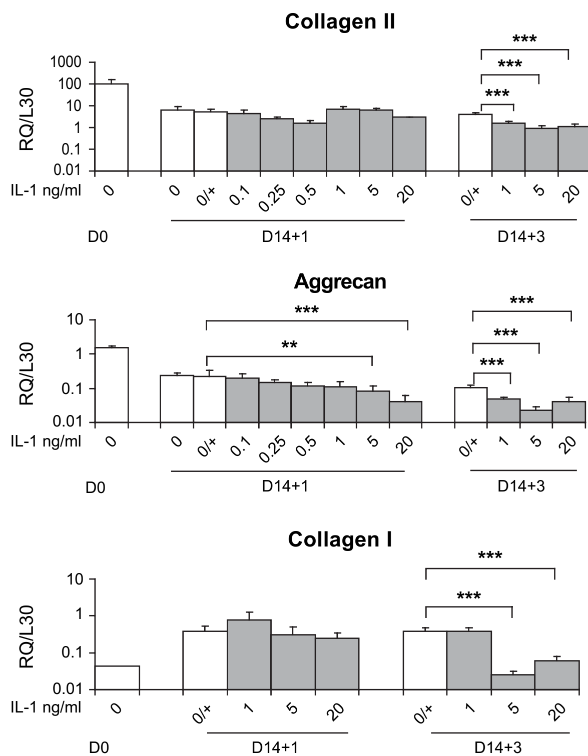

Fig. 2. Effect of the presence of IL-1β on the expression of cartilage matrix specific genes, when added on day 14 to 10^7 bovine chondrocytes seeded in collagen sponges. Cultures were grown in serum-free medium for 1 or 3 days containing IL-1β at 0.1, 0.25, 0.5, 1, 5, 20 ng/ml and 0.2% ethanol. Control cultures received or not 0.2% ethanol noted 0/+ and 0. RNAs were extracted from sponges followed by real-time PCR as described in Materials and methods. The amount of mRNA obtained was normalised to the amount of L30. Results are expressed in relative quantity/L30 (RQ/L30) ± SD from three experiments carried out with three to six samples. Comparisons were performed using the Student's *t*-test: **, $P < 0.01$; ***, $P < 0.001$).

As Fig. 2 shows, a decrease in the expression of COL2A1 (around 20-fold) and aggrecan (around 6.5-fold) and an increase in the COL1A2 expression (10-fold) were observed after 15 and 17 days of culture. As a consequence the relative expression ratio of COL2A1/COL1A2, as high as 2550 for the freshly isolated chondrocytes (day 0), reached 12.5 and 10 after 15 and 17 days of culture, respectively. However type II collagen remained the main collagen expressed under these culture conditions. In the presence of IL-1β, a significant decrease in expression of aggrecan was observed after 1 day of culture and with a concentration ≥5 ng/ml and after 3 days of culture for COL2A1 and aggrecan (concentration ≥1 ng/ml) and for COL1A2 (concentration ≥ 5 ng/ml). Interestingly the relative ratio of the expression level of COL2A1/COL1A2 remained similar.

In Fig. 3, we compared the expression of α1, α2, α10 and α11 integrin sub-unit genes. In freshly isolated chondrocytes we detected low levels of α10 (8×10^{-3}/L30) and α1 (3×10^{-3}/L30) and very low levels of α2 (7×10^{-4}/L30) and α11 (1.5×10^{-4}/L30) integrin sub-unit mRNAs. After 15 days of culture the α1 integrin sub-unit mRNA level increased (7.5-fold) whereas it decreased for the α2 and α10 sub-units (24- and 9-fold, respectively). The level of expression of the α11 sub-unit was not changed. After treatment for 1 day with 1 or 5 ng/ml IL-1β the expression of these genes were not significantly changed.

Figure 4 shows the gene expression level of four metalloproteinases (MMP-1, -2, -3 and -13) and aggrecanases-1 and -2 (ADAMTS-4 and -5). In freshly isolated cells the expression of the MMP-2 and MMP-3 genes was similar and at a relatively high level (8×10^{-2}/L30) whereas for the other genes expression was very low: 2×10^{-3}/L30 for ADAMTS-5, 9×10^{-4}/L30 for MMP-13 and ADAMTS-4 and only 4×10^{-5}/L30 for MMP-1. After 15 days of culture the variations were not very large.

In the presence of IL-1β, with the exception of the MMP-2 gene, all the other genes presented dose-dependent and significant increases in their expression. After 1-day treatment, a significant increase (between 4- and 9-fold) was first obtained with 0.5 ng/ml IL-1β. With 1 ng/ml IL-1β the expression levels reached a plateau with increases as high as 450-fold for MMP-1, 60-fold for MMP-13, 54-fold for ADAMTS-4 and MMP-3 and 10-fold for ADAMTS-5. After 3 days of culture, a dose-dependent increase was

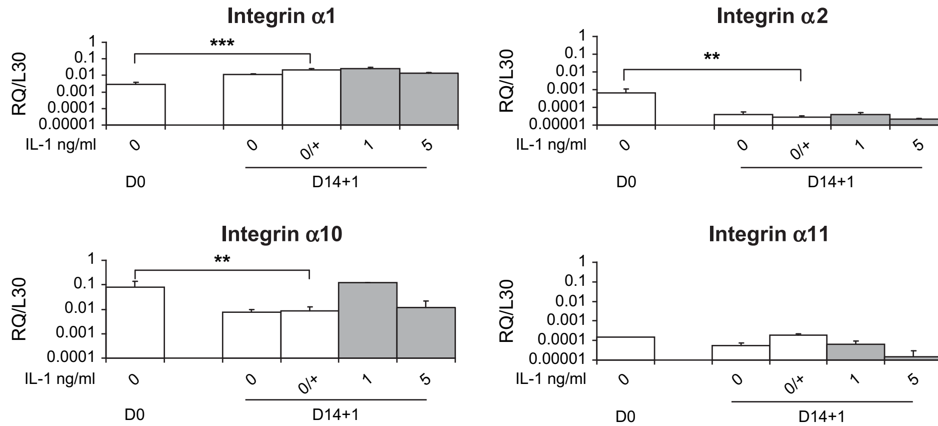

Fig. 3. Effect of the presence of IL-1β on the expression of collagen receptor integrin sub-unit genes, when added on day 14 to 10^7 bovine chondrocytes seeded in collagen sponges. Cultures were grown in serum-free medium for 1 day containing IL-1β at 1 and 5 ng/ml and 0.2% ethanol. Control cultures received or not 0.2% ethanol noted 0/+ and 0. RNAs were extracted from sponges followed by real-time PCR as described in Materials and methods. The amount of mRNA obtained was normalised to the amount of L30. Results are expressed in relative quantity/L30 (RQ/L30) ± SD from two experiments carried out with three to six samples. Comparisons were made using the Student's *t*-test: **, $P < 0.01$; ***, $P < 0.001$.

also observed for these genes, the highest levels being obtained with 20 ng/ml IL-1β for MMP-3 (8/L30), MMP-1 (0.5/L30), MMP-13 (0.36/L30), ADAMTS-5 (0.2/L30) and ADAMTS-4 (0.016/L30).

The levels of expression of the TIMP-1 and TIMP-2 genes are shown in Fig. 5: similar levels were observed in freshly isolated cells (0.09/L30 and 0.15/L30, respectively), values only slightly increased after 15 days of culture. After

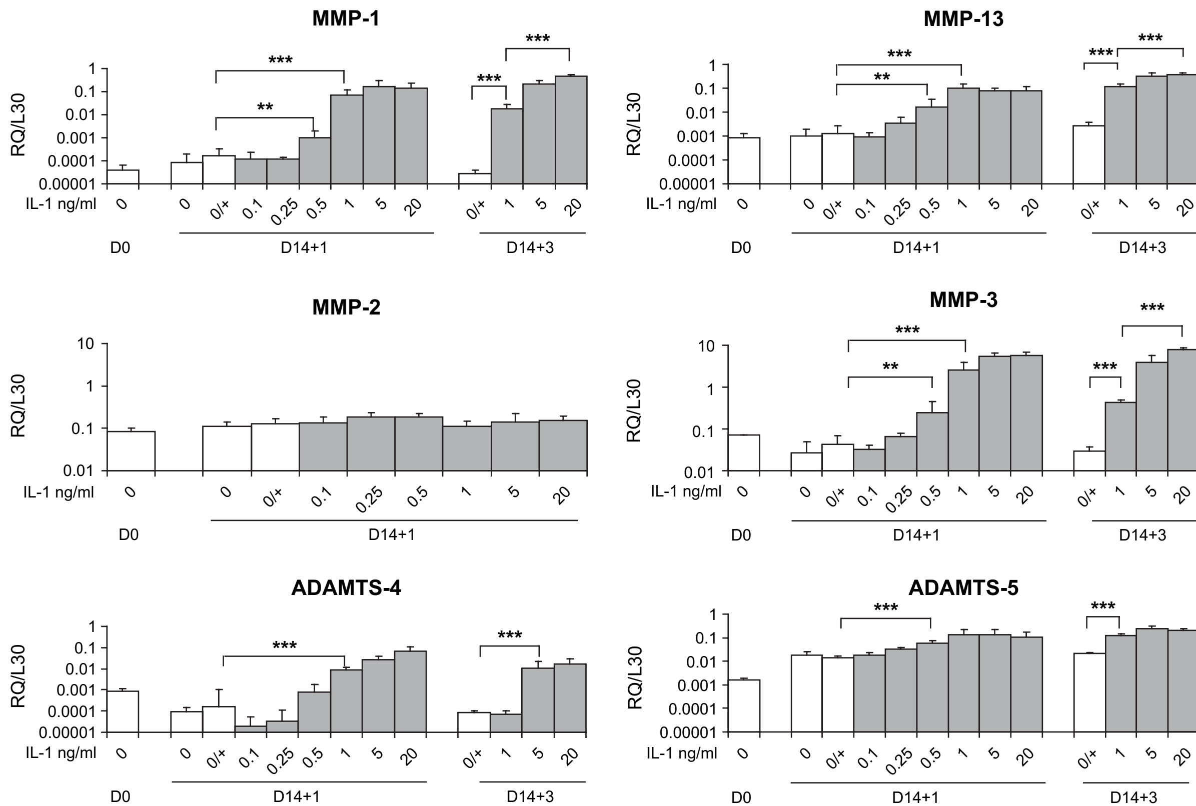

Fig. 4. Effect of the presence of IL-1β on the expression of MMP and ADAMTS genes, when added on day 14 to 10^7 bovine chondrocytes seeded in collagen sponges. Cultures were grown in serum-free medium for 1 or 3 days containing IL-1β at 0.1, 0.25, 0.5, 1, 5, 20 ng/ml and 0.2% ethanol. Control cultures received or not 0.2% ethanol noted 0/+ and 0. RNAs were extracted from sponges followed by real-time PCR as described in Materials and methods. The amount of mRNA obtained was normalised to the amount of L30. Results are expressed in relative quantity/L30 (RQ/L30) ± SD from three experiments carried out with three to six samples. Comparisons were performed using the Student's *t*-test: **, $P < 0.01$; ***, $P < 0.001$.

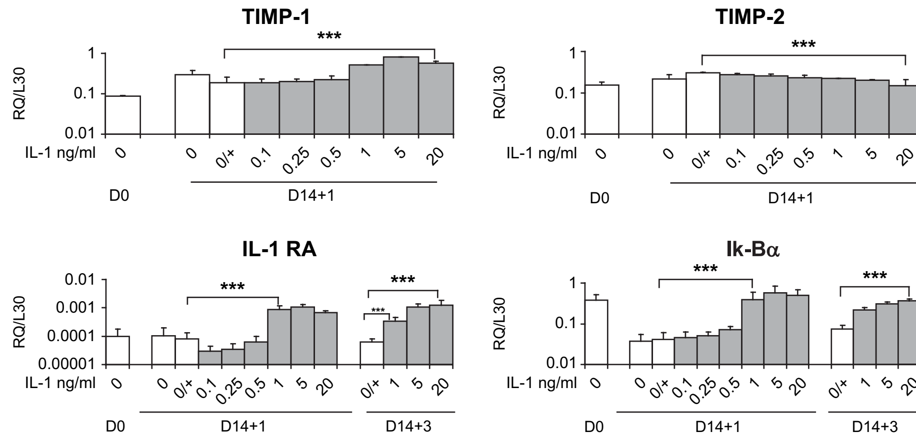

Fig. 5. Effect of the presence of IL-1β on the expression of TIMP-1 and -2 and IL-1Ra and Iκ-Bα genes, when added on day 14 to 10^7 bovine chondrocytes seeded in collagen sponges. Cultures were grown in serum-free medium for 1 or 3 days containing IL-1β at 0.1, 0.25, 0.5, 1, 5, 20 ng/ml and 0.2% ethanol. Control cultures received or not 0.2% ethanol noted 0/+ and 0. RNAs were extracted from sponges followed by real-time PCR as described in Materials and methods. The amount of mRNA obtained was normalised to the amount of L30. Results are expressed in relative quantity/L30 (RQ/L30) ± SD from three experiments carried out with three to six samples. Comparisons were performed using the Student's *t*-test: **, $P < 0.01$; ***, $P < 0.001$.

1-day treatment with IL-1β, a slight dose-dependent increase was noted for the TIMP-1 gene (3-fold with 20 ng/ml), whereas the level of expression of the TIMP-2 gene was not significantly changed.

For the interleukin-1 receptor antagonist protein gene (IL-1Ra), a very low level of expression was detected in freshly isolated cells and after 15 days of culture (10^{-4}/L30), whereas a significant increase was observed in the presence of IL-1β with a maximum (20-fold) after 3 days with 20 ng/ml IL-1β (Fig. 5). For Iκ-Bα, a net decrease (10- and 6-fold) in the expression was noted after 15 and 17 days of culture, respectively (Fig. 5), when compared to the value obtained with freshly isolated chondrocytes (day 0). In the presence of IL-1β, a dose-dependent increase in the expression level was shown to reach, with 20 ng/ml IL-1β, the level obtained with freshly isolated cells.

MMP-1, -2, -9 AND -13 PROTEIN LEVEL AND ACTIVITY

In order to confirm, at the protein level, the data obtained at the gene expression level we used zymography for the analysis of MMP-2, -9 and -13 [Fig. 6(A, B)] and specific fluorogenic substrates for MMP-1 and MMP-13 [Fig. 6(C–F)].

Zymography performed on the serum-free medium after 15 or 17 days of culture demonstrated the presence of MMP-2, more than 95% of which was in the inactive proMMP-2 form and the absence of MMP-9 and MMP-13. In the presence of increasing doses of IL-1β and notably after 3 days of treatment, it was possible to show an increase in the amount of proMMP-2 and to detect bands corresponding to proMMP-9, MMP-2 and proMMP-13.

Cleavage of the two specific fluorogenic peptides by media non-treated with trypsin measured the amount of the active forms of MMP-1 and MMP-13 [Fig. 6(C, E)], whereas, after trypsin treatment, both proMMP and MMP forms were measured, noted here as total MMP [Fig. 6(D, F)]. After 15 and 17 days the values obtained for both active and total forms of MMP-1 and MMP-13, present in the medium, were very low, at the limit of detection of the method. In the presence of IL-1β, large dose-dependent increases in these values for both MMP-1 and MMP-13 were observed after 1 and 3 days. For example [Fig. 6(C, D)] after 1 day with 20 ng/ml IL-1β, 6- and 18-fold increases in the amounts of the active and total forms of MMP-1, respectively, were noted. For both MMP-1 and MMP-13, the active forms corresponded to less than 15% of the total forms.

Discussion

In this study we present a model culture system that facilitated the exposure of chondrocytes to catabolic stimulators such as IL-1β and could be used for the determination of the metabolic responses of the cells in the presence of compounds with anti-inflammatory potential.

The choice of calf chondrocytes isolated from the metacarpophalangeal joint of 6-month-old animals was dictated by the easy access to the tissue and the large amount of cells available (more than 100×10^6 cells/joint). In our previous paper using the same cells[17] we have shown that the mRNA levels of the genes studied here were not very different for chondrocytes present in native cartilage or isolated after matrix release (noted here as the freshly isolated cells). Furthermore for more than ten experiments using cells isolated from different animals of the same age, we have always obtained reproducible levels of expression for most of the genes studied. For example, as expected for differentiated chondrocytes, high levels of COL2A1, COL9A1, aggrecan and α10 integrin sub-unit mRNAs and very low levels of COL1A2 and α11 integrin sub-unit mRNAs were measured in all the native cartilage extracts and the freshly isolated cells[17].

After a high initial cell-seeding density (10^7 cells) in collagen sponges, the cell number remained unchanged after 15 days of culture, whereas the sGAG content (170 μg/sponge) demonstrated the neoformation of a cartilage-like extracellular matrix. Furthermore as the extracellular matrix deposition, depending on the culture duration as previously shown[16] remained low under these conditions, the construct had a high diffusivity, necessary for optimal molecular

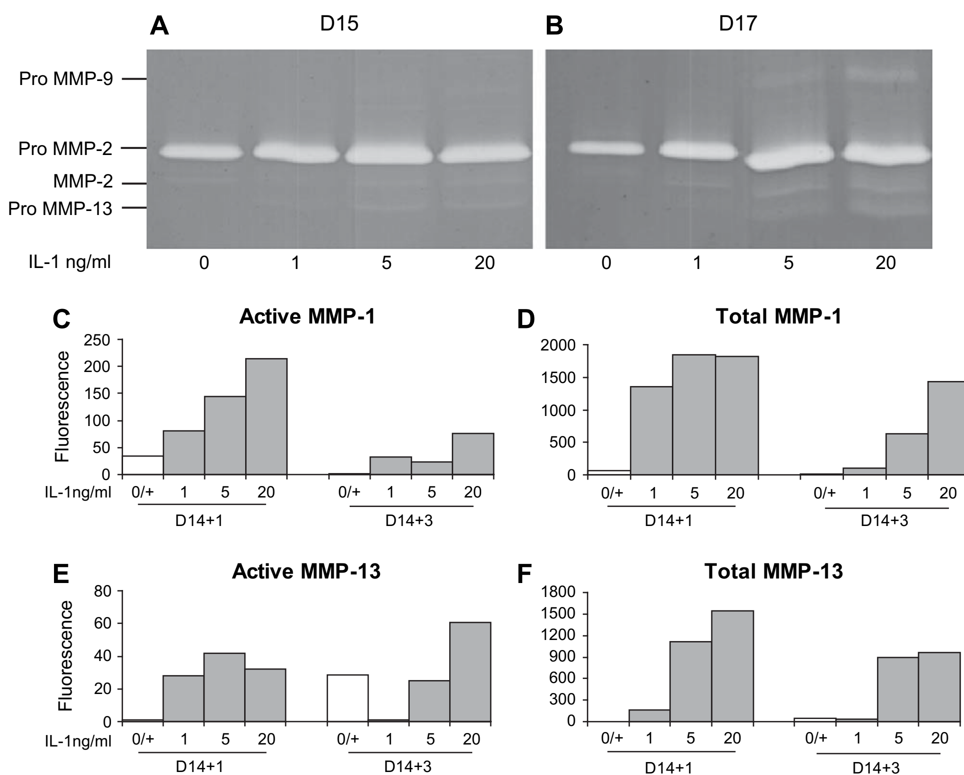

Fig. 6. Effect of the presence of IL-1β on the secretion of MMP-1, -2, -9 and -13 into the medium. IL-1β at 1, 5 and 20 ng/ml was added on day 14 to 10^7 bovine chondrocytes initially seeded in collagen sponges. Cultures were further grown on for 24 or 72 h in serum-free medium. Control media were obtained from non-seeded sponges grown in parallel cultures. Culture media were subjected to gelatine zymography for MMP-2, -9 and -13 as described in Materials and methods. Measurements of MMP-1 and MMP-13 activities were performed using the fluorometric method. The medium was incubated with trypsin (5 μg/ml) for activation of proMMP (total activity = active MMP + proMMP) as described in Materials and methods. In the medium without trypsin, active MMP levels were measured. The activities were evaluated after 4 h incubation at 37°C of 50 μl of conditioned medium with the respective fluorogenic substrate for MMP-1 and MMP-13 as described under experimental procedures. Fluorescence values of active and total MMPs represent the average level of duplicate samples.

diffusion and for the cells to have fewer nutrient limitations than chondrocytes in native cartilage[20].

After 15 days of culture within the collagen sponge, in spite of a decrease in COL2A1 and an increase in COL1A2 expression when compared to freshly isolated cells, the COL2A1/COL1A2 ratio remained high (12.5) demonstrating that the cells still expressed a cartilage-like phenotype. These results are consistent with the high expression of the aggrecan and α10 integrin sub-unit genes and the low expression of the α11 integrin sub-unit gene. We confirm here our previous data obtained with the same chondrocytes cultured in monolayer or within type I collagen gels[17] showing that the ratio of COl2A1/COl1A2 chain mRNA levels varied as the ratio of the α10/α11 integrin sub-unit mRNAs, depending on the differentiation/dedifferentiation state of the chondrocytes. This result is in good accordance with the non-overlapping expression pattern of α10β1 (cartilage) and α11β1 (non cartilaginous tissues) integrins[25,26]. It is thus important to note that in our model the main collagen-binding integrins expressed are α1β1 and α10β1 whereas the expression of α2β1 and α11β1 integrins was very low.

In the presence of high doses of IL-1β and after 3 days of treatment there were significant decreases in the expression of COL2A1 and aggrecan (1 ng/ml) and COL1A2 (5 ng/ml) as previously shown for chondrocytes in monolayer[27,28] or alginate bead culture[29]. The COL2A1/COL1A2 expression ratio had not changed with these treatments.

Concerning the expression of gene markers of matrix degradation, at day 0 (freshly isolated cells) and after 15 or 17 days of culture, the order of decreasing mRNA level of most metalloproteinases is the following: MMP-3 = MMP-2 > ADAMTS-5 > ADAMTS-4 = MMP-13 > MMP-1. It is interesting to note that Bau *et al.*[30] obtained the same order of mRNA levels for these enzymes for chondrocytes present in human articular cartilage (from donor ages 40–83 years). Important differences appear after culture *in vitro*: we have observed in our model (15 days of culture within a collagen sponge) only a very slight increase in the expression of these genes, whereas Bau *et al.*[30] observed very strong induction of the same genes (190–1000-fold) after 2 days of culture in alginate beads or in high-density monolayer. Further addition of 1 ng/ml IL-1β in their cultures did not lead to a great induction of the metalloproteinase genes (less than 10-fold for MMP-1 or MMP-13). In our model we observed a dose-dependent induction with IL-1β of all these genes with the exception of MMP-2. Significant increases started after 1 day with 0.5 ng/ml IL-1β. With 1 ng/ml and at 1 day we observed very large induction (54–450-fold for ADAMTS-4 and MMP-1, -3, and -13 and 10-fold for ADAMTS-5). Even higher expression of these genes was obtained with the highest dose of IL-1β used (20 ng/ml) and the longest treatment time (3 days), corresponding to the following order of decreasing mRNA expression levels: MMP-3 > MMP-1 > MMP-13 > ADAMTS-5 > ADAMTS-4.

It also worth noting that with or without addition of IL-1β, aggrecanase 2 (ADAMTS-5) is more highly expressed (>10-fold) than aggrecanase 1 (ADAMTS-4). Recent data[31,32] have shown that ADAMTS-5 was the major aggrecanase in mouse cartilage *in vivo* and *in vitro* and that the ADAMTS-5 null mice are protected from joint destruction in a model of OA (surgically induced joint instability) and in a model of experimental inflammatory arthritis. In humans, as in our bovine model, ADAMTS-5 is expressed at higher levels than ADAMTS-4 in both normal and arthritic cartilage[30,33] . However, further work will be required to determine the exact role played by these two enzymes during the progression of OA in human.

In contrast to the preceding enzymes analysed, the expression of MMP-2 was notable in the freshly isolated chondrocytes and after 15 days of culture and was not upregulated by IL-1β as previously shown in monolayer cultures of human[34] and rabbit chondrocytes[35] or human tendon cells[36].

For MMP-1 and MMP-13, we confirmed, at the protein level, their large and dose-dependent induction by IL-1β using fluorogenic substrate assays. As the values measured without IL-1β were within the detection limits of this method, it was difficult to quantify the increase in the inactive and active forms of MMP-1 and MMP-13. However, with or without IL-1β, the ratio between the active (MMP) and inactive (proMMP) forms of MMP-1 and MMP-13 had not changed (less than 15% of the enzymes were present in the culture medium in the active form).

Furthermore by using zymography we were only able to detect with the highest dose of IL-1β (20 ng/ml) and after 3 days a slight increase in proMMP-2 and the presence of faint bands on the zymogram corresponding to proMMP-9, MMP-2 and proMMP-13.

As a first attempt to determine the signalling pathway involved in the increased expression of MMPs and ADAMTSs by IL-1β in our model, we measured the expression of two important genes coding for the IL-1Ra and for an inhibitor of NF-κB activity (Iκ-Bα). IL-1Ra prevents the interaction between IL-1 and its cell surface receptor and thus competitively inhibits the pro-inflammatory activities of IL-1[37].

Recombinant human IL-1Ra has recently been approved for use in patients with RA[38]. In our culture model we observed a net, dose-dependent increase in the expression of IL-1Ra in the presence of IL-1β. We thus confirm here, as previously reported with human articular chondrocytes in monolayer culture[39,40], that IL-1Ra production might be part of a negative feedback mechanism initiated by IL-1.

Inflammatory responses induced by IL-1β are largely coordinated by NF-κB, a complex group of heterodimeric and homodimeric transcription factors. These molecules are trapped by Iκ-Bα in the cytoplasm as an inactive complex[41]. Indeed, blocking NF-κB activity by the overexpression of Iκ-Bα, a member of the Iκ-B family, reduces inflammation and tissue destruction by MMP-1 and MMP-3 in rheumatoid synovial tissue and dermal fibroblasts[42,43]. Here we measured the expression level of Iκ-Bα, and showed that its expression by freshly isolated chondrocytes (day 0) largely decreased after 15 or 17 days of culture without IL-1β, whereas in the presence of IL-1β, a dose-dependent and significant increase in its expression was observed, reaching its initial value (day 0) for 20 ng/ml IL-1β. It is obvious that these increases in both IL-1Ra and Iκ-Bα gene expressions are not sufficient to counteract the activity of NF-κB as high increases of MMPs gene expression are measured after only 24 h treatment with IL-1β and concentration equal or higher than 0.5 ng/ml. However, for concentration ≥1 ng/ml 3 days treatments are necessary, in our model, to significantly decrease the expression of COL2A1 and aggrecan genes. It is worth noting that in OA and RA patients, concentrations of IL-1β measured in synovial fluids averaged 0.028 ng/ml and 0.1 ng/ml, respectively[44,45].

This culture model is currently used to check the effect of drugs such as ASU and their active fractions as well as molecules whose ability to antagonize the IL-1-stimulated catabolic activity of chondrocytes was previously demonstrated using different *in vitro* chondrocyte culture models[46–48] .

Acknowledgments

The authors would like to thank Amanda Le Berre for her technical assistance in real-time PCR. This work was funded by the Laboratoires Expanscience (Epernon), by a "Programme Thématique Prioritaire" (085113) from the Rhône-Alpes region, by grants from ANVAR (J0011507V), from CNRS-INSERM "Ingénierie Tissulaire" (2000-03) and IT2b and from the "Fondation de l'Avenir pour la Recherche Appliquée" (Paris, ET2-320 et ET4-359).

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