Selective COX-2 inhibition prevents proinflammatory cytokine-induced cartilage damage

S. C. Mastbergen, F. P. J. G. Lafeber and J. W. J. Bijlsma

Department of Rheumatology and Clinical Immunology, University Medical Center Utrecht, Utrecht, The Netherlands

Abstract

Objectives. This study evaluated the in vitro effect of the selective cyclooxygenase-2 (COX) inhibitor celecoxib on cartilage matrix turnover under normal and inflammatory conditions.

Methods. Healthy human articular cartilage tissue alone, in co-culture with peripheral blood mononuclear cells (PBMC) or in the presence of interleukin 1 (IL-1β) plus tumour necrosis factor α (TNF-α) was cultured for 7 days in the presence of celecoxib. Changes in cartilage matrix turnover were measured.

Results. No direct effects of celecoxib on healthy normal cartilage were found. Both PBMC and IL-1β plus TNF-α induced strong inhibition of cartilage proteoglycan synthesis and significant enhancement of the release of proteoglycans, diminishing proteoglycan content. Celecoxib was able to reverse these adverse effects up to complete normalization.

Conclusions. The results suggest that, under the influence of inflammation, COX-2 is up-regulated, which results in disturbance of cartilage matrix turnover. Celecoxib, by diminishing COX-2 activity, prevents these adverse effects without having a direct effect on healthy cartilage.

KEY WORDS: Selective COX-2 inhibitor, Human cartilage tissue, Proteoglycan turnover, Inflammation; IL-1β, TNF-α.

An important prerequisite for the maintenance of the structural integrity of articular cartilage is the ability of chondrocytes to synthesize and degrade proteoglycans to maintain a functionally intact matrix. Small perturbations in cartilage metabolism may lead to increased or decreased local concentrations of matrix molecules, which can alter the physiological properties of the tissue. During joint diseases, such as rheumatoid arthritis (RA) and osteoarthritis (OA), chronic joint inflammation causes or contributes, primarily or secondary, to cartilage damage by influencing chondrocytes. This leads to erosion of articular cartilage, injury of surrounding tissue and eventually permanent loss of joint function [1].

Cyclooxygenase (COX), also known as prostaglandin synthase [2], is a potent mediator in inflammation. The anti-inflammatory effects of non-steroidal anti-inflammatory drugs (NSAIDs) are mainly due to their ability to inhibit prostaglandin production by COX suppression. As recently reviewed by Crofford et al. [3], two distinct forms of COX have been identified [4] on the basis of DNA sequence and expression. A constitutive form (COX-1) has been linked to the production of prostaglandins, which are physiologically important for the maintenance of organ and tissue homeostasis [5, 6]. The expression of a second form of COX (COX-2) is induced by proinflammatory cytokines and growth factors [7]. It is clear that COX-2 is the isoform responsible for the enhanced production of the prostaglandins that mediate inflammation, pain and fever and is the target enzyme for the anti-inflammatory activity of NSAIDs [8]. However, it has been suggested that COX-2 might also be involved in normal physiology, e.g. tissue repair [3].

The commonly used NSAIDs, however, are non-selective inhibitors and therefore they also inhibit COX-1 [9, 10]. Lack of selectivity is thought to account for the increased incidence of the gastric ulceration and other deleterious side-effects that accompany the chronic use of NSAIDs [11–13]. This has sparked interest in the development of drugs that specifically target COX-2, because such compounds would be associated with a lower incidence of adverse effects [14]. Celecoxib, being one of the selective COX inhibitors, at the right pharmacological dose inhibits COX-2 but not COX-1 [12, 15–17]. Analyses of data pooled from several trials suggest that celecoxib is associated with fewer clinically symptomatic ulcers and ulcer...
complications than are traditional NSAIDs, such as naproxen, diclofenac and ibuprofen [18].

Because NSAIDs diminish inflammation and inflammatory mediators, NSAIDs are indirectly beneficial for cartilage under inflammatory conditions. However, the direct effects of NSAIDs on cartilage have frequently been reported to be adverse, though beneficial and neutral effects have also been reported [19, 20–22]. Because these direct effects are not visible during clinical evaluation and are shadowed by the effects on inflammation, they are generally ignored. In addition, during X-ray analysis, changes in cartilage that are directly induced by NSAIDs remain undetectable because the processes concerned are very slow and irreversible harm can be done long before it becomes clinically evident. Nevertheless, in the long term such direct effects may be essential for the effectiveness of the NSAID treatment of chronic joint diseases, such as RA. Such direct effects may be even more important when the joint disease is mediated secondarily by RA. Such direct effects may be essential for the effectiveness of the NSAID treatment of chronic joint diseases, such as RA.

The local expression of COX-1 and COX-2 in joint tissues has been studied [23]. COX-1 is expressed in synovial lining cells derived from patients with OA and patients with RA. COX-2 expression is marked in synovium from RA patients but not OA patients [24]. On the other hand, in chondrocytes of both OA and RA cartilage, COX-2 has been shown to be up-regulated [25]. As a consequence of this up-regulation of COX-2, in diseased cartilage elevated levels of prostaglandin E2 (PGE2) are found, but the role of this elevation is poorly understood [26, 27]. The selective COX-2 inhibitors may contribute to the unravelling of the role of COX-2 and its product PGE2 in cartilage turnover, directly and under inflammatory conditions.

Although there is likely to be considerable enthusiasm for the development of NSAIDs that relieve pain in a wide variety of conditions but do not cause gastrointestinal toxicity on prolonged use, at least as important is the cartilage-protective effect of such drugs. Therefore, we studied the in vitro effect of celecoxib (SC-58635; 4-[5(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzene-sulphonamide), a selective COX-2 inhibitor, on human articular cartilage with respect to cartilage matrix turnover under normal and inflammatory conditions.

Material and methods

Cartilage culture

Human articular cartilage tissue was obtained post-mortem from knee condyles within 24 h of death. The donors were eight males and six females aged 67 ± 3 yr (s.e.m.) and were without a known history of joint disorders. The cause of death was related to cardiac failure or neurological disorders. A glossy, white, completely smooth surface and a healthy appearance designated normal cartilage [28]. Slices of cartilage were cut aseptically from the articular surface, excluding the underlying bone, and kept in phosphate-buffered (pH 7.4) saline. Within 1 h of dissection, the slices were cut into square pieces, weighed aseptically (range 5–15 mg, accuracy ±0.1 mg) and cultured individually in 96-well round-bottomed microtitre plates (200 μl culture medium per well for each explant, 5% CO2 in air, 37°C). The culture medium consisted of Dulbecco’s modified Eagle medium supplemented with glutamine (2 mM), penicillin (100 U/ml), streptomycin sulphate (100 μg/ml), ascorbic acid (0.85 mM) and 10% heat-inactivated pooled human male AB* serum.

Preparation of inflammatory cells

Blood was collected in heparin tubes from four donors with active RA. Peripheral blood mononuclear cells (PBMC) as a source of inflammatory cells were isolated by density centrifugation using Ficoll–Paque, washed twice in culture medium [29] and resuspended in culture medium to a final concentration of 0.15 × 106 cells/ml [30].

The IL-1 and TNF-α production of these cells was measured in the culture medium using an enzyme-linked immunosorbsent assay (ELISA) (Biosource Europe, Nivelles, Belgium) according to the manufacturer’s instructions.

Experimental set-up

Healthy human articular cartilage tissue was cultured for 7 days alone, in co-culture with inflammatory cells (PBMC; 0.15 × 106 cells/ml) or in the presence of IL-1β (200 pg/ml; PHC0814; Biosource) plus TNF-α (800 pg/ml, 19761T; Pharmingen). Celecoxib (supplied by Pharmacia, USA) was added at the start of the culture at concentrations of 0.01, 0.1, 1 and 10 μM. The mean pharmacological plasma concentration is 5 μM [31]. After 4 days the medium was refreshed and the cartilage was cultured for 3 successive days with the same additions. Changes in cartilage matrix turnover (proteoglycan synthesis, retention, release and content) were determined. For each experiment, a separate cartilage donor was used; the n-values given in the figure legends are the numbers of these experiments.

In a separate set of experiments, prostaglandin E2 levels in culture supernatants of normal cartilage with or without IL-1 plus TNF-α and with or without celecoxib were measured with a commercially available enzyme immunoassay kit (514010; Cayman Chemicals, USA).

Proteoglycan analysis

Sulphate incorporation rate was determined, as a measure of the proteoglycan synthesis rate, during the last 4 h of the first 4-day culture period, as described previously [32]. Before addition of 35SO42− (Na235SO4, 14.8 kBq/200 μl, carrier-free; NEX-041-H; DuPont), culture medium was replaced by fresh medium. After 4 h of labelling, the cartilage explants were rinsed three times for 45 min in culture medium under culture conditions and then incubated for an additional period of 3 days. After this second culture period, medium was removed and stored at −20°C for further analysis.
Cartilage tissue samples were digested (2 h, 65°C) in papain buffer as described previously [33] (Papain, P-3125; Sigma; 25 mg/ml in 50 mm phosphate buffer, pH 6.5, containing 2 mm N-acetyl cysteine and 2 mm Na3-EDTA). Papain digests were diluted and glycosaminoglycans (GAG) stained and precipitated with Alcian Blue dye solution (Alcian Blue 8GX, A-5268; Packard). The amount of 35SO4 was determined after centrifugation (9000 g, 10 min) was washed once (NaAc buffer containing 0.1 M MgCl2; 30 min, 37°C [34]). The pellet obtained after centrifugation (9000 g, 10 min) was then washed once (NaAc buffer containing 0.1 M MgCl2; 30 min, 37°C [34]). The pellet obtained was then washed once containing 0.3 M MgCl2; 30 min, 37°C (Packard). The amount of 35SO4 was then measured by liquid scintillation analysis after addition of Picofluor-40 (Packard). The amount of 35SO4 in the papain digest plus the amount in the 3-day culture medium after pulse labelling was normalized to the specific activity of the medium, labelling time and wet weight of the cartilage samples. The proteoglycan synthesis rate was expressed as nanomoles of sulphate incorporated per hour per gram wet weight of cartilage (nmol/h/g).

As a measure of proteoglycan content of the cartilage samples the amount of GAG was determined as previously described [32]. The GAG in the papain digest of cartilage samples was precipitated and stained with Alcian Blue as described above. Blue staining was quantified photometrically by the change in absorbance at 620 nm. Chondroitin sulphate (C4383; Sigma) was used as a reference. Values were normalized to the wet weight of the cartilage and expressed as milligrams of GAG gram wet weight of cartilage (mg/g).

Release of newly formed proteoglycans as a measure of retention of these proteoglycans was determined similarly. GAG was precipitated from the medium obtained from day 4 to day 7 and stained with Alcian Blue dye solution [34]. The radio-labelled GAG was measured by liquid scintillation analysis and normalized to the proteoglycan synthesis rate and expressed as the percentage release of newly formed proteoglycans.

To measure the total release of proteoglycans, the blue staining of the medium was quantified spectrophotometrically by the change in absorbance at 620 nm. Chondroitin sulphate (C4383; Sigma) was used as a reference. Values were normalized to the GAG content of the explants and expressed as the percentage release of GAG.

Calculations and statistical analysis

Because of focal differences in the composition and bioactivity of the cartilage on the femoral condyles, the results for at least eight cartilage samples per parameter per donor, obtained randomly and handled individually, were averaged and taken as a representative value for the cartilage of that donor. Several experiments with cartilage from different donors were performed. To make dose–response curves, a representative experiment was chosen and the intra-intra-assay variation (+ s.e.m.) of the eight samples per parameter is shown. On the bar graphs, the average of at least four individual experiments with the inter-assay variation (+ s.e.m.) is given; the n values are the exact numbers of experiments performed. Statistical evaluation of differences between treatments was performed with a non-parametric test for correlated data. P values less than or equal to 0.05 were considered statistically significant.

Results

Effect of celecoxib on normal human articular cartilage

Celecoxib, up to a concentration of 10 μM, did not change the proteoglycan turnover of normal healthy human articular cartilage (Fig. 1). The proteoglycan synthesis rate (Fig. 1A), the release of newly formed proteoglycans as a measure of retention of these newly formed proteoglycans (Fig. 1B) and the total release of proteoglycans (newly formed plus resident proteoglycans) (Fig. 1C) were not influenced significantly by celecoxib at concentrations of 0.01–10 μM.

Effect of celecoxib on human articular cartilage explants under the influence of inflammatory cells

Addition of RA PBMC to articular cartilage, mimicking inflammation, resulted in strong inhibition of cartilage proteoglycan matrix synthesis up to almost complete inhibition (always less than 10% of control synthesis, on average 0.2 ± 0.02 nmol/h per g remained). As a result, the release of newly formed proteoglycans was negligible (on average 0.02 ± 0.01 nmol/h per g in 3 days). Addition of celecoxib up to a concentration of 10 μM was unable to recover this disproportionately inhibited proteoglycan synthesis.

GAG release was increased by addition of PBMC (2-fold, 2.2 ± 1 to 4.9 ± 2 mg/g; P = 0.072) and could be normalized by celecoxib (to 2.3 ± 1 mg/g; P = 0.034). Percentage GAG release (GAG release normalized to the GAG content) was significantly increased by adding the inflammatory cells (on average 3-fold in 3 days) (Fig. 2A) and could be normalized completely in a dose-dependent way by addition of celecoxib.

As a result of the significantly increased GAG release and the strong inhibition of synthesis, a decrease in proteoglycan content was observed (11% in 7 days) (Fig. 2B). This decrease in proteoglycan content could be normalized completely by addition of celecoxib.

These effects of celecoxib could have depended entirely on inhibition of the catabolic properties of the inflammatory cells. However, it appeared that the proinflammatory cytokine levels produced by the cells were not significantly influenced by celecoxib (without celecoxib and with 10 μM celecoxib: 70 and 97 ng/ml respectively for IL-1, and 442 and 602 ng/ml for TNF-α; neither difference was statistically significant).

Effect of celecoxib on human articular cartilage explants under the influence of proinflammatory cytokines

To evaluate whether the effect of celecoxib was evoked indirectly by changing inflammatory cell activity or
directly by changing chondrocyte activity, cartilage was exposed directly to IL-1 plus TNF-α, the major mediators of inflammation-induced cartilage damage. Under the influence of IL-1 plus TNF-α, proteoglycan synthesis was significantly inhibited (on average 75% inhibition, $P < 0.022$) (Fig. 3A). Celecoxib was able to reverse this inhibition of proteoglycan synthesis significantly and dose-dependently, but not completely. Although a two-fold increase of proteoglycan synthesis was observed at the maximum concentration tested (10 μM), 46% inhibition remained (Fig. 3A).

Release of newly formed proteoglycans, both absolute (1.1 ± 0.2 to 0.6 ± 0.3 nmol/h/g) and as a percentage (normalized to the proteoglycan synthesis rate), was significantly increased by the addition of IL-1 plus TNF-α (on average a 2.5-fold increase, $P < 0.022$) (Fig. 3B), indicating impaired retention of the newly formed proteoglycans. Celecoxib was able to reverse this impaired retention of newly formed proteoglycans completely and dose-dependently (Fig. 3B).

Release of all GAG, newly formed plus resident GAG, absolute (0.8 ± 0.16 to 1.2 ± 0.16 mg/g) and as a percentage (normalized to the GAG content), was increased by addition of IL-1 plus TNF-α (on average...
an almost 2-fold increase, \( P < 0.022 \) (Fig. 3C). Celecoxib was able to reverse this increased release in a dose-dependent way and completely for the absolute (0.8 ± 0.05 mg/g) and percentage loss of GAG.

The decreased synthesis induced by IL-1 plus TNF-\( \alpha \) and the retention of proteoglycan and increased loss of GAG resulted in a lower GAG content of the cartilage (on average 20% loss, \( P < 0.040 \)) (Fig. 3D). Recovery of the disturbed cartilage matrix turnover induced by the proinflammatory cytokines upon addition of celecoxib resulted in dose-dependent normalization of the GAG content, which was statistically significant at 10 \( \mu \)M celecoxib.

PGE\(_2\) levels of cartilage were significantly elevated under the influence of IL-1 plus TNF-\( \alpha \) (2 ± 0.2 and 32 ± 9 ng/g for control and stimulated conditions respectively; \( n = 3; P < 0.02 \)). Addition of celecoxib led to significant reduction in the PGE\(_2\) level to the control level (1 ± 0.3 ng/g; \( n = 3; P < 0.02 \)).

**Discussion**

The structural integrity of the matrix of human articular cartilage is maintained by a dynamic equilibrium between synthesis and degradation. In RA and OA, small quantities of the proinflammatory cytokines—mainly IL-1 and TNF-\( \alpha \) from inflamed synovial tissue—are able to inhibit matrix synthesis [35–38]. This leads to impaired potential for repair and consequently increased susceptibility to mechanical damage [39]. Both cytokines have also been shown to increase the release of matrix components by the induction of matrix proteases (synthesis, release and activation) from chondrocytes by stimulation of the catabolic properties of these cells [40]. Also, the inflamed synovial tissue produces significant amounts of cartilage-destructive enzymes [38, 41]. Adverse changes in both the anabolic and catabolic processes of matrix turnover will be significantly damaging for cartilage.

There is a significant amount of evidence that cartilage itself is sensitive to certain NSAIDs. Mostly adverse effects, but also no effects or positive effects, have been reported. NSAIDs have been demonstrated to inhibit the synthesis of cartilage proteoglycans [20, 21, 42] and to increase the release of proteoglycans [43]. Also, recovery from the adverse effects of proinflammatory cytokines such as IL-1 has been reported to be diminished [39, 44, 45]. These are direct effects on cartilage and should be seen in the context of the
significant anti-inflammatory effects of these NSAIDs and hence of the indirect beneficial effect on cartilage integrity.

In animal models of arthritis, selective COX-2 inhibitors not only prevent the appearance of the characteristic features of inflammation but also prevent cartilage destruction [46–50]. However, this does not exclude the adverse direct effects on cartilage, as the effects mentioned above may arise by inhibition of cartilage-destructive inflammation. In the case of prolonged treatment, such adverse effects might, in the long term, dominate the anti-inflammation-mediated protection of cartilage.

Although the present study used an in vitro model, it is the first to show that celecoxib has no direct effects on normal human articular cartilage. This is in contrast to several other NSAIDs tested under comparable conditions, which showed adverse effects [42, 43, 51]. This absence of an adverse effect of celecoxib was to be expected in the light of selective inhibition of COX-2, as this isoform is not expected to be produced in significant amounts in normal human cartilage. However, it is not known if the adverse effects on cartilage of some of the conventional NSAIDs result from inhibition of COX-1, which could be essential for normal chondrocyte function, or if other mechanisms are also involved.

The present study shows that celecoxib does not interfere with chondrocyte function under normal conditions. In addition, celecoxib is able to restore cartilage proteoglycan turnover when it is influenced adversely by inflammation. Inflammatory cell-induced loss of GAG could be reversed, normalizing proteoglycan content despite the absence of a noticeable effect on proteoglycan synthesis. This suggests that inhibition of new formation contributes significantly less to the decrease in proteoglycan content than the increased loss of resident proteoglycans. These effects of celecoxib could depend entirely on inhibition of the catabolic properties of the inflammatory cells. However, it appeared that proinflammatory cytokine levels produced by the inflammatory cells were not influenced significantly by celecoxib. Although not conclusive, this suggests that celecoxib has a direct effect on chondrocytes when they are under the influence of PBMC. This was proved by the effect of celecoxib on cartilage exposed to IL-1 plus TNF-α. Assuming that celecoxib is unable to neutralize the activity of both cytokines directly, for example by binding, the effect of the selective COX-2 inhibitor must indeed have been directly on the chondrocytes. Interestingly, this suggests that the disturbed proteoglycan turnover of cartilage, diminished proteoglycan synthesis, diminished retention of newly formed proteoglycans and increased proteoglycan release, under the influence of IL-1 plus TNF-α, is mediated mainly by COX-2. Because synthesis is not normalized completely, additional pathways must be involved. In the gastrointestinal tract, COX-2 is suggested to be involved in tissue repair [52–56]. The present data show that COX-2 is involved in breakdown of cartilage under the influence of inflammation.

The induction of COX-2 by IL-1 plus TNF-α might be directly linked to excessive PGE2 production, which would provide an explanation of the disturbed proteoglycan turnover. Few data on the effects of prostaglandins on cartilage have been published. The effect of PGE2 on cartilage metabolism is still controversial, and depends on the type [26] and stage of differentiation [57–60] of the target cells. The detection of high concentrations of PGE2 in rheumatoid synovial fluid [61–63] and synovial tissue [64] and the stimulation of PGE2 synthesis by articular cartilage chondrocytes cultivated in conditioned media of rheumatoid and non-rheumatoid synovial tissue cells [65 and present observations] suggest the involvement of PGE2 in the catabolic processes of articular cartilage in RA. An adverse effect of excessive PGE2 on cartilage is supported indirectly by our experiments.

In conclusion, the present study suggests that the inflammation-induced adverse effects on cartilage are mediated by COX-2 and that celecoxib, a selective COX-2 inhibitor, has cartilage-protective properties as it can restore the integrity of the cartilage matrix under inflammatory conditions. Although elevated PGE2 levels were found in the present experimental set-up, additional studies with OA and RA cartilage should be performed. Such studies could show if selective COX-2 inhibition is beneficial to cartilage in these pathological conditions. Nevertheless, the present results suggest that, in addition to its gastroprotective properties, celecoxib may be a drug of choice in the treatment of chronic destructive joint diseases where anti-inflammatory drugs need to be used for a prolonged period.

Acknowledgements
This study was supported by a grant from Pharmacia.

References
Selective COX-2 inhibitors prevent cartilage damage 807

41. Clark JM, Powell LA, Ramsey S, Hazleman BL, Cawston TE. The measurement of collagenase, tissue inhibitor of metalloproteinases (TIMP), and collagenase–TIMP complex in synovial fluids from patients with...