Effects of celecoxib on human chondrocytes - enhanced production of chemokines

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Abstract

Objective

The purpose of this study was to examine the effects of a selective cyclooxigenase-2 (COX-2) inhibitor (celecoxib) comparing diclofenac.

Methods

Using chondrocytes derived from cartilage of non-arthritic (NA) subjects or patients with osteoarthritis (OA) or rheumatoid arthritis (RA), we examined the effects of celecoxib on incorporation of ^{3}H-thymidine and ^{35}S-sulfate, apoptosis, and production of matrix metalloproteinase (MMP)-1, MMP-3, MMP-13, and regulated upon activation, normal T cell expressed and secreted (RANTES), macrophage inflammatory protein (MIP)-1α and nitric oxide (NO).

Results

Celecoxib and diclofenac tended to reduce ^{3}H-tymidine incorporation of chondrocytes. Celecoxib induced apoptosis in a dose-dependent manner, but to a lesser degree than diclofenac. Celecoxib inhibited proteoglycan synthesis (indicated by ^{35}S-sulfate incorporation) in NA chondrocytes, but not in OA and RA chondrocytes. Celecoxib increased interleukin-1 (IL-1)-induced production of RANTES and MIP-1α by chondrocytes and decreased IL-1-induced NO production by chondrocytes, whereas it did not affect MMP production.

Conclusion

Celecoxib had both beneficial and adverse effects on chondrocytes. RA, OA and NA chondrocytes showed different responses. Interestingly, celecoxib enhanced the production of chemokines.

Key words

Selective COX-2 inhibitor, diclofenac, chondrocytes, osteoarthritis, rheumatoid arthritis, chemokines, MMP, nitric oxide.

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Introduction

Osteoarthritis (OA) is a chronic disease clinically characterized by pain and inflammation. Non-steroidal anti-inflammatory drugs (NSAIDs) are commonly used to treat symptoms of OA, and in many cases they are used long-term for pain relief. Whereas conventional NSAIDs have serious adverse gastrointestinal effects, selective COX-2 inhibitors have little effect on the gastrointestinal system. There have been conflicting reports about the effects of NSAIDs on articular cartilage. Some reports indicate that NSAIDs can aggravate articular destruction (1-3), whereas other reports demonstrate that they have chondroprotective effects (4, 5). Rheumatoid arthritis (RA) is a chronic, systemic inflammatory disease. Although the main trend in treatment of RA is toward disease-modifying drugs (DMARDs) and biological agents, non-steroidal anti-inflammatory drugs (NSAIDs) and selective COX-2 inhibitors are still used for long-term improvement of the quality of life of RA patients. (6). There have been few reports of the effects of selective COX-2 inhibitors on chondrocytes derived from OA or RA patients, or healthy subjects.

The purpose of the present study was to examine the direct effects of a selective COX-2 inhibitor (celecoxib) on human articular cartilage *in vitro*. Using human chondrocytes, we examined the effects of celecoxib on proliferation, apoptosis, proteoglycan synthesis, and production of matrix metalloproteases (MMPs), chemokines, and nitric oxide (NO) comparing those of diclofenac.

Materials and methods

Chondrocyte culture

Chondrocytes were prepared from surgical specimens obtained during athroplasty for OA and RA. Specimens obtained from femoral neck fractures were used as non-arthritic (NA) controls. The OA patients (n = 5) had an average age of 78.0 years and were diagnosed as having primary OA of the knee or the hip according to the criteria of ACR (7). The RA patients (n = 5) fulfilled the ACR criteria for RA (8) and had an average age of 67.3 years. The RA pa-tients were treated with predonisolone (= < 5 mg/day) and disease-modifying drugs including methotrexate. The average age of the fracture patients (n = 6) was 80.3 years. The articular cartilage of their specimens showed no arthritic aspects and radiological findings. Cartilage was carefully cut away from the subchondral bone, finely minced and digested in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, NY) with 1 mg/ml bacterial collagenase (Sigma, MO). The cells were then filtered, washed, and seeded into 75-cm^2 flasks (Becton Dickinson Labware, Bedford, UK). The cells were cultured in DMEM containing 10% fetal calf serum (FCS; Gibco BRL, NY), 100 units/ml penicillin (Gibco BRL), and 100 μg/ml streptomycin (Gibco BRL). The cells were expanded once and used for the following experiments.

^{3}H- thymidine incorporation

Chondrocytes were seeded in 96-well plates and incubated with conditioned medium until they became confluent. Then, the medium was aspirated, and the cells were cultured for 48 hours at 37°C in conditioned medium containing celecoxib (10 nM, 100 nM, 1 μM or 10 μM), diclofenac (10 μM) or actimomycine D (100 nM). After 44 hours of culture, the medium was supplemented with 78 kBq/ml ^{3}H-thymidine (Daiichi Kagaku Yakuhin, Japan). The cells were harvested onto a glass fiber using Harvester 9600 (TOMTEC, Connecticut, USA), and the radioactivity was measured using a liquid scintillation counter (MicroBeta TriLux; WALLAC, Turku, Finland). Each experiment was quadruplicated, and each value used for analysis was the mean of the 4 measurements.

Cell viability assay

Chondrocytes were cultured in 6-well plates with conditioned medium until they became confluent. Then, the medium was aspirated, and the cells were cultured in conditioned medium with serial concentrations of celecoxib or diclofenac for 24 hours at 37°C. Chondrocytes were collected by trypsinization with 0.5 mg/ml trypsin for 5 minutes, and were then washed

twice in phosphate-buffered saline (PBS). Cell viability was evaluated using the Guava ViaCout Assay system (Guava Technologies, USA) according to the manufacturer's instructions. Briefly, cells were suspended in 50 µl of PBS in 1.5-ml tubes and ViaCount Reagent was added to each tube, followed by incubation for 30 min in the dark. The cells were analyzed using a Guava personal cytometer that could count apoptotic cells automatically. Our previous study showed that the frequency of apoptotic cells counted by Guava staining was in accordance with annexin V positive cells analyzed by a conventional flow cytometry (9).

Proteoglycan synthesis
Chondrocytes were seeded in 24-well plates and cultured in conditioned medium until they became confluent. Then, the medium was aspirated, and the cells were cultured in conditioned medium containing serial concentrations of celecoxib, diclofenac (10 µM) or actimomycine D (100 nM) for 48 hours. After 42 hours of culture, 370 kBq/ml $Na_2[^{35}S]O4$ (Daiichi Kagaku Yakuhin, Japan) was added to the medium. The supernatant was aspirated and stored for later detection of labeled proteoglycan. The cells were dissolved in 500 µl of papain solution (25 µg/ml papain, 0.01 M sodium acetate, 0.05 M disodium EDTA, 0.005M L-cystein, HCl [pH 5.8]) for 16 hours at 60°C. We assayed proteoglycan synthesis by measuring ^{35}S-sulfate incorporation using a dye-binding technique (10). Briefly, 25 µl of cellular sample and supernatant was applied onto a filtered 96-well plate (Multiscreen-HV MAHV N4510; MILLIPORE, USA), followed by addition of 75 µl/well dilution buffer (0.05 M sodium acetate [pH 5.8], 0.5% Triton X-100) and 100 µl/well Alcian blue dye solution (0.2% Alcian blue, 0.05 M sodium acetate, 0.085 M $MgCl_2$). Each plate was agitated for 30 min at room temperature, and the aqueous fraction was filtered through the membrane using a Millopore vacuum manifold apparatus (MILLIPORE, USA). Each well was washed with 200 µl of washing buffer (0.05 M sodium acetate, 0.1 M sodium sulfate, 0.05 M

$MgCl_2$ [pH 5.8]) 3 times. The membrane in the bottom of each well was punched out, and the radioactivity was measured using a liquid scintillation counter (TAURUS No 36010; MICROMEDIC SYSTEM INC., USA). Total ^{35}S-sulfate incorporation was the sum of labeled proteoglycan in the cells and the supernatant.

Production of MMPs, chemokines and nitric oxide
Chondrocytes exposed to arthritic conditions such as OA have been shown to be affected by interleukin-1 (IL-1), and IL-1 induces production of MMPs, chemokines and NO by chondrocytes (11, 12). Therefore, we exposed chondrocytes to IL-1 and then treated them with various concentrations of celecoxib or diclofenac. Chondrocytes were seeded in 24-well plates and cultured in conditioned medium until they became confluent. Then, the medium was replaced with 2.5% FCS/DMEM containing 5ng/ml IL-1β (Rosch, Mannheim, Germany) and either serial concentrations of celecoxib or diclofenac (10 µM), and the cells were cultured for 48 hours. Supernatant was then collected to measure the levels of MMP-1, MMP-3, MMP-13, MIP-1α and RANTES by enzyme-linked immunosorbent assay (ELISA) using commercially available kits (Amersham Pharmacia Bioteck, UK [MMP-1, MMP-13 and MIP-1α]; Daiichi Kagaku Yakuhin, Japan [MMP-3]; R&D systems Quantikine [RANTES]). Nitric oxide was measured using the Gliess method (Nitrite Colorimetric Assay Kit; BioDynamics Laboratory, Japan). Each value used for analysis represents the average of duplicate or triplicate experiments for each individual subject.

Statistical analysis
The values used for analysis were expressed as the mean ± SEM. As values of proteoglycan synthesis and those from ELISA were dependent based on the samples from individual patients, statistical evaluation were performed using the Friedman test followed by Wilcoxon's signed rank test for pairwise comparison between control and treatment groups. Values of prolifera-

tion assay and cell viability assay were independent, thus statistical analysis was made using Kruskal-Wallis rank sum test followed by the Mann-Whitney test for pairwise comparison between groups.

Results
³H- thymidine incorporation
There was a similar tendency in 3H-thymidine incorporation between OA, RA and NA samples. With all 3 sample types, celecoxib reduced ³H- thymidine incorporation in a dose-dependent manner and statistical significance was found in OA and RA chondrocytes. Diclofenac (10 µM) significantly reduced ³H- thymidine incorporation only in RA chondrocytes and the reduction was less compared to celecoxib at the same concentration as celecoxib (Fig. 1).

Apoptosis induction
Both celecoxib and diclofenac increased the percentage of apoptotic cells in a dose-dependent manner in all cell types. However, celecoxib induced apoptosis less than diclofenac in RA and NA. The percentages of apoptotic cells treated with celecoxib remained within 10% and significantly less compared to diclofenac at a concentration of 10 µM. (Fig. 2).

Proteoglycan synthesis
Proteoglycan synthesis was not affected by celecoxib and diclofenac in OA chondrocytes, whereas proteoglycan synthesis was suppressed by 10 µM of diclofenac of RA chondrocytes and by both celecoxib and diclofenac in NA chondrocytes (Fig. 3). Thus, suppressive effect on proteoglycan synthesis of celecoxib was less than diclofenac in RA patients.

Production of MMP-1, MMP-3, MMP-13, MIP-1a, RANTES and NO by chondrocytes.
Celecoxib did not affect production of MMP-1, MMP-3 and MMP-13 by IL-1 stimulated chondrocytes or unstimulated chondrocytes. While the levels of RANTES and MIP-1α were below the limit of detection in unstimulated chondrocytes, those in IL-1 stimulated chondrocytes were more than 200pg/ml
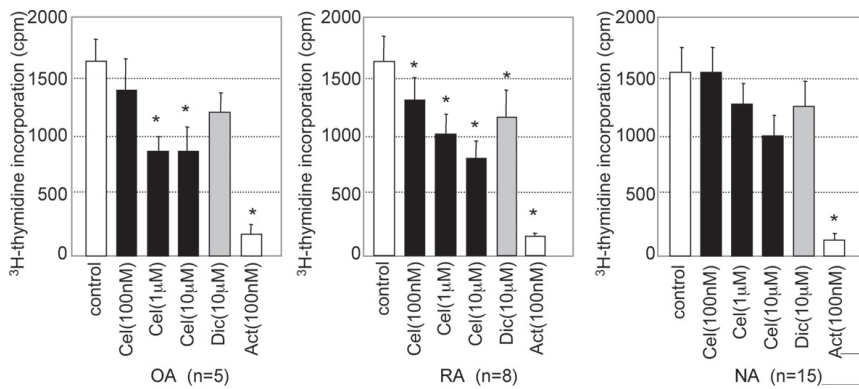
Fig. 1. ^{3}H-thymidine incorporation was examined in chondrocytes derived from OA, RA and NA samples. Cel: celecoxib, Dic: diclofenac, Act: actinomycine D, OA: osteoarthritis, RA: rheumatoid arthritis, NA: non-arthritic. Values are expressed as the mean ± SEM. *: $p < 0.05$ compared to control [Kruskal-Wallis rank sum test and Mann-Whitney test].
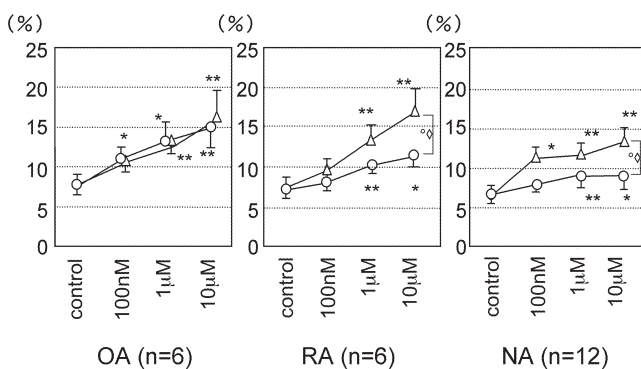

Fig. 2. The percentage of apoptotic cells defined by Guava Viacout Assay system. Open circle indicates celecoxib. Triangle indicates diclofenac. Values are expressed as the mean ± SEM. *: $p < 0.05$; **$p < 0.01$ compared to controls [Kruskal-Wallis rank sum test and Mann-Whitney test]; §: $p < 0.05$ celecoxib vs diclofenac [Mann-Whitney test].
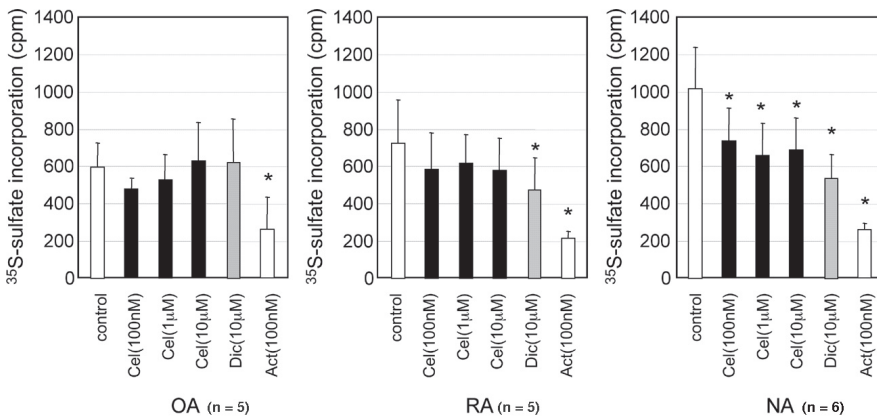

Fig. 3. Proteoglycan synthesis expressed by 35S-sulfate incorporation. Cel: celecoxib; Dic: diclofenac; Act: actinomycine D; OA: osteoarthritis; RA: rheumatoid arthritis; NA: non-arthritic. Values are expressed as the mean ± SEM. *: $p < 0.05$ compared to control [Friedman test and Wilcoxon's signed rank test].

on average. Interestingly, IL-1-induced RANTES production was significantly increased by 100 nM, 1 µM and 10 µM of celecoxib and 10 µM of diclofenac in OA chondrocytes, by 100 nM of celecoxib and 10 µM of diclofenac in RA chondrocytes, and by 100 nM and 1 µM of celecoxib in NA chondrocytes (Fig. 4). With all cell types, IL-1-induced MIP-1α production was signifi-

cantly increased by 100 nM, 1 µM and 10 µM of celecoxib. With NA chondrocytes, IL-1-induced MIP-1α production was also increased significantly by 10 µM of diclofenac (Fig. 5). Celecoxib did not affect MMP production in all cell types (data not shown).

Celecoxib and diclofenac have a tendency to suppress IL-1-induced NO production. The suppression of IL-1-

induced NO production was significant in RA chondrocytes treated with 1 µM of celecoxib and in NA chondrocytes treated with 1 µM and 10 µM of celecoxib. The suppressive effect of diclofenac was not siginificant (Fig. 6).

Discussion

NSAIDs and selective COX-2 inhibitors are often used for the treatment of chronic arthritis such as OA and RA over the long term, and gastrointestinal adverse effects of conventional NSAIDs and cardiovascular events of some coxibs have been critical issues. With long-term administration of coxibs or NSAIDs, their direct effects on chondrocytes should be taken into consideration as well. Mean peak plasma concentrations of diclofenac were 2.2 to 4.7 µM after a single dose of 50 mg (13) and a mean peak plasma concentration of celecoxib was 1.85 µM after a single dose of 200 mg. (14). Thus, the concentrations that used in the present study approximately reflected the therapeutic dosage.

Chondrocyte proliferation, indicated by ^{3}H-thymidine incorporation, is slightly but significantly suppressed by celecoxib and diclofenac. Though the clinical significance of chondrocyte proliferation is unclear, celecoxib has been shown to have negative effects on it in this study. In growth plate chondrocytes, it has been reported that, PGE_2 is necessary for DNA and matrix synthesis (15). Thus, suppression of ^{3}H-thymidine incorporation may be due to an inhibitory effect of celecoxib on PGE_2 synthesis by chondrocytes.

Depletion of chondrocytes in OA cartilage can be mediated by apoptosis (16). As for apoptosis, there has been conflicting findings regarding prostaglandins and its derivatives. Whereas exogenous PGE_2 induced chondrocyte apoptosis through cAMP-dependent pathway (17), 15d-PGJ_2 (another metabolite of arachidonic acid) has anti-apoptotic effects (18). Thus, the induction of apoptosis by celecoxib and diclofenac may be due to the altered balance of arachidonic cascade. The present study also showed that the response of chondrocytes to celecolxib and diclofenac was different between
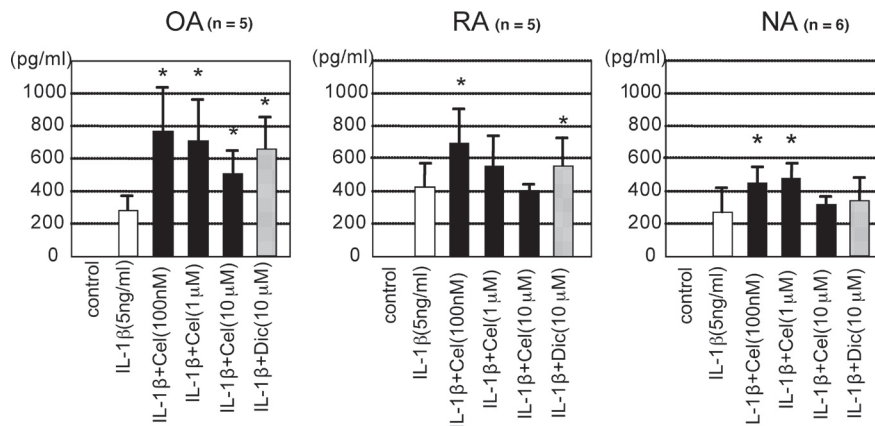
OA (n = 5)

RA (n = 5)

NA (n = 6)

Fig. 4. RANTES production by OA, RA and NA chondrocytes and effects of celecoxib and diclofenac. Each value represents the results of individual samples. Cel: celecoxib; Dic: diclofenac; OA: osteoarthritis; RA: rheumatoid arthritis; NA: non-arthritic. Values are expressed as the mean ± SEM. *: $p < 0.05$ compared to IL-1β [Friedman test and Wilcoxon's signed rank test].

OA (n = 5)
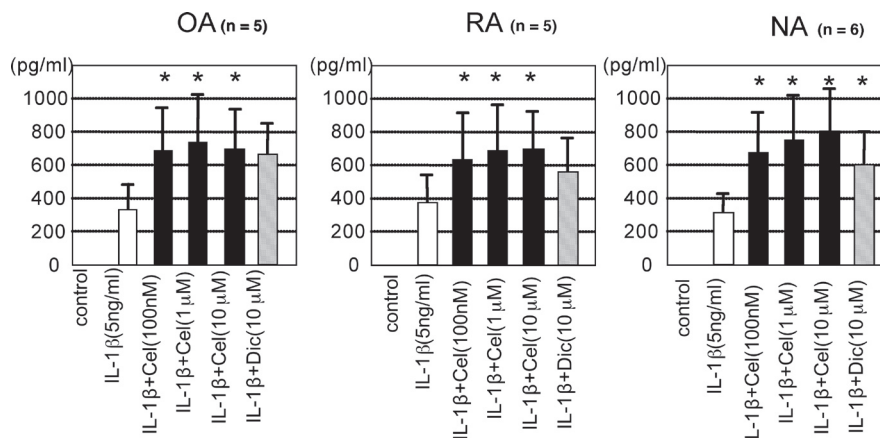

RA (n = 5)

NA (n = 6)

Fig. 5. MIP-1α production by OA, RA and NA chondrocytes and effects of celecoxib and diclofenac. Each value represents the results of individual samples. Cel: celecoxib; Dic: diclofenac; OA: osteoarthritis; RA: rheumatoid arthritis; NA: non-arthritic. Values are expressed as the mean ± SEM. *: $p < 0.05$ compared to IL-1β [Friedman test and Wilcoxon's signed rank test].

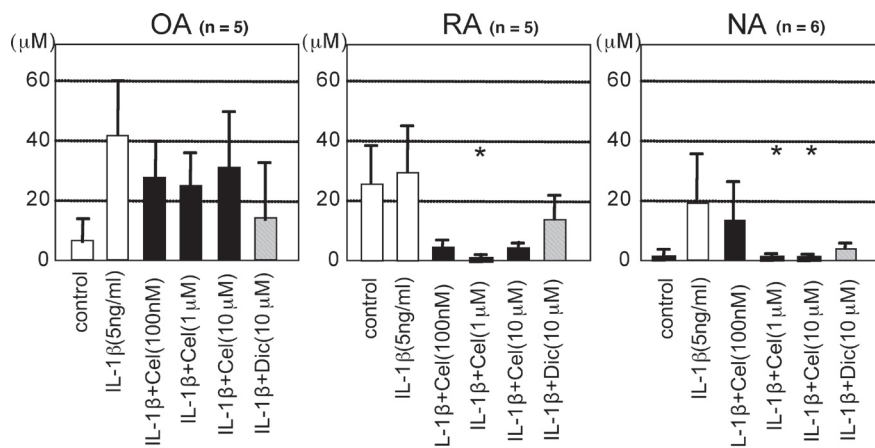## OA (n = 5)

RA (n = 5)

NA (n = 6)

Fig. 6. Nitric oxide production by OA, RA and NA chondrocytes and effects of celecoxib and diclofenac. Each value represents the results of individual samples. Cel: celecoxib; Dic: diclofenac; OA: osteoarthritis; RA: rheumatoid arthritis; NA: non-arthritic. Values are expressed as the mean ± SEM. *: $p < 0.05$ compared to IL-1β [Friedman test and Wilcoxon's signed rank test].

OA, RA and NA.

There have been reports of both beneficial and adverse effects of NSAIDs on proteoglycan or glycosaminoglycan (GAG) synthesis of chondrocytes. Bjelle *et al*. (19) and Collier *et al*. (20)

reported that some NSAIDs, including diclofecnac, significantly inhibited total ^{35}S incorporation in GAG both in matrix and in medium. In contrast, Masterbergen *et al*. (21) reported that celecoxib increased the proteoglycan content in explant culture. In the present study, celecoxib and diclofenac exerted various degrees of suppression of proteoglycan synthesis by chondrocytes. The discrepancies between this study and previous studies conducted by other researchers seem to be largely due to differences in experimental design. Proteoglycan synthesis of OA chondrocytes was not affected by celecoxib and diclofenac, whereas proteoglycan synthesis in RA chondrocytes was suppressed exclusively by diclofanac and that in NA chondrocytes was suppressed both by celecoxib and diclofenac. The OA chondrocytes which phenotypes presumably were altered by exposure to various cytokines and growth factors *in vivo* might be relatively resistant to celecoxib from the view point of proteoglycan synthesis.

It has been reported that celecoxib inhibits the production of MMP-1, MMP-2 and MMP-3 by stimulated rheumatoid synovial cells (22). In the present study, celecoxib and, diclofenac had no positive or negative effect on MMP production by chondrocytes.

In our previous study, we found that ckemokines (which are inflammatory and immune mediators) are involved in degradation of articular cartilage (12). In that study, chondrocytes were shown to express RANTES, MIP-1α, MIP-1β and MCP-1, and their receptors, and these chemokines suppressed proteoglycan synthesis. In the present study, RANTES and MIP-1α were upregulated by IL-1 stimulation and, interestingly, the expression of these chemokines was further increased by celecoxib. In this view point, celecoxib might have adverse effects on articular cartilage.

Nitric oxide is expressed in OA chondrocytes, directly breaks down cartilage matrix and induces chondrocyte apoptosis, thereby damaging articular cartilage (23, 24). Thus, suppression of chondrocyte NO production by celecoxib is beneficial for OA patients.

In conclusion, celecoxib has both beneficial and adverse effects on chondrocytes *in vitro*, and the effects depend on the combination of cell type and drugs. Interestingly, celecoxib increases IL-1-induced chemokine production by chondrocytes.

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