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Long-term NSAID treatment directly decreases COX-2 and mPGES-1 production in the articular cartilage of patients with osteoarthritis

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Summary

Objective: To simultaneously study the effect of a selective cyclooxygenase-2 (COX-2) inhibitor and that of a classic non-steroidal anti-inflammatory drug (NSAID) on the expression of pro-inflammatory genes in the cartilage of patients with severe knee osteoarthritis (OA) and in cultured human OA chondrocytes.

Methods: A 3-month clinical trial was carried out on 30 patients with severe knee OA scheduled for knee replacement surgery. Patients were randomized into two groups: patients treated with celecoxib (CBX) and patients treated with aceclofenac (ACF). OA patients who did not want to be treated served as the control group. After surgery, cartilage was processed for molecular biology studies. We also employed cultured chondrocytes from different OA patients to examine NSAID effects on pro-inflammatory gene expression in cells stimulated with interleukin (IL)-1β.

Results: Both CBX and ACF inhibited COX-2, microsomal prostaglandin E synthase-1 (mPGES-1) and inducible nitric oxide synthase (iNOS) synthesis in the articular cartilage of OA patients. In cultured chondrocytes, both NSAID decreased COX-2 and mPGES-1 synthesis and prostaglandin E2 (PGE2) release induced by IL-1β, while no effect was observed on nitric oxide or iNOS synthesis. In OA patients, only CBX decreased tumor necrosis factor alpha and IL-1β expression in the cartilage, while both NSAID diminished IL-1β induced cytokine synthesis in cultured OA chondrocytes.

Conclusions: Both NSAID diminished PGE2 release and induced a decrease in COX-2 and mPGES-1 synthesis in the cartilage from OA patients and in OA chondrocytes. These data suggest that prolonged therapy with PGE2 blocking agents decreases PGE2 production not only by direct inhibition of COX-2 activity, but also by down-regulating COX-2 and mPGES-1 synthesis in the cartilage. However, CBX and ACF seem to have a different anti-inflammatory profile in controlling pro-inflammatory gene expression in the cartilage.

Key words: Osteoarthritis, COX-2, Articular cartilage, NSAID, Celecoxib.

Introduction

Osteoarthritis (OA) is characterized by the degeneration of cartilage matrix components together with an excessive production of different cytokines. Interleukin (IL)-1β increases the synthesis of many pro-inflammatory mediators such as the inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), microsomal prostaglandin E synthase-1 (mPGEs-1), and matrix metalloproteinases, and, hence, it promotes the release of nitric oxide (NO) and prostaglandin E2 (PGE2)[1–4]. Similarly, IL-1β also retards anabolic activities in chondrocytes that leads to a decline in proteoglycan and collagen synthesis[5,6].

NO is also implicated in joint inflammation and the structural deterioration observed during OA[7–9]. NO inhibits matrix synthesis and induces its degradation, and promote cell injury[7–9]. iNOS is the key enzyme responsible for NO generation in inflammatory and pathological processes, including OA. NO synthesis seems to take place mostly in the cartilage during OA[10], and its accumulation correlates with the joint destruction[11].

PGE2 is the eicosanoid found at the highest concentrations in OA joints, and it can be synthesized by chondrocytes, synoviocytes and macrophages[12]. PGE2 regulates many processes, including the release of inflammatory mediators, cell apoptosis, neoangiogenesis, the synthesis of structural mediators, and even symptoms of OA[12–14]. In fact, for decades, elements that control PGE2 synthesis have been the main targets in OA therapy. In the cartilage, PGE2 can exert anabolic and pro- or anti-catabolic effects[13]. PGE2 inhibited proteoglycan degradation[15] and prevented the release of metalloproteases[16]. At the same time, PGE2 inhibition prevented cytokine-induced cartilage damage[17] and increased proteoglycan synthesis in OA cartilage[18,19]. The different effects induced by PGE2 in the cartilage may largely depend on its binding to different cell surface prostaglandin E receptors (EP receptors), each coupled to a distinct cell response[20]. However,

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despite the clear importance there is still little known about the regulation of EP receptors during OA.

Non-steroidal anti-inflammatory drug (NSAID) are commonly used to treat symptoms of OA and, in many cases, they are employed over extended periods[21]. The therapeutic efficacy of NSAID in modulating pain and inflammation has been attributed to its ability to impair PGE2 release by inhibiting COX enzyme activity, although their direct effects on cartilage are still to be fully defined. There is growing interest in the effects of NSAID and of specific COX-2 inhibitors on cartilage, although these effects cannot be easily studied in clinical trials since the changes in cartilage during OA are rather slow process. To date, most of the information on how NSAID act comes from short-term cell or explants cultures studies and although many patients with OA have taken these drugs for several years, few studies have evaluated the long-term effects of NSAID.

Therefore, the aim of this study was to examine the effect of a specific COX-2 inhibitor, as well as that of a classic NSAID, on the expression of pro-inflammatory genes in the articular cartilage of patients with severe knee OA and in human OA chondrocytes in culture.

Patients and methods

PATIENTS

In this study, we evaluated the effect of the NSAID in the articular cartilage of the same OA patients included in a previous work in which we analyzed synovial inflammation associated to OA[22]. In the study were included 30 patients with clinical and radiological evidences of knee OA that had been scheduled for total knee replacement surgery (Hospital Virgen de la Cinta, Tortosa, Tarragona, Spain). The patients included in the study that agreed to NSAID therapy were randomized into two groups that received either celecoxib (CBX) (200 mg/24 h) or aceclofenac (ACF) (100 mg/12 h) for 3 months[22]. Ten patients who agreed to participate in the study but preferred not to be treated with NSAID were also recruited to the study and used as control (CTR) group[22]. Because of different technical problems five of the 30 patients enrolled into the study were excluded. So, the number of patients that completed the study was as follows: nine for the CBX group, seven for the ACF group and nine for the CTR group[22]. As previously described, OA patients at the baseline visit had similar clinical characteristics, and the Western Ontario and McMaster Universities Index of Osteoarthritis (WOMAC) questionnaire rendered identical results for the three groups[22]. During surgery, two different fragments from the medial femoral condyle of each patient were excised and immediately frozen to carry out further gene and protein expression studies. Two additional specimens from the same region were dissected to perform histopathological studies.

CARTILAGE HISTOPATHOLOGICAL STUDIES

In order to carry out histopathological studies on cartilage, two fragments from the medial femoral condyle of each patient were dissected. After macroscopic evaluation, the fragments were fixed for 24 h in 4% paraformaldehyde, decalcified for 6 weeks in an ethylenediaminetetraacetic acid (EDTA) solution (2 mM EDTA, 0.5 mM tartrate sodium potassium, pH 1) and embedded in paraffin.

The cartilage sections (4 μm) were stained with hematoxylin–eosin and Alcian blue, and the histopathological alterations were evaluated using the Mankin's grading system[23] in a blinded fashion. The histopathological study for each patient was carried out in the most damaged zone of the cartilage. A partial score for each category of the Mankin scale (structure abnormalities, cellularity, matrix staining and tidemark integrity) was attributed, and these scores were combined for each section.

CELL CULTURE

Chondrocytes were obtained from the knee articular cartilage of different OA patients included in the *in vivo* study (fulfilling American College of Rheumatology (ACR) criteria for functional class III or IV) and that underwent joint replacement surgery at the Orthopaedic Surgery Department of the Fundación Jiménez Díaz[24]. The global Mankin scores for the cartilage used to obtain chondrocytes cultures ranged from 11.5 to 12.5. Informed consent was obtained from each patient, as was the approval of the local ethical committee.

In each experiment, cells were deprived of serum for 48 h and then they were stimulated with 10 u/ml IL-1β (Immunogenex, LA, CA, USA). Where indicated, the cells were incubated with CBX (10^{-6} M, Pfizer, NY, USA) or diclofenac (DCF, 10^{-6} M, Sigma, St. Louis, MO, USA) for 60 min prior to stimulation, and the cells were maintained in the presence of these compounds throughout the experiment period. In the *in vitro* studies DCF was used instead of ACF because it is the main active metabolite of ACF[25]. NSAID were tested at the concentration which correspond to the mean peak plasma concentration after oral administration of a single therapeutic dose[26,27].

GENE EXPRESSION STUDIES USING REAL-TIME POLYMERASE CHAIN (PCR)

For gene expression studies, quiescent cells were incubated with 10 u/ml IL-1β in the presence or absence of the drugs. Total RNA from the cartilage or from the cultured chondrocytes was obtained using the RNeasy Lipid Tissue Kit (Qiagen Inc, Valencia, CA, USA). The High-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) was used to generate cDNAs[22]. RNA quantification was performed by single-reporter real-time PCR using the ABI Prism 7500-Sequence Detection system (Applied Biosystems)[22]. We used specific TaqMan assays inventoried for COX-2, mPGES-1, iNOS, EP1, EP2, EP3, EP4, IL-1β, and tumor necrosis factor alpha (TNFα) (all from Applied Biosystems). A pre-designed 18S rRNA assay (Applied Biosystems) was also used as an endogenous control. mRNA expression was normalized to that of 18S rRNA in each well. Moreover, each patient or experimental point for each gene was then normalized relative to the calibrator value (one of the CBX patients or unstimulated cells in each experiment were chosen as calibrator = 1).

WESTERN BLOT ANALYSIS

For protein studies, quiescent cells were incubated with 10 u/ml IL-1β in the presence or absence of drugs for 24 h. Cartilage proteins from each patient or from cultured chondrocytes were homogenized with ice-cold lysis buffer [1% Nonidet P-40 (Sigma); 0.5% sodium dodecyl sulfate, 0.1 M EDTA, 1 M dithiothreitol, and 1 M phenylmethylsulphonylfluoride in phosphate buffered saline]. The lysates were transferred to Eppendorf tubes and centrifuged twice at 12,000*g* for 10 min. The protein concentration was determined by the Bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). Western blot studies were performed as described previously[24]. The extracts were probed with primary antibodies against human COX-2 (Santa Cruz Biotech, Heidelberg, Germany), mPGES-1 (Cayman Chemicals Ann Arbor, MI, USA), iNOS (Chemicon, CA, USA), TNFα (Peprotech, London, UK) and IL-1β (MBL, Woburn, MA, USA). Each membrane was probed with anti-α-tubulin (Sigma) to ensure equal protein loading, and the immunoreactivity of each protein was then fully corrected for that of α-tubulin. The data are shown as the mean ± s.e.m. of arbitrary densitometry units for each group.

PGE2 ASSAY

PGE2 was determined in cell supernatants as described previously[28].

NITRITE ASSAY

The quantity of nitrites in the culture medium was measured by the Griess method. Quiescent cells were incubated for 24 h with 10 u/ml IL-1β in the presence or absence of drugs, and equal volumes of supernatant and Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamide dihydrochloride in 5% H_3PO4) were incubated at room temperature for 10 min. The absorbance was measured at 570 nm and the concentration of nitrites was calculated using a standard curve.

STATISTICAL ANALYSIS

All statistical analyses were performed using SPSS 11.0 (SPSS Inc) and the results were expressed as the mean ± S.E.M. Data from multiple groups were compared by Kruskal–Wallis non-parametric analysis, and a pairwise comparison using the Mann–Whitney test was applied when overall differences were identified. Differences were considered significant when the *P*-value was less than 0.05.

Results

CARTILAGE HISTOPATHOLOGY

All cartilage specimens stained with hematoxylin–eosin and Alcian blue were evaluated. The predominant histopathological