Metabolism of Human Articular Chondrocytes Cultured in Alginate Beads. Longterm Effects of Interleukin 1ß and Nonsteroidal Antiinflammatory Drugs

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ABSTRACT. ***Objectives***. To investigate the longterm effects (12 days) of nonsteroidal antiinflammatory drugs [NSAID: aceclofenac (ACECLO), sodium diclofenac (DICLO), indomethacin (INDO), nimesulide (NIM), rofecoxib (ROFE), celecoxib (CELE), piroxicam (PIROX), and ibuprofen (IBUP)] on the metabolism of human chondrocytes cultured in alginate beads.

Methods. Enzymatically isolated osteoarthritic (OA) chondrocytes were cultured in alginate beads in a well defined culture medium for 12 days. The DNA content was measured according to a fluorimetric method and cell proliferation was determined by the incorporation of ^{3}H-thymidine in the newly synthesized DNA. Interleukin 6 (IL-6) and IL-8, stromelysin [matrix metalloproteinase-3 (MMP-3)], and aggrecan (AGG) production were assayed by specific enzyme amplified sensitivity immunoassays, and prostaglandin E_2 (PGE_2) production by specific radioimmunoassay. All NSAID were tested at the mean peak plasma concentration (C_{max}) obtained after oral administration of a therapeutic dose.

Results. In alginate beads, chondrocytes synthesized high amounts of AGG, which were largely (98%) immobilized in the alginate matrix. A large amount (43%) of the IL-8 produced was stored in the alginate beads, whereas almost all IL-6 production (94%) was released in the culture supernatant. At the therapeutic concentration, all NSAID tested fully blocked PGE_2 production. ACECLO, DICLO, INDO, NIM significantly inhibited basal and IL-1ß stimulated IL-6 production; CELE and IBUP only inhibited IL-1ß stimulated IL-6 production; and ROFE and PIROX had no significant effects. No NSAID showed significant effects on basal and IL-1ß stimulated IL-8 production, except CELE and IBUP, which slightly increased basal IL-8 production. ACECLO and INDO increased AGG content by 25% in the alginate beads, while the other NSAID were without significant effect. No NSAID were able to modify the inhibitory effect of IL-1ß on AGG production. NSAID did not modify MMP-3 production.

Conclusion. The mechanism of action of NSAID seems to be multifactorial and not limited to the inhibition of cyclooxygenases. Further, in our culture conditions, at the C_{max} and by comparison with other NSAID, ACECLO and INDO show an advantageous activity profile. They fully blocked PGE_2 production, inhibited IL-6 synthesis, and increased aggrecan synthesis. These effects appear advantageous for the longterm treatment of chronic joint diseases such as osteoarthritis. (J Rheumatol 2002;29:772–82)

Key Indexing Terms:

CHONDROCYTES OSTEOARTHRITIS INFLAMMATION
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Articular cartilage is essentially composed of an extensive hydrated matrix, synthesized and turned over by chondrocytes, the only type of cell found in the cartilage. Cartilage matrix is principally composed of aggrecan (AGG) and type II collagen whose synthesis is controlled by hormones, cytokines, and mechanical stimuli. In normal conditions, cartilage matrix remodeling is a slow process, consisting of a phase of chondrocyte resorption, directly compensated for by a phase of chondrocyte repair[1-3]. In pathological conditions such as osteoarthritis (OA), cartilage turnover may

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accelerate, leading to an early repair reaction followed by cell decompensation and cartilage degradation. Repair reaction is characterized by cartilage hypertrophy and increased synthesis of matrix components and metalloproteases[4,5]. This homeostatic reaction is not efficient, is transient, and ultimately fails to repair cartilage defects, although the etiological factors persist. Cartilage resorption is mediated by enzymes and free radicals produced by neighboring tissues, but also by chondrocytes themselves[6]. These processes are induced and controlled by potent autocrine and paracrine mediators, mainly interleukin 1ß (IL-1ß) and tumor necrosis factor-α, and probably other cytokines such as IL-6 and IL-8[6,7]. *In vitro,* IL-1 is a potent inhibitor of proteoglycans and collagen type II synthesis, and a stimulator of metalloproteases, IL-6, IL-8, prostaglandin E_2 (PGE_2), nitric oxide and reactive oxygen species production[7-11]. For these reasons, IL-1ß is commonly used in culture models to mimic the circumstances leading to *in vivo* cartilage degradation.

Nonsteroidal antiinflammatory drugs (NSAID) are the most commonly used drugs in the treatment of OA[12]. Until now, NSAID were considered to be symptom modifying drugs that acted by inhibiting prostaglandin synthases. There is little doubt that certain NSAID can provide more than symptomatic relief. Several *in vitro* and animal studies have shown that some NSAID may reduce cartilage degradation or stimulate proteoglycan (PG) synthesis, whereas others may have a deleterious effect[13-15]. These observations suggest that NSAID could interfere with the cartilage metabolism and modulate disease progression[16-18]. Further, recent studies have revealed that the mechanism of action of NSAID is multifactorial and involves pathways other than the inhibition of PG synthesis. Indeed, some NSAID exert antioxidant properties[19-21], inhibit cytokine production[22,23], decrease the expression of adhesion molecules by human neutrophils[24,25], modulate enzymatic activity[16,26], or induce tissue inhibitor of metalloproteases (TIMP-1) synthesis[27]. Further, in experimental dog OA, carprofen reduces growth of osteophytes, the severity of cartilage lesions, and the remodeling of subchondral bone, suggesting that this NSAID could prevent cartilage degradation by preventing the abnormal metabolism of subchondral osteoblasts[28]. Together, these observations might suggest modifying recommendations for use of NSAID to protect cartilage in OA.

At this time, most knowledge of the actions of NSAID comes from short term cell or explant cultures[29-32]. While many patients with OA have taken these drugs for several years, few studies studied the longterm effects of NSAID[33–35]. Further, different NSAID were rarely compared in the same experiment[36]. Longterm studies have faced challenges, because chondrocyte dedifferentiation occurs when these cells are cultivated in monolayer[37,38]. Chondrocytes cultured in alginate beads permit the chondrocyte phenotype to remain stable for a long period of time[39-43].

We used this culture model to investigate the metabolism of chondrocytes by assessing AGG, IL-6, IL-8, PGE_2, and stromelysin [matrix metalloproteinase (MMP-3)] production and the longterm influence of IL-1ß and several NSAID within these parameters.

MATERIALS AND METHODS

Chondrocyte culture in alginate beads. Cartilage was obtained from the knees of cadavers immediately after death, being excised from the superficial and medium layers of cartilage and avoiding the calcified layer. Upon dissection, the femoral, patellar, and tibial articular surfaces were evaluated for gross pathological cartilage modifications. Using the scale of Moskowitz, *et al*[44], the severity of pitting was recorded for each donor. Four different grades were considered: 0, normal white cartilage in all areas examined; I, the presence of a yellow-grey area with some superficial fibrillations on one or more articular surfaces; II, irregular surface with deep fibrillations on one or more articular surfaces; and III, ulcers penetrating to the subchondral bone on one or more articular surfaces. Experiments were performed with OA cartilage from 4 donors (3 male, one female) with a mean age of 49 (39–69) years. All donors showed OA cartilage lesions of grade I (n = 3) or III (n = 1).

Cartilage was cut into small fragments and then subjected to enzymatic digestions sequentially with hyaluronidase, pronase, and collagenase as described[9,10,23]. The cells were then filtered through a nylon mesh with a pore diameter of 70 µm, and then washed 3 times with sterile water 0.9% NaCl. Cell viability was estimated by trypan blue exclusion test and in all cases was superior to 95%. Chondrocytes were suspended in 1.2% low viscosity alginate (Sigma-Aldrich, Bornem, Belgium) in 0.9% NaCl solution at a density of 4×10^6 cells/ml, which was slowly passed through a 25 gauge needle in a dropwise fashion into a 102 mM $CaCl_2$ solution (Sigma-Aldrich). After instantaneous gelation the beads were allowed to polymerize further for 10 min in this $CaCl_2$ solution. Then, they were washed with saline solution and 18 beads were cultured in 1 ml of culture medium per well in a 24 well plate. Culture medium was DMEM supplemented with 1% ITS+ (ICN Biomedicals, Asse-Relegem, Belgium), 10 mM HEPES, penicillin (100 U/ml) and streptomycin (100 U/ml), 200 µg/ml glutamine (Biowhittaker Europe, Verviers, Belgium), 50 µg/ml ascorbic acid (Sigma-Aldrich), 2 mM proline (Gibco, Merelbeke, Belgium). ITS+ is a premixed cell growth system containing in 1 ml: 0.625 mg insulin, 0.625 mg transferrin, 0.625 µg selenious acid, 0.125 g bovine serum albumin, and 0.535 mg linoleic acid. Cells remained in this culture medium (washout medium) for 48 h as a precaution against *in vivo* contamination with drugs that donors may have taken before death. After this washout period, culture medium was changed and the various drugs added. Culture medium was changed every 3 days and the collected supernatants were kept at –20°C until analysis. Chondrocytes were cultured in alginate beads for a period of 12 days.

Treatment. Chondrocytes were cultured for 12 days in the absence or in presence of IL-1ß 10^{-10} M (Boehringer, Mannheim, Germany) and with or without NSAID. NSAID were tested at the concentration corresponding to the mean peak plasma concentration (C_{max}) obtained after oral administration of a therapeutic dose. The C_{max} used in this study were 7.4 µg/ml for aceclofenac (ACECLO)[45,46], 1.4 µg/ml for diclofenac (DICLO)[47], 2 µg/ml for indomethacin (INDO)[48], 3 µg/ml for nimesulide (NIM)[49,50], 1 µg/ml for rofecoxib (ROFE)[51], 0.7 µg/ml for celecoxib (CELE)[52], 7 µg/ml for piroxicam (PIROX)[53,54], and 25 µg/ml for ibuprofen (IBUP)[55,56]. All the NSAID tested were first dissolved in N,N-dimethylformamide (DMF; Sigma-Aldrich), and then diluted in culture medium to achieve the required final concentration. The final concentration of DMF was 0.1%. Three wells were used for each concentration of drugs and for the corresponding controls, and the experiments were repeated 3 times with the cartilage of 3 different donors. All NSAID were tested on cartilage specimens coming from the same donors.

End of the culture. The beads of each well were dissolved in 1 ml 0.1 M citrate for 10 min. The resulting suspension was centrifuged at 1200 rpm for 10 min. With this method, 2 fractions were collected: the supernatant containing macromolecules from the further-removed matrix (FRM) compartment, and a pellet containing cells with their associated cellular matrix (CM). These 2 fractions were studied separately. The cell pellets were washed with phosphate buffered saline (PBS; Biowhittaker Europe), and then homogenized in 1 ml of PBS by ultrasonic dissociation at 4˚C for DNA assay. CM and FRM were kept at –20˚C until analysis.

DNA assay. The DNA content of the cultures was measured according to the fluorimetric method of Labarca and Paigen[57].

^{3}H-Thymidine incorporation analysis. At the end of different culture periods (0, 24 h, 48 h, 5, 8, 11 days), 1 µCi of methyl-^{3}H-thymidine (50 Ci/mmole; ICN Biomedicals) was added to the culture supernatant for 24 h. Afterward, the beads were dissolved in a citrate solution as described in the previous paragraph. Cell pellets obtained by centrifugation were washed with PBS and dissolved in 1 ml of 10% trichloroacetic acid for 15 min at 4˚C. After ultrasonic dissociation and centrifugation at 2500 rpm, the remaining pellet containing chromatin was homogenized in 500 ml NaOH 1 N. After 30 min, the volume was adjusted to 1 ml by addition of HCl and the pH was neutralized. Incorporated radioactivity was then counted twice by scintillation with a beta counter.

PGE_2 radioimmunoassay. PGE_2 was assayed in the different compartments (culture supernatants, FRM, and CM) without previous extraction according to a radioimmunoassay[58]. In this assay, polyclonal antiserum obtained from rabbit does not cross react with other prostanoids (TxB_2, 6-keto-PGF1a, PGA_2) or fatty acids (arachidonic, linoleic, oleic). ^{3}H-labeled PGE_2 was purchased from New England Nuclear (Brussels, Belgium) and the standard molecule (PGE_2) from Sigma-Aldrich. Intra- and interassay coefficients of variation were 6 and 10%, respectively. The limit of detection of the radioimmunoassay was 20 pg/ml.

Immunoassays for AGG, IL-6, IL-8, and MMP-3. AGG, IL-6, IL-8, and MMP-3 were measured in the different compartments (culture supernatants, FRM, CM) by specific enzyme amplified sensitivity immunoassays (Biosource Europe, Fleurus, Belgium). The assays were based on the oligonal system in which several monoclonal antibodies were directed against distinct epitopes of the molecules. In the case of MMP-3, the antibodies are directed against pro-MMP-3, activated-MMP-3, and MMP-3 bound to TIMP-1 and TIMP-2. The intra- and interassay coefficients of variation were less than 5% for all immunoassays.

Histology. At the end of the 12 day period, alginate beads were processed for light microscopy by the method described by Petit, *et al*[43]. The alginate beads were fixed for 4 h at 4˚C with 4% paraformaldehyde in 0.1 M sodium-cacodylate buffer (pH 7.4) to which 10 mM $CaCl_2$ was added to prevent disintegration of the beads. After washing overnight at 4˚C in 0.1 M sodium-cacodylate buffer containing 50 mM $BaCl_2$, the beads were dehydrated in a series of graded methanol washes followed by a xylene wash and finally embedded in paraffin. Five millimeter sections were cut with a microtome, rehydrated, and stained with hematoxylin and eosin.

Calculation and statistical analysis. The results (mean ± SD) were expressed as the concentration of AGG, MMP-3, IL-6, IL-8, and PGE_2 in the culture supernatants, CM, and FRM per µg of DNA. The total amount AGG, MMP-3, and cytokines present in alginate bead was obtained by the addition of the quantities found in the CM and in the FRM. Comparison of mean values was performed using the Mann-Whitney U test. Values were considered significant at $p < 0.05$.

RESULTS

Effects of IL-1ß and NSAID on cell proliferation and viability. As observed by light microscopy, cells retain their spherical shape (Figure 1). We noted that ^{3}H-thymidine incorporation reached a maximum the first day and then decreased as a function of culture duration (Figure 2A). Cell proliferation resulted in an enhancement of DNA content during the first 3 days of the culture, followed by a slow but progressive increase between Day 3 and Day 12 (Figure 2B). The viability of the cells was > 90% as estimated by trypan blue exclusion after their release from alginate beads. Cell viability and DNA content were not significantly affected by drug or IL-1ß treatments.

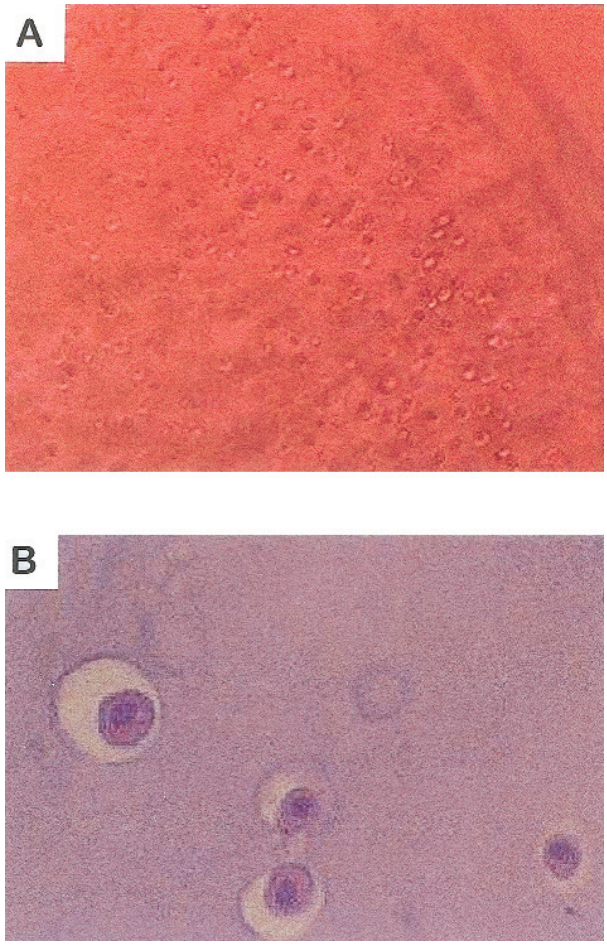

Figure 1. A. Microphotography of an alginate bead after 12 days of culture in the control conditions (magnification 200×). B. Hematoxylin-eosin staining of alginate bead slices (magnification 1200×).

Effect of IL-1ß and NSAID on AGG production and distribution. AGG were quantified in the CM, FRM, and culture supernatants. We observed that the total amounts of AGG contained in the beads (sum of AGG contained in CM and FRM) increased essentially during the first 6 days of the culture and then remained stable until Day 12 (Figure 3). During the first 6 days of culture, the AGG manifested mainly in the CM and then slowly distributed themselves through the FRM (Figure 3). At Day 6, AGG content increased in FRM but decreased in the CM, suggesting that newly synthesized AGG progressively migrated from the CM to the FRM. After 12 days of culture, only 2% of AGG

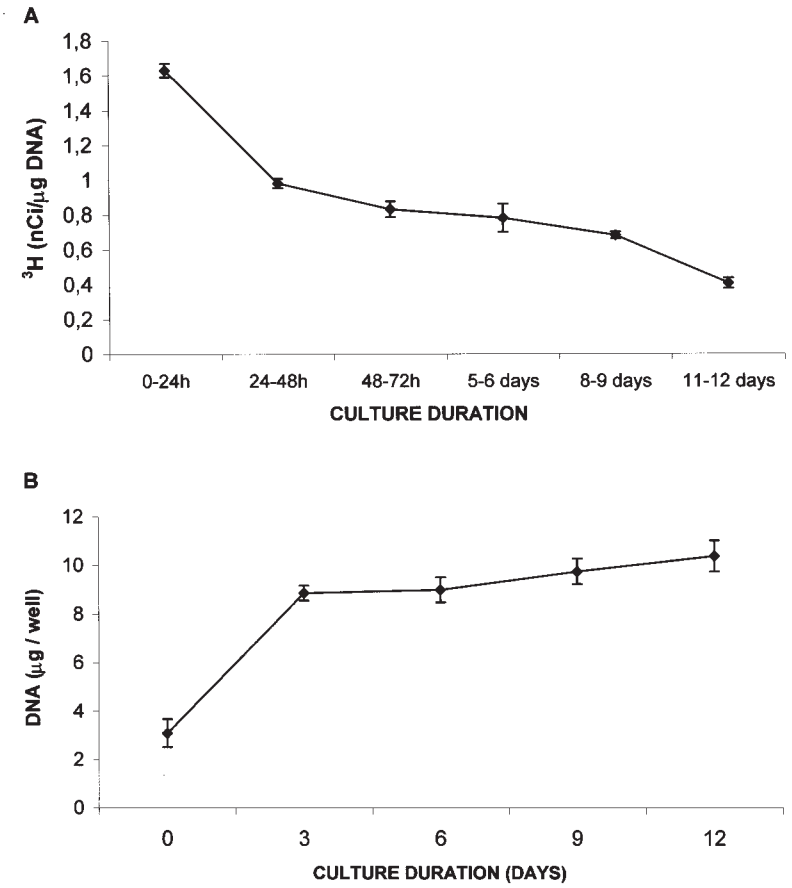

Figure 2. Cell proliferation. A. ^{3}H-thymidine incorporation according to culture duration. Results are expressed in nCi/µg DNA/24 h. The means ± SD values of triplicate wells are represented. B. Evolution of DNA content present per well based on culture duration. Results are the mean ± SD values of 4 independent experiments.

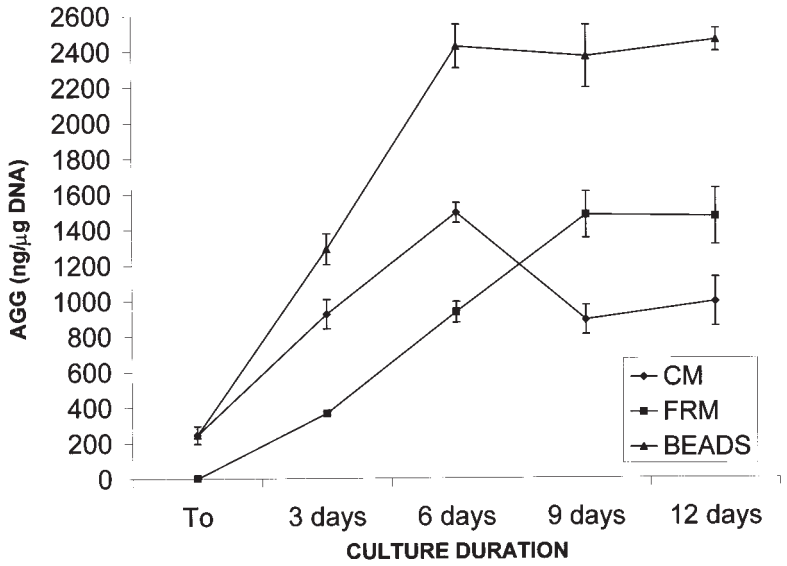

Figure 3. Kinetic curves of aggrecan (AGG) contained in cellular matrix (CM), further-removed matrix (FRM), and in the beads. Bead content corresponds to the total production resulting from addition of the amount of AGG contained in the CM and in the FRM. Results are expressed in mean ± SD values of 4 independent experiments. T0: time 0.

production was released in the culture supernatants (Figure 4). The amount of AGG accumulated in the beads at the end of the culture was drastically decreased by IL-1ß 10^{-10} M (Figure 5). This inhibitory effect was observed in both the CM and the FRM compartments (Figure 5). The amount of AGG found in the culture supernatant was also reduced by IL-1ß in comparison with controls; however, the ratio of AGG released into the culture supernatant was doubled (Figure 4).

In the absence of IL-1ß 10^{-10} M, ACECLO and INDO increased the quantity of AGG contained in the beads at the end of the culture by 22.3% and 25.9%, respectively. The other NSAID did not show any significant effects at the tested doses (Figure 6A). In the presence of IL-1ß 10^{-10} M, no NSAID significantly affected AGG production (Figure 6A).

Effects of IL-1ß and NSAID on IL-6 and IL-8 production and distribution. OA chondrocytes spontaneously produced IL-6 and IL-8. Production increased as a function of the culture duration. In control conditions, the majority of IL-6 production (94%) was found in the culture supernatant, while a high proportion (43%) of the IL-8 production remained in the alginate beads (Figure 4).

In the presence of IL-1ß 10^{-10} M, the production of these 2 cytokines was markedly increased to 7.5 times for IL-6 and 10 times for IL-8. The distribution of IL-6 between the different compartments was identical to that observed in the controls. In contrast, the amount of IL-8 immobilized in the alginate beads was slightly, but not significantly, increased in the IL-1 treated culture and reached 50% of the total IL-8 production (Figure 4).

At the plasma concentrations, NSAID did not modify IL-8 production, except for CELE and IBUP, which increased basal IL-8 production by 25 to 30% (Figure 6B). However, CELE and PIROX modified IL-8 distribution. Indeed, in the basal conditions, CELE increased the IL-8 level found in the culture supernatant, but did not modify IL-8 content in the alginate bead. Therefore, the basal proportion of IL-8 contained in alginate decreased from 43% to 31% (Figure 7). PIROX had no significant effect on the distribution of the basal IL-8 production, but significantly modified the distribution of IL-8 in IL-1ß stimulated conditions. Indeed,

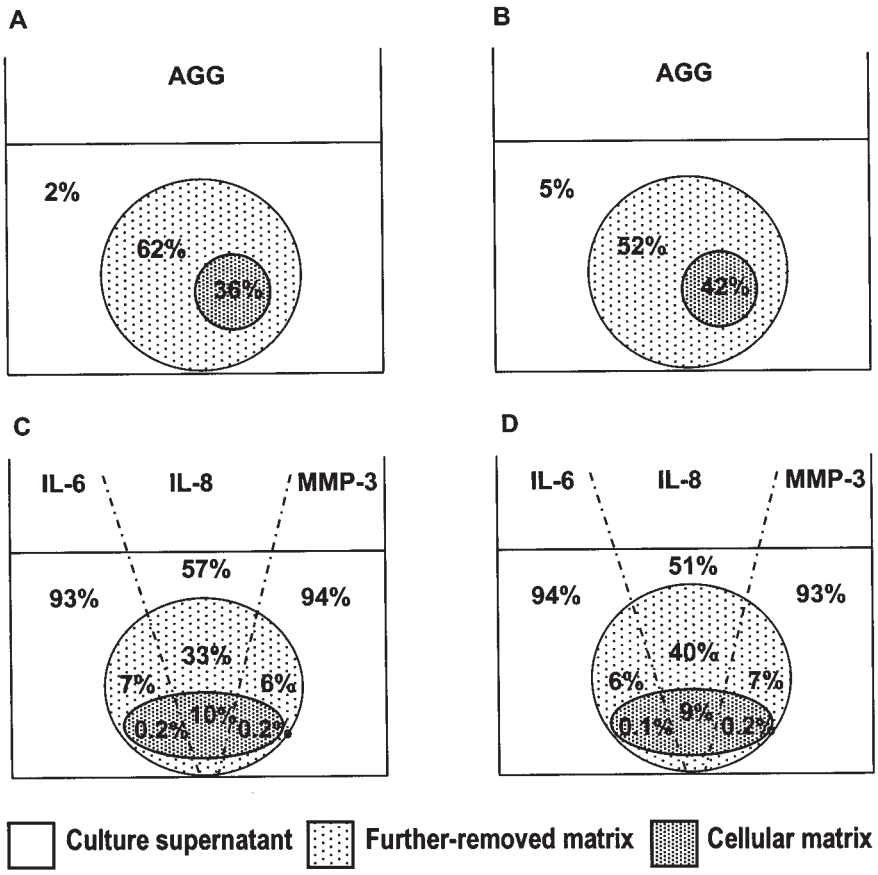

Figure 4. Distribution through the different culture compartments of aggrecan (AGG), interleukin 6 (IL-6), IL-8, and stromelysin [matrix metalloproteinase-3 (MMP-3)] in the absence (A, C) and presence of IL-1ß (10^{-10} M) (B, D). C and D. The production percentage present in culture supernatant and in the beads (further-removed matrix and cellular matrix) for IL-6, IL-8, and MMP-3. A and B. Percentage present in culture supernatant, further-removed matrix, and cellular matrix for AGG.

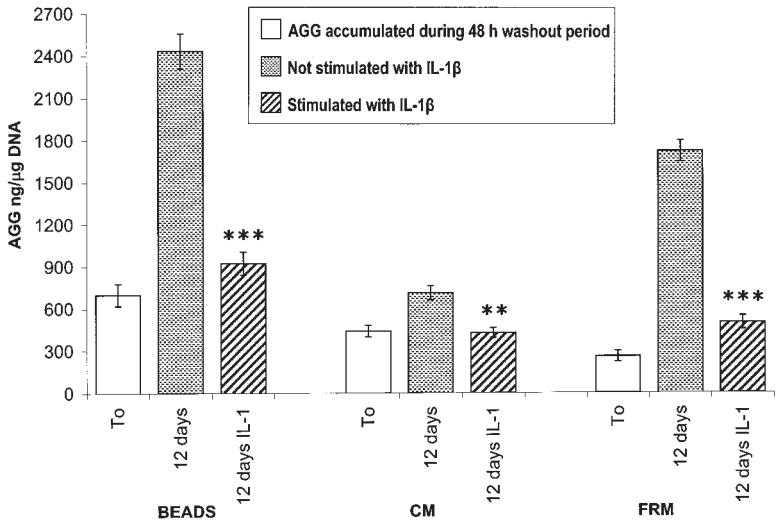

Figure 5. Effect of AGG accumulated in beads after 12 days of culture in the presence or absence of IL-1ß 10^{-10} M. Effect on cellular matrix (CM) and further-removed matrix (FRM) compartments is illustrated, and the total amount present in the beads (FRM + CM). Time 0 (T0, white bars) represents the amount of AGG accumulated during the 48 h washout period before IL-1 treatment. Results are expressed as means ± SD values of 3 experiments performed with cartilage from 3 different donors. **p < 0.01 vs not stimulated with IL-1ß; ***p < 0.001 vs not stimulated with IL-1ß.

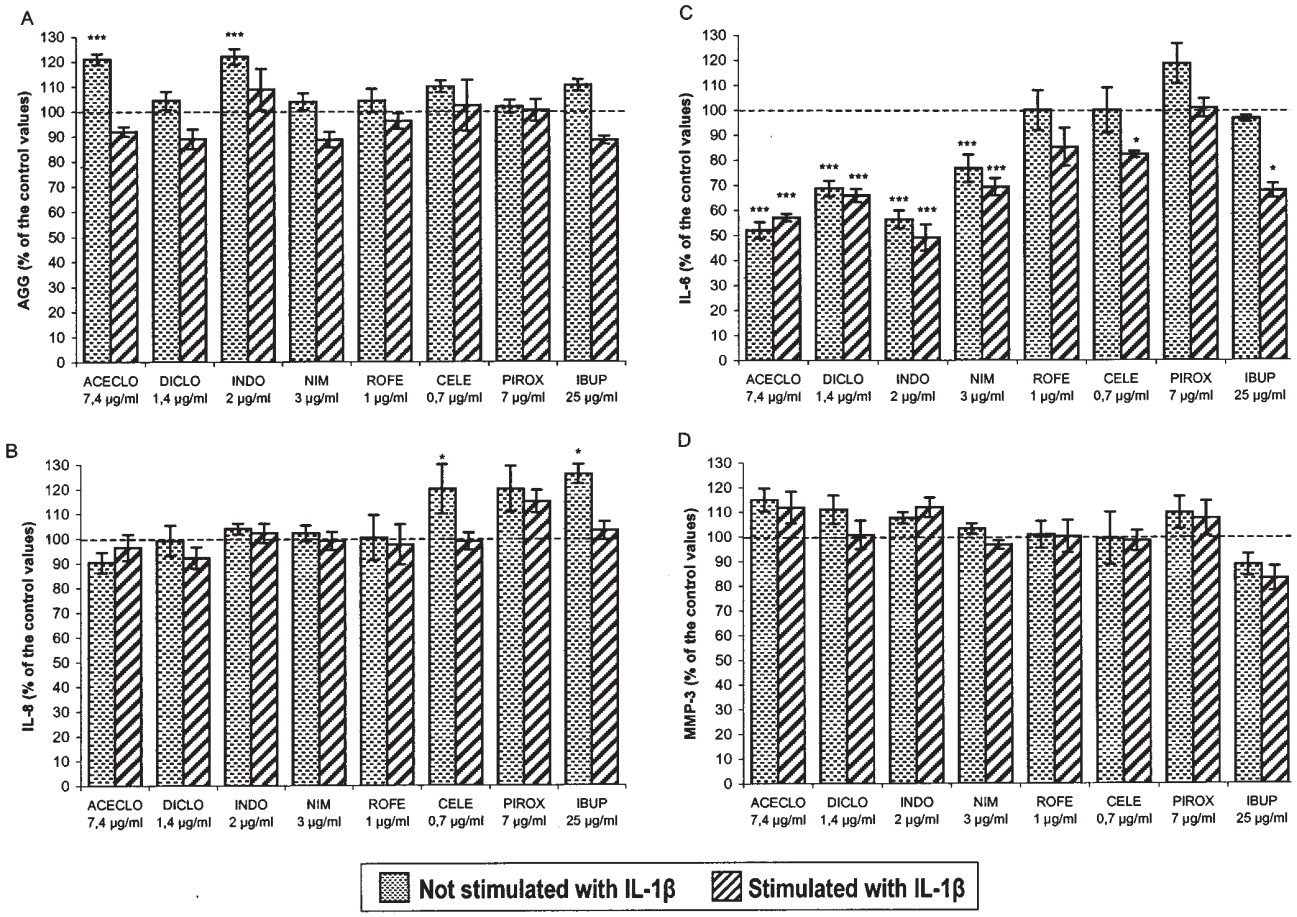

Figure 6. Effect of NSAID on aggrecan (AGG) (A), IL-8 (B), IL-6 (C), and stromelysin (MMP-3) (D) total production (cellular matrix + further-removed matrix + culture supernatant) after 12 day culture in the presence or absence of IL-1ß 10^{-10} M. Results are expressed as a percentage of control values. NSAID were added at the following doses: ACECLO 7.4 µg/ml, DICLO 1.4 µg/ml, INDO 2 µg/ml, NIM 3 µg/ml, ROFE 1 µg/ml, CELE 0.7 µg/ml, PIROX 7 µg/ml, IBUP 25 µg/ml. Results are expressed as means ± SD values of 3 experiments performed with cartilage from 3 different donors. *p < 0.05, ***p < 0.001.

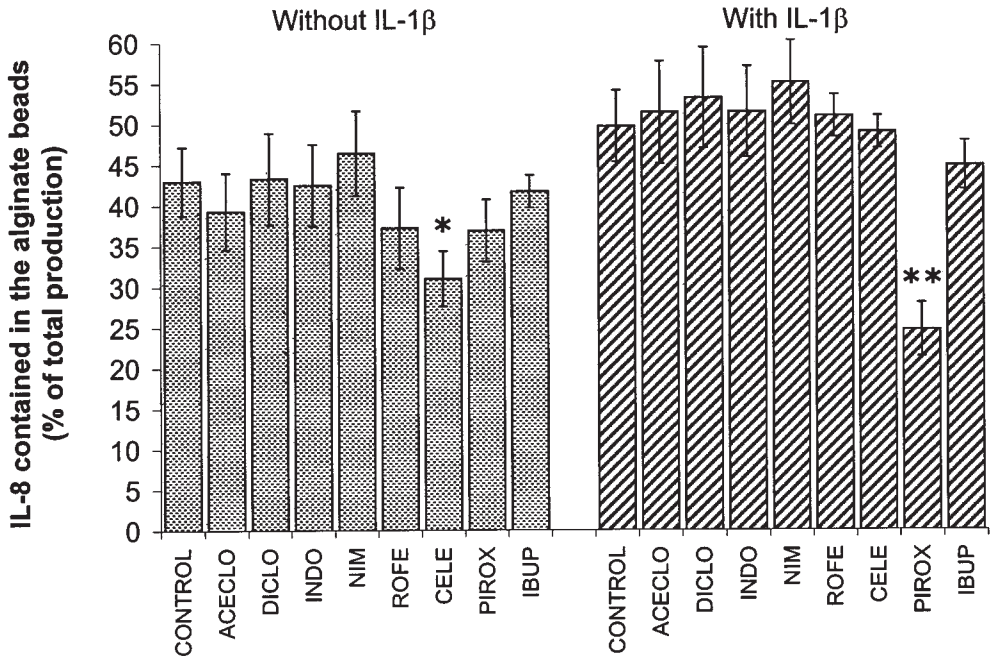

Figure 7. Effect of NSAID on IL-8 distribution through the different culture compartments after 12 day culture period. Results are expressed as percentage of total IL-8 production contained in the beads (further-removed matrix + cellular matrix) in the presence or absence of IL-1ß 10^{-10} M. Results are expressed as means ± SD values of 3 experiments performed with cartilage from 3 different donors. *$p < 0.05$, **$p < 0.01$.

while the ratio of IL-8 present in the alginate bead decreased from 50% to 25%, 75% of the IL-1ß stimulated IL-8 production was released in the supernatant (Figure 7). ACECLO, DICLO, INDO, and NIM significantly reduced both basal and IL-1ß stimulated IL-6 production, while CELE and IBUP decreased only IL-1ß stimulated IL-6 production (Figure 6C).

Effects of IL-1ß and NSAID on MMP-3 production and distribution. At each culture period, more than 90% of the MMP-3 spontaneously produced by human OA chondrocytes were released in the culture supernatant. IL-1ß stimulated MMP-3 production without affecting its distribution (data not shown). In our experimental conditions, NSAID did not significantly affect MMP-3 synthesis and distribution even in the presence of IL-1ß (Figure 6D).

Effects of IL-1ß and NSAID on PGE_2 production. In basal conditions, the production of PGE_2 was very low overall during the culture period. In the presence of IL-1ß 10^{-10} M, PGE_2 synthesis increased markedly. When chondrocytes were incubated with a therapeutic dose of each NSAID, production became undetectable even in the presence of IL-1ß.

DISCUSSION

In normal articular cartilage, chondrocytes express a specific phenotype characterized by low rate of proliferation, the spherical shape of cells, and by the synthesis of highly specialized macromolecules, mainly aggrecan and type II collagen, which form the extracellular matrix[1-3]. In our study, the quantitative analysis of AGG synthesis showed that (1) the majority (98%) of the newly synthesized AGG were immobilized in the alginate matrix; (2) AGG content in the alginate bead increased during the first days of culture and then remained stable, suggesting that matrix remodelling reached a steady state; and (3) the distribution of AGG in the alginate was progressing and migrated from the cellular matrix to the further-removed matrix. Nevertheless, after 12 days of culture, CM represented about 35% of total production. These findings are in agreement with other studies showing that AGG is the major PG synthesized (over 95%) by chondrocytes in alginate culture, and that one-third of these newly synthesized molecules remain in the CM beyond the 3 week culture period[40]. In 1998, Van Osch, *et al*[42] evaluated the relative volume occupied in alginate. In a 13 day culture, cells, CM, and FRM represented 3.5%, 5.2%, and 91.3% of the relative volume, respectively.

When IL-1ß 10^{-10} M was added to the culture medium, the AGG contained in the beads were significantly reduced. The reduction was more evident in the FRM compartment than in the CM compartment. Further, in parallel to the decrease of AGG contained in the alginate bead, an increase of the AGG released into the culture supernatants was recorded. This effect probably resulted from either the degradation of AGG and then the release to the supernatant of AGG fragments, or from an increase of non-aggregating molecules that could more readily leave the alginate beads.

For the first time, we have shown that IL-6 and IL-8 were diversely distributed between the various matrix compartments. In normal conditions, 43% of IL-8 production remained in the FRM, while IL-6 production was essentially (94%) released in the culture supernatant. Further, IL-1ß widely stimulated production of IL-6 and IL-8, but while the IL-6 distribution was unaffected by IL-1 stimulation, IL-8 accumulated in the FRM. The role played by IL-8 in the pathophysiology of OA remains obscure. However, in rheumatoid arthritis, IL-8 is an important chemoattractant factor for neutrophils and T lymphocytes towards the inflammatory center[59]. Recently, it was reported that IL-8 in the cartilaginous matrix *in vivo*, creating a gradient of concentration in the matrix, promotes the migration of neutrophils through the degraded cartilage towards the subchondral bone[60-62]. At this level, IL-8 induces neutrophil activation and degranulation, triggering the release of free radicals and lysosomal enzymes[63,64] by these cells, which are mediators of cartilage degradation.

In this model, we also studied the effects of different NSAID (Table 1). Until now, no study has investigated the effects of NSAID on chondrocyte metabolism using the alginate culture model. Except ACECLO and INDO, which slightly enhanced AGG production by chondrocytes, the other NSAID showed a neutral effect on AGG synthesis. These findings must be compared with results of Dingle[14,65], which classified NSAID into 3 groups according to their effects on sulfated glycosaminoglycan (S-GAG) synthesis by human OA explants cultured for 6 days in the presence of fetal calf serum or insulin-like growth factor I. The first group included NSAID that stimulated GAG synthesis, such as ACECLO. The second group, including DICLO and PIROX, exerted neutral effects. The third group was composed of NSAID that reduced GAG synthesis, including INDO, NIM, and IBUP. Concerning ACECLO, our results corroborate Dingle's conclusions. On the other hand, we are in disagreement regarding the effects of INDO, NIM, and IBUP. In the Dingle study, these NSAID were classified as inhibitors of PG synthesis. In our study, INDO was shown to increase PG synthesis, whereas NIM and IBUP were without significant effects. This discrepancy can be explained by the drug concentrations used in both studies. Indeed, in Dingle's study, INDO and NIM were tested at 30 and 50 µg/ml, respectively, doses largely superior to therapeutic concentrations used in our study. It was more surprising to find that INDO increased AGG synthesis. This finding contradicts *in vivo* studies that describe a deleterious effect of INDO on cartilage[16,17]. Other *in vitro* studies have reported a depressive action of INDO on GAG synthesis[66–68]. On the other hand, other authors have shown a neutral effect of therapeutic concentrations of INDO in canine[13], pig[31], or human explants[32]. Another explanation is

Table 1. Summary of the results, expressed in significant variations of total amount of aggrecan produced in 12 days of culture compared to control conditions.

Drug Tested	AGG	IL–6	IL–8	MMP–3	PGE_2
Aceclofenac, 7.4 µg/ml					
Without IL–1β	↑↑↑	↓↓↓	0	0	↓↓↓
With IL–1β	0	↓↓↓	0	0	↓↓↓
Diclofenac, 1.4 µg/ml					
Without IL–1β	0	↓↓↓	0	0	↓↓↓
With IL–1β	0	↓↓↓	0	0	↓↓↓
Indomethacin, 2µg/ml					
Without IL–β	↑↑↑	↓↓↓	0	0	↓↓↓
With IL–1β	0	↓↓↓	0	0	↓↓↓
Nimesulide, 3µg/ml					
Without IL–β	0	↓↓↓	0	0	↓↓↓
With IL–1β	0	↓↓↓	0	0	↓↓↓
Rofecoxib, 1µg/ml					
Without IL–1β	0	0	0	0	↓↓↓
With IL– 1β	0	0	0	0	↓↓↓
Celecoxib,0.7 µg/ml					
Without IL–β	0	0	↑	0	↓↓↓
With IL– 1β	0	↓	0	0	↓↓↓
Piroxicam, 7µg.ml					
Without IL–β	0	0	0	0	↓↓↓
With IL–β	0	0	0	0	↓↓↓
Ibuprofen, 25 µg/ml					
Without IL–1β	0	0	↑	0	↓↓↓
With IL–IL–1β	0	↓	0	0	↓↓↓

0: no significant effect, ↑ ($p < 0.05$), ↑↑, ↑↑↑ ($p < 0.001$): significant increase; ↓ ($p < 0.05$), ↓↓, and ↓↓↓ ($p < 0.001$): significant decrease. AGG: aggrecan; IL: interleukin; MMP-3: stromelysin (matrix metalloproteinase - 3); PGE_2: prostaglandin E_2.

the difference in culture medium composition. Dingle and collaborators used fetal calf serum, whereas our culture medium was serum-free. Serum contains hormones and growth factors that create different patterns of cell stimulation and then modify the response of cells to the drugs.

Our data reveal that, at therapeutic plasma concentration, ACECLO, DICLO, INDO, and NIM significantly inhibited baseline and IL-1ß stimulated chondrocyte IL-6 production, whereas CELE and IBUP inhibited only IL-1ß stimulated production. These results confirm our previous report that NIM and DICLO are potent inhibitors of IL-6 synthesis by human OA chondrocytes in short term cultures[23]. Depending on the exact role of IL-6, the clinical relevance of this observation remains unclear. Indeed, IL-6 is a cytokine with multiple and varied effects, some proinflammatory and others antiinflammatory. Interestingly, ROFE and CELE, which both preferentially inhibit cyclooxygenase-2 (COX-2) at therapeutic dose, were ineffective on IL-6 production compared with classical NSAID. The inefficiency of COX-2 specific inhibitors on IL-6 production can be explained by the low dose of the drug tested. In our study, NSAID were tested at concentration corresponding to the mean plasma concentration (C_{max}) obtained after oral administration of a therapeutic dose. For ROFE and CELE, these concentrations were very low and correspond to 1.0 and 0.7 µg/ml, respectively. At these concentrations, they selectively inhibit COX-2 but weakly inhibit IL-6[69]. On the other hand, PIROX, which at the concentration of 7 µg/ml inhibits both COX-1 and COX-2, is also inefficient on IL-6 production. Taken together, these results suggest that the effect of NSAID on IL-6 production is not PGE_2 dependent and is probably related to the structure of the NSAID. Interestingly, we observed that no NSAID reduce IL-8 production by chondrocytes. Nevertheless, PIROX reduced the proportion of IL-8 accumulated in the presence of IL-1.

We conclude that alginate beads constitute an interesting model for studying effects of drugs on production of aggrecans and cytokines by chondrocytes, but also on their distribution in the extracellular matrix. We have also demonstrated *in vitro* that the effects of NSAID on chondrocytes differ according to their chemical structure and that the mechanism of action of NSAID is multifactorial (Table 1).

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