COX-2 inhibition in osteoarthritis: effects on cartilage

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COX-2 INHIBITION IN OSTEOARTHRITIS: EFFECTS ON CARTILAGE

COX-2 INHIBITIE IN ARTROSE:

EFFECTEN OP KRAAKBEEN

(MET EEN SAMENVATTING IN HET NEDERLANDS)

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Chapter 1

Introduction

Osteoarthritis

Osteoarthritis, or cartilage degeneration, is a widely prevalent chronic disabling condition. Clinically, osteoarthritis is characterized by joint pain, tenderness, li mitation of movement, crepitus, occasional effusion, and local inflammation secondary to the disease process (1). Pain, joint deformation, and stiffness of the joint capsule may lead to severe restriction of function and in the long term to disability. The origin of pain varies and is not completely understood. It is thought to include, inflammation of the synovium, distension of the joint capsule, periarticular muscle spasm, stretching of nerve endings in the periosteum around osteophytes, and microfractures in the bone (2). The main pathological characteristic of osteoarthritis is the progressive destruction of the articular cartilage. In many cases, subchondral cysts and sclerosis, osteophyte formation and mild synovial inflammation are observed (3). Diagnosis of osteoarthritis is mostly based on clinical symptoms and physical examination combined with radiographic evaluation (4). The American College of Rheumatology defines the most commonly accepted clinical classification criteria for hip, knee, and hand osteoarthritis (5-7).

Osteoarthritis is the most frequent form of musculoskeletal disorder, affecting over 10% of the adult population (8). This disease causes significant pain and disability in 25% of people over the age of 60 and accounts for half of all chronic conditions in people aged over 65 years. Osteoarthritis has serious medical, social, and economic impact, since it causes many restrictions in daily life specifically for the aged population. The number of individuals over the age of 50 is expected to double between 1990 and 2020. For the first time in history, by 2010, there will be more people in Europe over 60 years of age than people less than 20 years of age, resulting in an escalation of medical costs, due to diseases related to age such as osteoarthritis.

Ample evidence suggests a multifactorial etiology of osteoarthritis by combinations of biomechanical, biochemical, inflammatory and genetic factors (9, 10). The initial event that triggers the pathological process is unclear and it is still being debated whether the initial changes occur in the cartilage, subchondral bone, or even in the synovium (11). Risk factors that are identified for osteoarthritis are diverse and can be separated in systemic factors that increase the susceptibility to osteoarthritis (gender, race, genetical predisposition) and in factors that determine the site and severity of osteoarthritis such as joint trauma, obesity, congential or acquired anatomical deformities, chondrocalcinosis and occupation (10, 12-15). It has been suggested that risk factors differ from joint to joint and at different sites within the same joint (16). Furthermore risk factors for the development of osteoarthritis may be different from those for the progression of osteoarthritis.

Tissues involved in the pathogenesis of osteoarthritis

Osteoarthritis is a joint disease with different etiologies but with similar biological, morphological, and clinical outcomes. The disease processes do not only affect the articular cartilage of the synovial joint, but also the adjacent bone, ligaments, capsule, synovial membrane, and peri-articular muscles. It is even been proposed that osteoarthritis is a systemic disorder and not a disease in which only the joint is affected (17).

Synovial joints facilitate mobility by allowing bones to articulate with one another. In synovial joints the bone ends are covered with a layer of hyaline cartilage. Joint capsule and ligaments connect the two opposing bone ends forming the joint. The joint cavity is lined by a vasculized synovium and is firmly enclosed by the fibrous capsule. It is filled with synovial fluid, which is an ultra-filtrate derived from plasma, supplemented with components, in particular hyaluronic acid, produced by the lining cells. Hyaluronic acid makes the fluid viscous, which is important for joint lubrication. Furthermore, the joint fluid is important for nourishment of the articular cartilage. The specific combination of bone, cartilage, synovial fluid, synovial lining, and joint capsule with surrounding ligaments, tendons, and muscles makes the synovial joint capable of resisting the biomechanical stresses that develop during joint movement and loading.

Articular Cartilage

Articular cartilage is an avascular, aneural connective tissue, important in resistance against compressive forces and shear stresses during joint use. It is also capable of absorbing stress by deforming under mechanical load, and thereby minimizing peak stresses on subchondral bone (18). In addition, the shock absorbing capacity of the subchondral bone results in less mechanical stress on the cartilage (19). The cartilage has great durability since in most people it provides normal joint function for over eighty years or even longer. Although cartilage seems a simple inert tissue, upon closer inspection it contains an elaborate, highly structured extracellular matrix that is deposited and maintained by a relative small number of highly specialized cells: the chondrocytes. Chondrocytes are considered to be highly differentiated mesenchymal cells, which produce the extracellular matrix and surround themselves with it. This results in the absence of cell-to-cell contact. Most chondrocytes probably cease to divide under healthy conditions when skeletal maturation has been achieved (20)

The major component of the extracellular matrix of articular cartilage are water (70-75% of the weight in adult tissue), collagen and proteoglycans (17-19% and 5-10% of the wet weight, respectively) (18, 21). The collagen network defines the form and tensile strength of articular cartilage, while the highly hydrophilic proteoglycans are responsible for the resilience of cartilage. These proteoglycans are immobilized in the collagen network, which results in fixation of a large negative charge within the cartilage matrix. To balance this negative charge, cations are drawn into the tissue, thereby creating a large osmotic potential. As a consequence, water is absorbed into the tissue, which results in a large swelling

force. In the unloaded condition, swelling of the tissue is constrained by tensile stiffness of the collagen network (22). On compressive loading of the joint, water is squeezed out of the cartilage. During unloading, because of the osmotic pressure of the proteoglycans, this water is absorbed again, providing the unique resilience of articular cartilage (18, 23). The matrix, where the turnover of proteoglycans is much higher than that of collagen, is maintained by chondrocyte activity. The principal collagen, type II collagen, accounts for 90 to 95 percent of the collagen in articular cartilage (24, 25). With increasing age there appears to be an increase in the non-enzymatic cross-linking between collagen molecules (26, 27). This will stiffen the cartilage matrix (28) and might limit the repair activity by chondrocytes (29) thus may contribute to cartilage damage with increasing age (30).

Proteoglycans are proteins to which are covalently attached one or more glycosaminoglycans. In cartilage, these glycosaminoglycans include hyaluronic acid, chondroitin sulphate, keratan sulphate, and dermatan sulfate (31). The predominant proteoglycan in articular cartilage is aggrecan, which is a large aggregating monomer. Most aggrecans are non-covalently associated with hyaluronic acid and link proteins to form large proteoglycan aggregates (32).

In healthy individuals, normal cartilage-matrix turnover exists in which synthesis and degradation are balanced. Chondrocytes respond to changes in the extracellular matrix, such as the presence of fragmented matrix molecules (breakdown products), the presence of cytokines and growth factors with catabolic and anabolic effects, and the frequency and intensity of joint loading (33-35). In osteoarthritis there is a progressive loss of articular cartilage accompanied by an attempt to repair the cartilage. Swelling is one of the first features in cartilage degeneration, indicating collagen damage (36). In turn this leads to a loss of tensile properties and to a loss of proteoglycans. Aging causes a decrease of the capacity of the cells to synthesize some types of proteoglycans. In addition, their response to stimuli, including growth factors, decreases (26). These age-related changes may limit the ability of the cells to maintain tissue integrity and thereby contribute to the development of degeneration of the articular cartilage.

Subchondral bone

Morphologic changes in the bone in osteoarthritis include the appearance of osteophytes at the margins of the joint, subchondral cysts, and subchondral sclerosis. Evidence of the involvement of subchondral bone in osteoarthritis originally comes from the inverse association between osteoarthritis and osteoporosis (37). The health and integrity of articular cartilage are at least dependent on the mechanical properties of the underlying bone. The subchondral bone is thought to be important in absorbing loads in the joint (38). In the initiation of cartilage damage, a sharp gradient of stiffness of the underlying bone may play a role. Progression of cartilage lesions was suggested to occur only in regions where subchondral bone has become stiffened, e.g. as a result of healing of microfractures (19).

Whether subchondral sclerosis precedes or follows injury to the articular cartilage is uncertain, both options have been suggested in animal models (39, 40) as well as in human osteoarthritis (41, 42). The role of subchondral bone in osteoarthritis appeared to be more complex, since osteoarthritic bone has also been described to be less stiff, to show a reduced mineral content and to be considerably less dense (43, 44). The apparent density of the subchondral bone seems to be increased in osteoarthritis probably because of the increased number of trabeculae and reduced separation between trabeculae, while the material density of the bone is significantly lower than in controls The reduction in mineral can be explained by the increased turnover rate in osteoarthritic bone, which results in younger, less mineralized bone.
In conclusion, although there is evidence for an important role of changes in subchondral bone in osteoarthritis (45) it remains unclear whether bony changes are important in the initiation and/or in the progression of human osteoarthritis and in what manner the changes are involved.

Synovial tissue
The synovial tissue lines the joint cavity and consists of one or two cell layers of synovial cells. The synovial lining cells play a role in removal of waste products from the joint cavity and in immunological defense. The cause of synovial inflammation and its role in the pathogenesis of osteoarthritis remains unclear. Osteoarthritis is commonly described as a non-inflammatory disease. Despite this, inflammation is increasingly recognized as contributing to the symptoms and progression of osteoarthritis (46, 47). Morning and inactivity stiffness are common symptoms in patients with the disease, and acute inflammatory flares, characterized by local warmth, tenderness and effusions are not uncommon. NSAIDs alleviate symptoms of osteoarthritis and may be more effective than simple analgesics, such as paracetamol (48). Serological and histological evidence of synovitis is commonly found in osteoarthritis, even though osteoarthritis has not been consistently associated with specific immune responses. Inflammation may contribute to the cartilage damage by producing catabolic cytokines, such as interleukine-1 (IL-1) and tumor necrosis factor α (TNFα), which in turn induce release of matrix metalloproteinases (MMPs), such as collagenases and stromelysins. MMPs have been localized in regions of degradation and they are elevated in synovial fluid and cartilage from osteoarthritic patients (20), suggesting a role of MMPs in the degradation of cartilage in osteoarthitis. MMP-1 and -13 (collegenase-1 and -3, respectively) are involved in degradation of type II collagen, while MMP-3 is one of the stromelysins, involved in the degradation of proteoglycans (49). The appearence of breakdown products of aggrecans in osteoarthritis pointed to the presence of aggrecanases (50, 51). These proteinases use a specific cleavage site in aggrecan and are considered to be of significance in cartilage degradation (20). All these metabolic mediators are also produced by chondrocytes and are involved in normal matrix turnover. Remarkably, they are produced in elevated amounts by osteoarthritic chondrocytes, contributing to joint

pathology. The role of synovial changes in cartilage damage in osteoarthritis remains minor compared to that in rheumatoid arthritis, where aggressive invasion of synovial tissue in cartilage and bone contributes significantly to joint damage.

Management of osteoarthritis

Current treatment of osteoarthritis is aimed at reducing pain, minimizing functional disability, and limiting progression of damage. Therefore a combination of both non-pharmacological and pharmacological treatment is needed. Non-pharmacological therapies include patient education -an essential part of any treatment of osteoarthritis-, lifestyle modification, weight reduction, regular exercise, physiotherapy and the use of orthopedic appliances (canes, insoles, braces) (52). Dependent on the cause of osteoarthritis, patients can modify their lifestyle. For example, patients who developed osteoarthritis due to overweight may experience a decrease in pain after weight loss. Patients, who developed osteoarthritis due to excessive use of a joint, may benefit from a decrease in loading of that particular joint in daily life. In addition, physical therapy is important in the treatment of osteoarthritis, since strengthening the muscles relating to the osteoarthritic joint may relieve pain and improve function (53). This effect is likely to be the result of an increase in the joint-stabilizing and shock-absorbing properties of the peri-articular muscles.

Pharmacological treatment includes the application of non-opioid or opioid analgesics, non-steroidal anti-inflammatory drugs (NSAIDs) or COXIBs and intra-articular glucocorticoids. Drugs for treatment of osteoarthritis have been classified according to the guidelines of the Osteoarthritis Research Society (54), as "symptom modifying" or "structure modifying" depending on their ability to relieve symptoms or to affect structural changes, respectively. Systemic treatment of osteoarthritis is mainly "symptom modifying" and is directed at relief of pain and at diminishing secondary inflammation. The first pharmacological line of treatment is analgesics. It has been recommended to prescribe NSAIDs only when analgesics do not result in symptom relief (55, 56). The use of NSAIDs or intra-articular corticosteroids is effective in diminishing secondary inflammation, thereby reducing pain. However, chronic use of NSAIDs is accompanied by an increased incidence of the gastric ulceration, erosions, perforation, hemorrhage, kidney, adverse renal effects including edema, and other deleterious effects (57-59).

Although much research is performed to find "structure modifying" drugs, or "disease modifying osteoarthritis drugs" (DMOADs), until now no convincing results have been described. Two components are considered to be possible candidates, namely Glucosamine sulphate and hyaluronan. Both these molecules are important cartilage matrix components and are lost from the matrix in osteoarthritic cartilage. In addition, hyaluronan in the synovial fluid is important for joint lubrication. There are several reports suggesting that these compounds are disease modifying (60, 61). Due to lack of a plausible mechanism of action, these compounds are still not generally accepted as a disease-modifying drug.

In the late stage of osteoarthritis, when analgesics, physical, and pharmacological

therapy do no longer provide sufficient pain relief and maintenance of function, surgical treatment of osteoarthritis is indicated. Joint replacement (endoprosthesis) and joint fusion (arthrodesis) are frequently used treatments, since both are effective in relieving pain. Joint implants, mainly used for hip or knee osteoarthritis, give good results. However, in later years the risk of loosening of the implant exists, which may make revision surgery with less good results necessary. Arthrodesis is at the expense of joint motion, which increases the risk of overloading adjacent or contralateral joints (62).
A relatively new approach is to treat severe OA patients with joint distraction (63). This treatment is based on avoiding mechanical contact between the articular surfaces while intra-articular intermittent fluid pressure is maintained. Significant clinical benefit was found in 75% of the 57 patients with severe ankle osteoarthritis in a open prospective study (64). Most interestingly, the improvement increased over time. Radiographic evaluation showed increased joint space width and decreased subchondral sclerosis. Moreover, joint distraction showed significantly better results than debridement (64). Although the prospective follow-up remains relatively short retrospective data have demonstrated efficacy for more than 7 years after treatment, which suggest joint distraction to be a serious alternative for treatment of severe ankle OA at relatively young age to delay the need for joint replacement or joint fusion. Whether joint distraction is also effective for other joints remains to be established.

NSAIDs and cartilage
Because NSAIDs diminish tissue inflammation and inflammatory mediators, NSAIDs are indirectly beneficial to cartilage under inflammatory conditions. These are indirect effects of NSAIDs on cartilage. The direct effects on cartilage are less clear. 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 synthesis of cartilage proteoglycans (60, 65, 66) and to increase the release of cartilage proteoglycans (67). Also, recovery from the adverse effects of proinflammatory cytokines such as IL-1 has been reported to be diminished by NSAIDs (68-70). These are all *in vitro* data and whether these effects of NSAIDs occurred also *in vivo* still needs to be established. *In vivo* data are very limited. Direct effects of NSAIDs are not visible during clinical evaluation and are shadowed by the effects on inflammation therefore 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.
The anti-infammatory properties of NSAIDs are responsible for their worldwide application. Their major mechanism of action lies in the inhibition of the cyclo-oxygenase (COX) enzyme. This enzyme has a crucial role in the synthesis of prostaglandin-G_2 out of arachidonic acid, after its liberation from membrane phospholipids. This step is followed by a peroxidase reaction, leading to

prostaglandin-H_2 production, and an isomerase reaction, resulting in the various active prostaglandins such as prostaglandin-E_2 (PGE_2).
In the past two decades, two isoenzymes of COX have been recognized. COX-1 is considered to be the housekeeping enzyme, being continuously expressed in multiple organ systems. The gastrointestinal mucosa, for example, is protected by the COX-1 dependent production of the prostaglandins-E_2 and -I_2 (prostacyclin). Prostaglandin-E_2 is also an important factor in renal function. Further, thromboxane, the prostaglandin formed by COX-1 in platelets, is responsible for platelet aggregation. In contrast, the other isoenzyme, COX-2, is not or only to a little extent produced in normal physiologic circumstances. However, during inflammation it is upregulated. Then, the prostaglandins- E_2 and - I_2 are produced abundantly, leading to pro-inflammatory activities and vasodilatation, facilitating and sustaining the inflammatory reaction.
It is clear that the isoenzyme COX-2 is responsible for inflammation, pain and fever and is the target for the anti-inflammatory activity of NSAIDs (71). However, the commonly used NSAIDs are non-selective COX inhibitors and therefore they also inhibit COX-1 (72, 73). Lack of selectivity is thought to account for the frequently described side-effects, such as gastrointestinal ulceration and decrease in platelet aggregation, with chronic use of NSAIDs (57-59). This has sparked interest in the development of drugs that specifically target COX-2. This led to a new class of designer drugs, the selective COX-2 inhibitors, or COXIBs. It has been demonstrated that this new class of drugs shows good anti-inflammatory effects with fewer complications regarding the gastrointestinal tract and platelet function (74-77).
Because of the role of NSAIDs in treatment of joint disease including osteoarthritis, the local expression of COX-1 and COX-2 in joint tissues has been studied (78). COX-1 is expressed in synovial lining cells derived from patients with both OA and rheumatoid arthritis (RA). COX-2 expression is marked in the synovium from RA, but not OA patients (79). On the other hand, in chondrocytes of both OA and RA cartilage COX-2 is shown to be upregulated (80). As a consequence of this upregulation of COX-2, in diseased cartilage elevated levels of prostaglandin-E_2 are found of which the role is hardly understood (81, 82). COXIBs may contribute in the unravelling of the role of COX-2 and its product prostaglandin E_2 in cartilage turnover, directly and under inflammatory conditions.

Recent developments of COXIBs
Introduction of COXIBs held a promise of improved treatment of arthritis and osteoarthritis without the gastrointestinal effects associated with other NSAIDs. Celecoxib, and rofecoxib were the first to be classified as COXIBs. The positive results of these inhibitors in several clinical trials with both rheumatoid arthritis and osteoarthritis patients in combination with extensive marketing programs, led to wide scale use of these inhibitors. However, new insights into the cardiovascular related side effects led to warning against the uncritical use of COXIBs. Inhibition of prostacyclin with relatively unopposed platelet thromboxane generation may

lead to thrombotic risk (83). The Vioxx Gastrointestinal Outcomes Research (VIGOR) trial (84) comparing rofecoxib to the NSAID naproxen in patients with RA showed a non-significant increase in atherothrombotic cardiovascular events associated with rofecoxib. More recently, more trials showed similar results with respect to cardiovascular side-effects of rofecoxib and other COXIBs (85). Most recently, the Adenomatous polyp prevention on Vioxx (APPROVE) trial, a prospective clinical trial, resulted in withdrawal of rofecoxib (Vioxx) based on information indicating that it increases the number of acute myocardial infarctions and sudden cardiac deaths. In contrast, the Celecoxib Long-Term Arthritis Saftey Study (CLASS) (86) showed no increase in cardiovascular events with celecoxib. A recent analysis of cardiac events in almost 4000 patients randomized to celecoxib versus either ibuprofen or diclofenac (87) showed no evidence for an increase in cardiovascular risk, irrespective of whether patients were treated with aspirin. These findings suggest that COXIBs as a class do not simply increase cardiovascular events.
However, a recent study showed a different perspective. In 1 of 2 long-term cancer-prevention trials, where patients taking 400 mg of celecoxib twice daily had a 3.4 times greater risk of cardiovascular events compared with placebo. For patients in the trial taking 200 mg twice daily, the risk was 2.5 times greater compared to placebo. Based on these statistically significant findings, the sponsor of the trial in collaboration with the manufacturer of celecoxib has suspended the dosing of the drug in the study. It is to note that findings with non-inflammatory conditions, such as the cancer prevention trials, might differ from the actual situation in an inflammatory condition such as arthritis. Until 2004, the advantages of COXIBs: notably fewer gastrointestinal complications than with the NSAIDs were considered to outweigh the adverse effects. Since the other COXIBs share properties with rofecoxib, such as the potential for a higher thrombotic response in treated patients, it now seems wise to refrain from prescribing coxibs in patients with an increased cardiovascular risk profile until further data are available.

Outline of the thesis

Although there is considerable enthusiasm for the usage of COXIBs that relieve pain in a wide variety of conditions but do not cause gastrointestinal toxicity on prolonged use, an important issue is also the direct effect of these drugs on cartilage. As mentioned above, in case of osteoarthritis inflammation is mostly mild and secondary. Although treatment with COXIBs will be beneficial to cartilage indirectly by controlling inflammation, their direct effect on cartilage, specifically in case of chronic use may be at least as important in the final outcome of treatment with COXIBs. Evaluation of these direct effects of COXIBs on cartilage remains difficult as outlined above. Therefore, several *in vitro* methods, an animal *in vivo* and a human *ex vivo* approach have been chosen for evaluation.
The purpose of the present thesis was therefore to evaluate the direct effects of a COXIB, celecoxib, on human osteoarthritic cartilage. An issue of major relevance to clinical practice, since it is essential that compounds used to treat joint disorders

do not impair the ability of the chondrocyte to repair its already damaged matrix. In **chapter 2** we described **the *in vitro* effects of COX-2 inhibition** by celecoxib on cartilage-matrix turnover under **normal and inflammatory conditions**. To see whether these results were applicable to osteoarthritic cartilage we tested this also ***in vitro* on preclinical (degenerated) and clinical osteoarthritic human cartilage**, as described in **chapter 3**. As we found only marginal effects of COX-2 inhibition for proteoglycan synthesis in both chapter 2 and 3, we evaluated whether human articular cartilage **proteoglycan synthesis and release** were **related to prostaglandin-E_2** production and/or another mediator involved in disturbance of cartilage-matrix turnover. This is described in **chapter 4**. As some NSAIDs have adverse direct effects on human cartilage we were interested as this holds true for the common used NSAIDs and whether the adverse effects were COX-1 related. In **chapter 5** we tested the **direct effects** of different **commonly used NSAIDs** on human **osteoarthritic cartilage**. Although such *in vitro* systems have the advantage that the direct effects on osteoarthritic cartilage, without interference of synovial inflammation can be studied, extrapolation to the *in vivo* situation has it limitations. Evaluation of joint tissue, specifically cartilage, in humans is difficult. Therefore we used an animal model of osteoarthritis, the canine groove model. This model is based on surgically applied damage of the articular cartilage followed by transient forced loading of the affected joint and shows characteristics of OA, mimicking human OA. However, to establish whether the observed characteristics of degeneration in this model represents the **surgically applied damage** or are the results of **progressive features of osteoarthritis** we evaluated this "groove" model shortly after surgery. This is described in **chapter 6**. In **chapter 7** we described the actual animal *in vivo* study, where we looked at **the effects of celecoxib** in the groove model an **experimental canine model of osteoarthritis**.
This model allows *in vivo* evaluation in an early stage of osteoarthritis but also here the extrapolation to the human situation remains restricted. As mentioned earlier, evaluation of human cartilage is difficult. One possibility of studying human osteoarthritic cartilage without performing large and long clinical trials is to evaluate the effects of shortterm treatment on osteoarthritic patients before joint replacement surgery. The cartilage available after surgery can be studied in all its aspects. In **chapter 8** we described **the effects of selective COX-2 inhibition after short-term treatment of patients with severe knee osteoarthritis**.

Chapter 2

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

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Abstract

Objectives: This study evaluates the *in vitro* effect of the selective COX-2 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 (PB MC) or in the presence of IL-1β plus TNFα was cultured for 7 days in the presence of celecoxib. Changes in cartilage matrix turnover were determined.

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

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

Introduction

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 physiological properties of the tissue. During joint diseases, such as rheumatoid arthritis (RA) and osteoarthritis (OA), chronic joint inflammation, primarily or secondary, causes or contributes to cartilage damage by influencing chondrocytes. This leads to erosion of articular cartilage, injury of surrounding tissue and eventually permanent loss of joint function (88).

Cyclooxygenase (COX) also known as prostaglandin synthase (89) is a potent mediator of inflammation. The anti-inflammatory effects of nonsteroidal anti-inflammatory drugs (NSAIDs) are mainly due to their ability to inhibit prostaglandin production by COX suppression. As recently reviewed by Crofford *et al* (90), two distinct forms of COX have been identified (91), based on 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 (92, 93). The expression of a second form of COX (COX-2) is induced by pro-inflammatory cytokines and growth factors (94). It is clear that COX-2 is the isoform responsible for the enhanced production of prostaglandins that mediate inflammation, pain, and fever and is the target enzyme for the anti-inflammatory activity of NSAIDs (71). However, it is suggested that COX-2 might be involved in normal physiology e.g. tissue repair as well (90).

The commonly used NSAIDs, however, are non-selective inhibitors and therefore inhibit COX-1 as well (72, 73). A lack of selectivity is thought to account for the increased incidence of gastric ulceration and other deleterious side effects that

accompany the chronic use of NSAIDs (57-59). This has sparked interest in the development of drugs that specifically target COX-2. The scope is that such compounds would be associated with a lower incidence of adverse effects (71). Celecoxib, being one of the selective COX inhibitors, at the right pharmacological dose, inhibits only COX-2 and not COX-1 (57, 95-97). Analyses of data pooled from several trials suggest that celecoxib is associated with fewer clinically symptomatic ulcers and ulcer complications than traditional NSAIDs are, such as naproxen, diclofenac or ibuprofen (98).

Because NSAIDs diminish inflammation, and with that inflammatory mediators, NSAIDs are indirectly beneficial for cartilage under inflammatory conditions. However, direct effects of NSAIDs on cartilage have frequently been reported to be adverse, though beneficial as well neutral effects have been reported as well (65, 66, 99, 100). 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 directly induced by NSAIDs remain undetectable because it concerns very slow processes and irreversible harm can be done long before it becomes clinically evident. Nevertheless, on the long-term such direct effects may be essential for the effectiveness of NSAID treatment of chronic joint diseases such as rheumatoid arthritis. Such direct effects may be even more important when the joint disease is secondarily mediated by inflammation, as is the case in osteoarthritis.

The local expression of COX-1 and COX-2 in joint tissues has been studied (78). COX-1 is expressed in synovial lining cells derived from patients with both OA and RA. COX-2 expression is marked in the synovium from RA, but not OA patients (79). On the other hand, in chondrocytes of both OA and RA cartilage COX-2 is shown to be upregulated (80). As a consequence of this upregulation of COX-2, in diseased cartilage elevated levels of prostaglandin E_2 (PGE_2) are found of which the role is hardly understood (81, 82). The selective COX-2 inhibitors may contribute in the unraveling of the role of COX-2 and its product PGE_2 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, in the present study the *in vitro* effect of celecoxib (SC-58635;4-[5(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl]benzene-sulfonamide), a selective COX-2 inhibitor, on human articular cartilage is studied with respect to cartilage-matrix turnover under normal and inflammatory conditions.

Material and Methods

Cartilage culture technique.

Human articular cartilage tissue was obtained *post mortem* from knee condyles within 24 hours of death. The donors, aged 67±3 years (SEM; m/f is 8/6); were without 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 (101). Slices of cartilage were cut aseptically from the articular surface, excluding the underlying bone, and kept in phosphate buffered (pH 7.4) saline (PBS). Within one hour 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 microtiter plates (200 μl culture medium per well for each explant, 5% CO_2 in air, 37°C). The culture medium consisted of Dulbecco's modified Eagle's medium (D-MEM), supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin sulfate (100 μg/ml), ascorbic acid (0,085 mM), and 10% heat inactivated pooled human male AB^+ serum.

Preparation of inflammatory cells.
Blood was collected from donors (n=4) with active rheumatoid arthritis in heparin tubes. Peripheral blood mononuclear cells (PBMC) as a source of inflammatory cells were isolated by density centrifugation using Ficoll-Paque, washed twice in culture medium (102) and resuspended in culture medium to a final concentration of $0.15x10^6$ cells/ml (103).
IL-1β and TNFα production of these cells was measured in the culture media using ELISA (Biosource Europe, Nivelles, Belgium) according to manufacturer instructions.

Experimental set-up.
Healthy human articular cartilage tissue was cultured for 7 days, alone, in co-culture with inflammatory cells (peripheral blood mononuclear cells, PBMC; $0.15x10^6$ cells/ml) or in the presence of IL-1β plus TNFα (200 pg/ml, Biosource, PHC0814, 800 pg/ml, Pharmingen, 19761T, respectively). Celecoxib (supplied by Pharmacia, US) was added at the start of the culture in concentrations of 0.01, 0.1, 1 and 10 μM. Mean pharmacological plasma concentrations are 5 μM (104). After 4 days medium was refreshed and cartilage cultured for a successive 3 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, as given in the figure legends, represent the number of these experiments.
In addition, in a separate set of experiments, prostaglandin E_2 levels in culture supernatants of normal cartilage with or without IL-1β/TNFα with or without celecoxib were measured by using commercial available EIA-kit (Cayman chemicals US; 514010)

Proteoglycan analyses.
Sulfate incorporation rate was determined, as a measure of the proteoglycan synthesis rate, during the last 4 hours of the first 4-day culture period, as described previously (105). Before addition of $^{35}SO_4^{2-}$ ($Na_2{}^{35}SO_4$, 14.8 kBq/200 μl, DuPont

NEX-041-H, carrier-free), culture medium was replaced by fresh medium. After 4 hours labeling, the cartilage explants were rinsed 3 times for 45 minutes in culture medium under culture conditions and 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 before (106) (Papain, Sigma P-3125, 25 mg/ml in 50 mM phosphate buffer pH 6.5 containing 2mM N-acetyl cysteine and 2 mM Na2-EDTA). Papain digests were diluted and glycosaminoglycans (GAGs) stained and precipitated with Alcian Blue dye solution (Alcian Blue 8GX, sigma A-5268, saturated in 0.1 M sodium acetate buffer pH 6,2 containing 0.3 M $MgCl_2$; 30 minutes, 37°C (107)). The pellet obtained after centrifugation (9000g, 10 min.) was washed once (NaAc-buffer containing 0.1 M $MgCl_2$) and subsequently dissolved (sodium dodecyl sulphate; SDS in 5 mM NaAc; pH 6.2; 2% w/v). The $^{35}SO_4^{2-}$ radioactivity of the sample was measured by liquid scintillation analysis after addition of picofluor-40 (Packard). The amount of $^{35}SO_4^{2-}$ of the papain digest plus the three day culture medium after pulse labeling, was normalized to the specific activity of the medium, labeling time and wet weight of the cartilage samples. Proteoglycan synthesis rate was expressed as nmoles sulfate incorporated per hour per gram wet weight of cartilage (nmol/h.g).
As a measure of PG content of the cartilage samples the amount of GAG was determined as described previously (105). The GAG in the papain digest of cartilage samples were precipitated and stained with Alcian Blue as described above. Blue staining was quantified photometrically by the change in absorbence at 620 nm. Chondroitin sulphate (Sigma C4383) was used as a reference. Values were normalized to the wet weight of the cartilage and expressed as mg 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. GAGs were precipitated from the medium obtained from day 4 to 7 and stained with Alcian Blue dye solution (107). The radio-labeled GAGs were measured by liquid scintillation analysis and normalized to the proteoglycan synthesis rate and expressed as percentage release of newly formed proteoglycans.
For the total release of proteoglycans the blue staining of the medium was quantified spectrophotometrically by the change in absorbence at 620 nm. Chondroitin sulphate (Sigma C4383) was used as a reference. Values were normalized to the GAG content of the explants and expressed as percentage release of GAGs

Calculations and statistical analysis.
Because of focal differences in composition and bioactivity of the cartilage on the femur condyles, the results of minimal 8 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 of different donors were performed. For the dose response curves shown, a representative experiment was chosen and intra intra-assay variation (±SEM) of the 8 samples per parameter shown. For the bar-graphs the average of at least 4 individual experiments, the n value representing the exact number of experiments performed with inter-assay variation (±SEM) is given. 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

The effect of celecoxib on normal human articular cartilage.

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

The effect of celecoxib on human articular cartilage explants under the influence of inflammatory cells.

Addition of rheumatoid arthritis peripheral blood mononuclear cells (MC) to articular cartilage, mimicking inflammation, resulted in a 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.g remained). As a result, release of newly formed proteoglycans was negligible (on average 0.02 ± 0.01 nmol/h.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 enhanced by addition of MC (two fold 2.2 ± 1 to 4.9 ± 2 mg/g; $p \leq 0.072$) 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 enhanced by adding the inflammatory cells (on average three fold in 3 days; figure 2A) and could be normalized completely in a dose dependent way by addition of celecoxib.

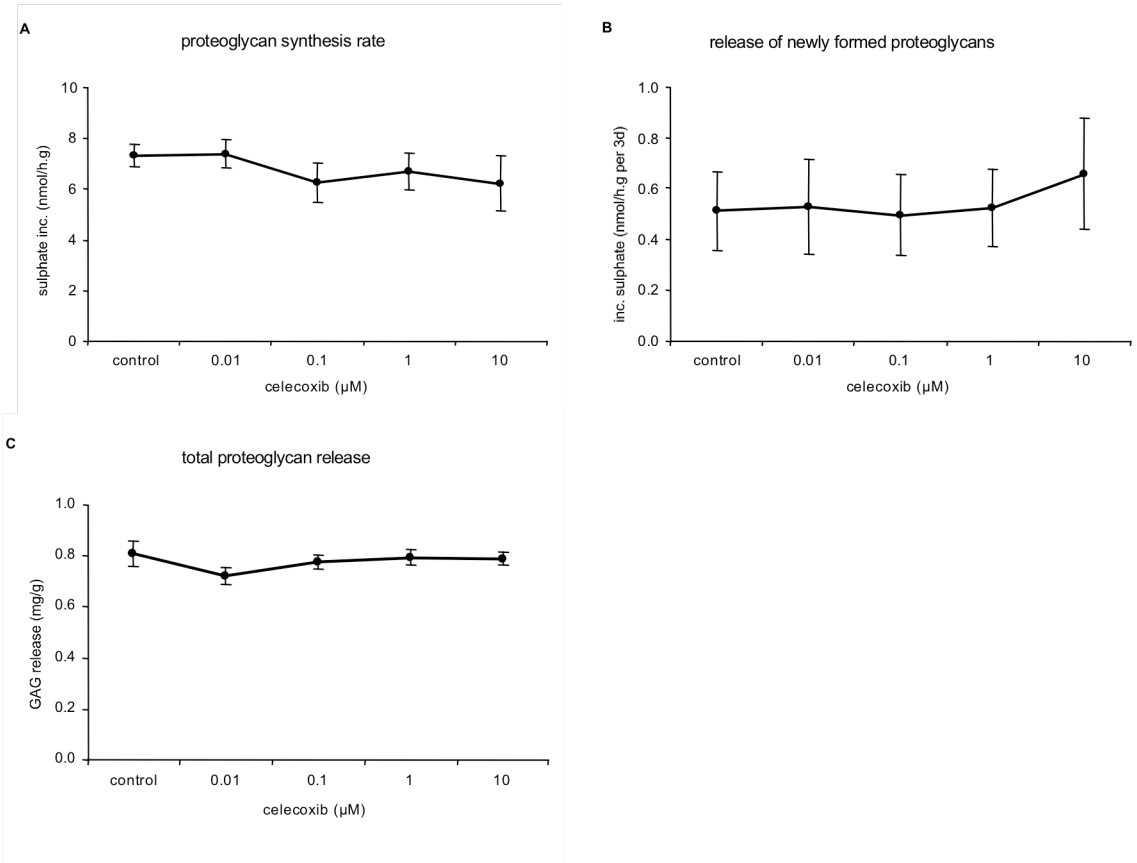


Figure 1. *The effect of celecoxib on human articular cartilage explants.*
Proteoglycan synthesis rate as measure of cartilage matrix synthesis expressed as sulphate incorporation rate per gram wet weight of cartilage tissue (1A), release of newly formed proteoglycans as a measure of retention of newly formed proteoglycans expressed as nmoles of incorporated sulphate released during three days per gram wet weight of cartilage tissue (1B) and total release of proteoglycans expressed as glycosaminoglycan (GAG) release per gram wet weight of cartilage tissue (1C). The results are presented as means ±SEM (n=6). Each experiment was performed with cartilage of a separate donor, with 8 tissue samples per parameter, each handled and cultured individually. No statistical significant changes were found upon addition of celecoxib.

As a result of the significantly enhanced GAG release and the strong inhibition of synthesis a decrease in proteoglycan content was observed (11% in 7 days; figure 2B). This decrease in proteoglycan content could be normalized completely by addition of celecoxib.
These effects of celecoxib could entirely depend on an inhibition of the catabolic properties of the inflammatory cells. However, it appeared that pro-inflammatory cytokine levels produced by the cells were not significantly influenced by celecoxib: 70 vs. 97 ng/ml and 442 vs. 602 ng/ml for IL-1β and TNFα respectively, for the cultures without vs. with 10 μM celecoxib, both not statistically significantly different.

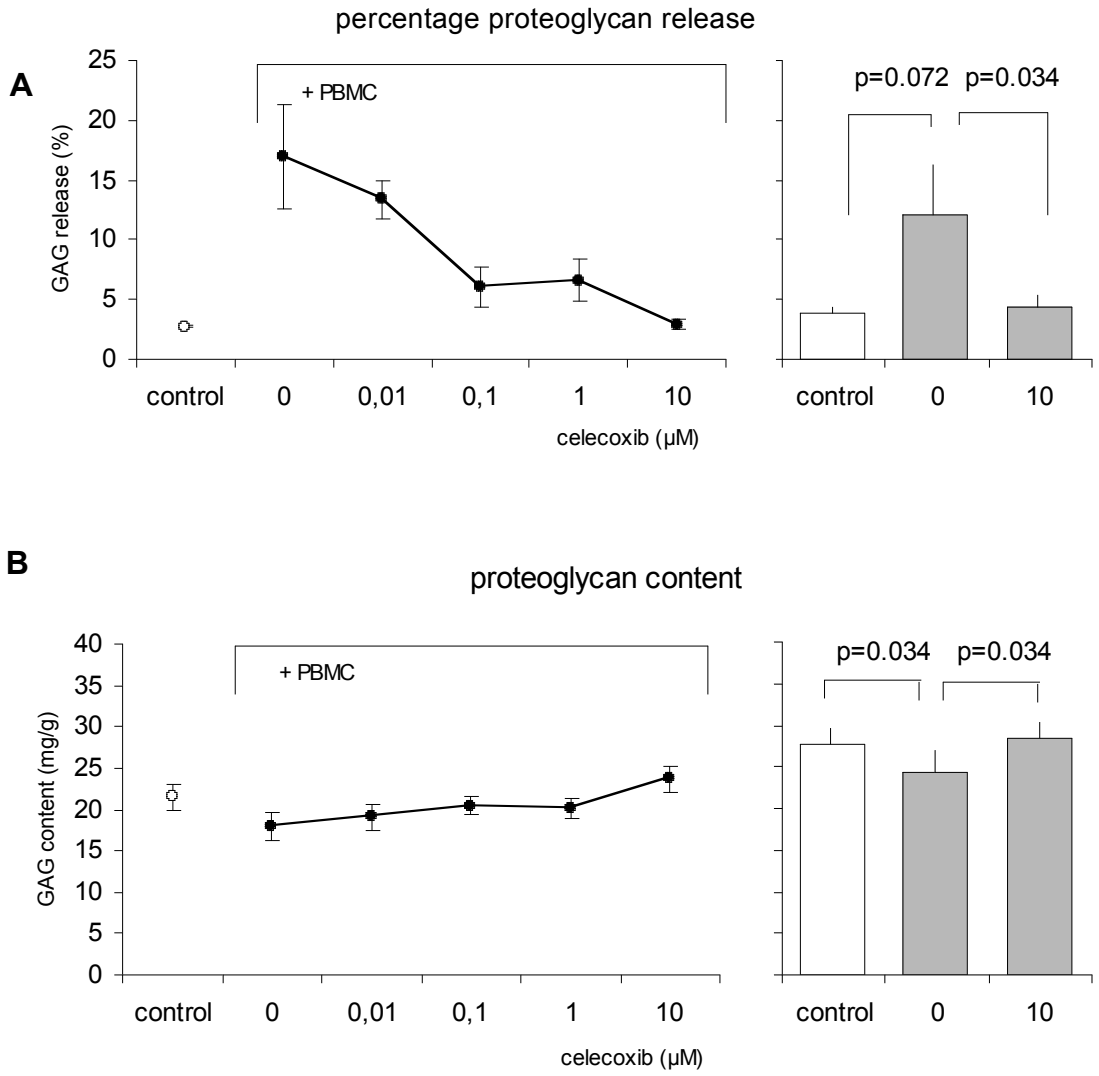


Figure 2. *The effect of celecoxib on human articular cartilage under the influence of inflammatory cells.*

Percentage GAG release (glycosamoniglycan release normalized to GAG content; %) as a measure of proteoglycan release in the upper panel (A). Proteoglycan content (expressed as GAG content) is depicted in the lower panel (B). Left panels show a dose response curve, a representative experiment of 2 (mean values of 8 explants of one donor with intra-assay variation (SEM) are given). Right panels show the average of 4 experiments each with cartilage of another donor (mean values with inter-asay variation (SEM) are given). Open symbol (left) and open bar (right) represent control condition in the absence of inflammatory cells. Filled symbols and gray bars represent the condition in the presence of peripheral blood mononuclear cells from RA patients (PBMC) without and with celecoxib. Celecoxib is able to normalize GAG release and GAG content in a dose dependent way (left panel) and on average (right panel).

The effect of celecoxib on human articular cartilage explants under the influence of pro-inflammatory cytokines.

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

Release of newly formed proteoglycans, absolute (1.1 ± 0.2 to 0.6 ± 0.3 nmol/h.g) and as percentage (normalized to the proteoglycan synthesis rate) was significantly enhanced by the addition of IL-1β plus TNFα (on average a 2.5 fold increase, $p \leq 0.022$; figure 3B), indicating an impaired retention of the newly formed proteoglycans. Celecoxib was able to completely and dose dependently reverse this impaired retention of newly formed proteoglycans (figure 3B).

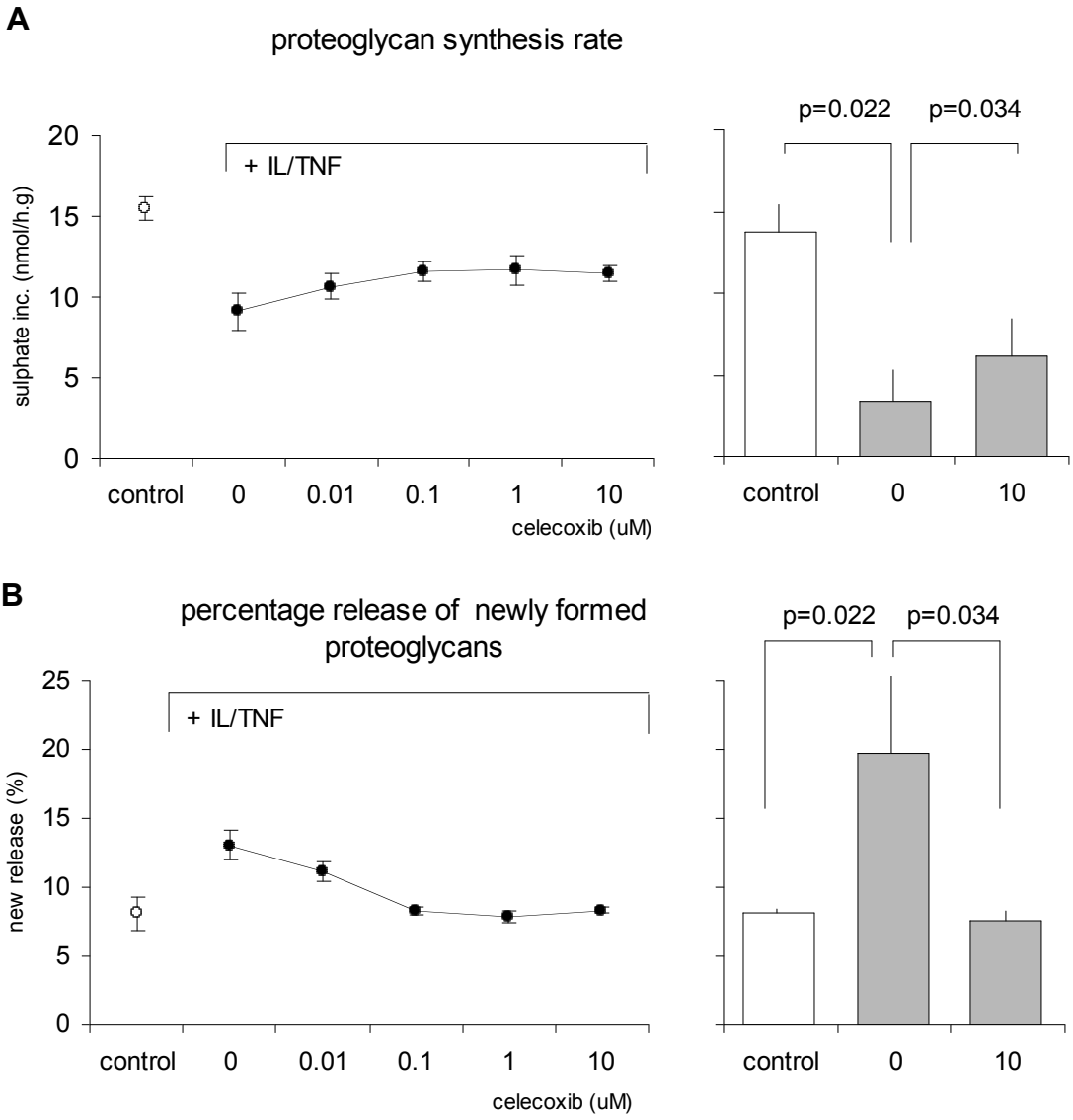
Release of all GAG, newly formed plus resident GAG, absolute (0.8 ± 0.16 to 1.2 ± 0.16 mg/g) and as percentage (normalized to the GAG content), was increased by addition of IL-1β plus TNFα (on average an almost two fold increase, $p \leq 0.022$; figure 3C). Celecoxib was able to reverse this increased release in a dose-dependent way and completely for the absolute (0.8 ± 0.05 mg/g) as well as percentage loss of GAG.

The by IL-1β plus TNFα induced decreased synthesis and retention of proteoglycan and increased loss of GAG resulted in a decreased GAG content of the cartilage (on average 20% loss, $p \leq 0.040$, figure 3D). Recovery of the disturbed cartilage matrix turnover induced by the pro-inflammatory cytokines upon addition of celecoxib resulted in a dose dependent normalization of the GAG content, statistically significant at 10 μM celecoxib.

PGE_2 levels of cartilage were significantly elevated under influence of IL-1β/TNFα (2 ± 0.2 and 32 ± 9 ng/g for control and stimulated conditions, respectively; n=3; $p \leq 0.02$). By addition of celecoxib PGE_2 levels were significantly decreased to control levels (1 ± 0.3 ng/g; n=3; $p \leq 0.02$).

Discussion

The structural integrity of the matrix of human articular cartilage is maintained by a dynamic equilibrium between synthesis and degradation. In rheumatoid arthritis but also osteoarthritis small quantities of the pro-inflammatory cytokines mainly IL-1β and TNFα from inflamed synovial tissue are able to inhibit matrix synthesis (108-111). This leads to impaired potential for repair and with that to an increased susceptibility to mechanical damage (68). Both cytokines have also been shown to enhance release of matrix components by the induction of matrix proteases (synthesis, release and activation) from chondrocytes by stimulation of the


A
proteoglycan synthesis rate
sulphate inc. (nmol/h.g)
20
15
10
5
0
+ IL/TNF
control
0
0.01
0.1
1
10
celecoxib (uM)
p=0.022
p=0.034
control
0
10
B
percentage release of newly formed proteoglycans
new release (%)
25
20
15
10
5
0
+ IL/TNF
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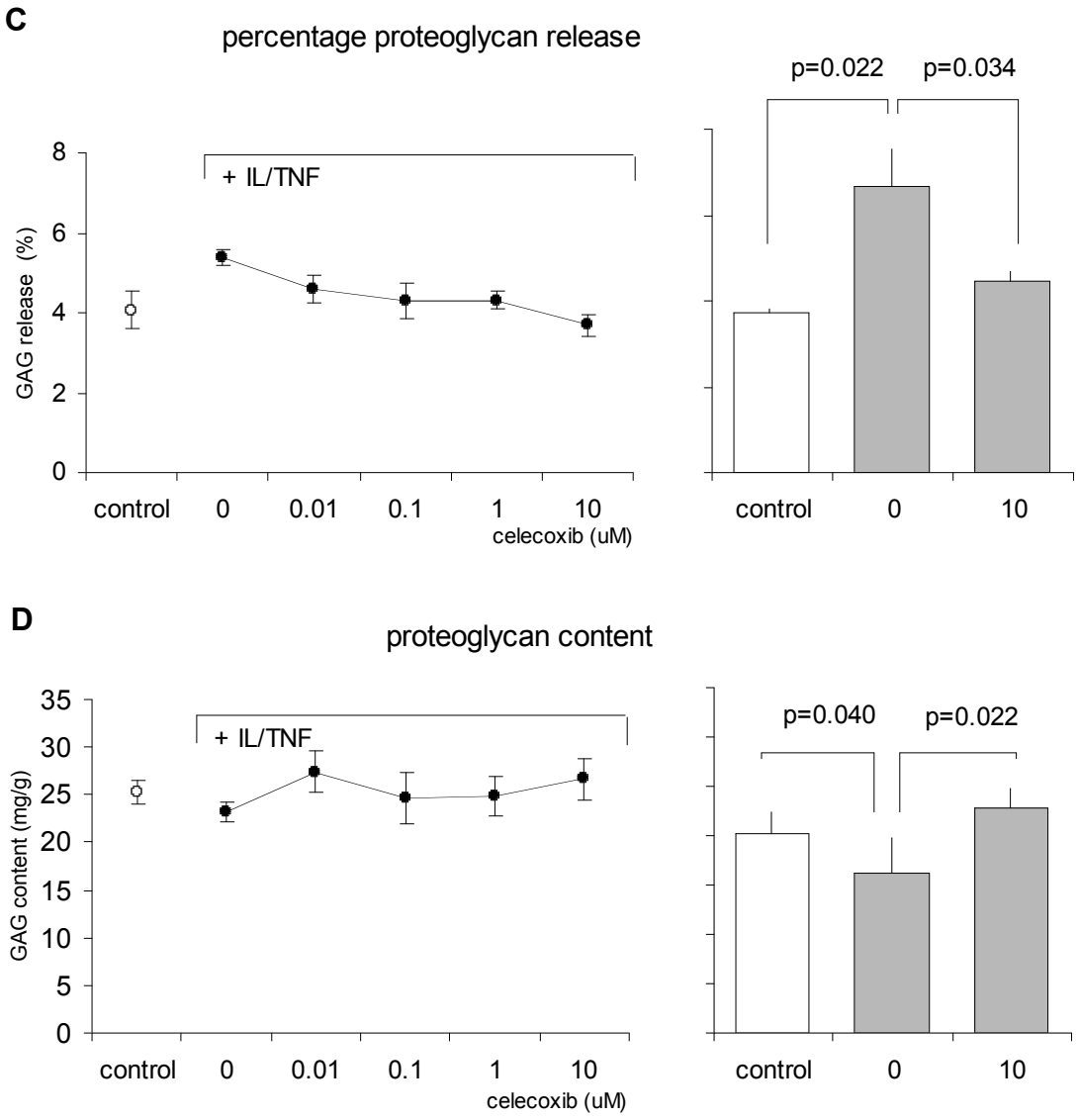

Figure 3. *The effect of celecoxib on human articular cartilage explants under the influence of inflammation related cytokines.* Sulphate incorporation rate as a measure of proteoglycan synthesis (3A), release of newly formed proteoglycans (normalized to the synthesis of these proteoglycans (3B), the percentage release of proteoglycans as measured by the percentage release of GAG (normalized to the GAG content; 3C) and GAG (glycosaminoglycan) content as a measure of proteoglycan content (3D) are shown. Left panels show a dose response curve, a representative experiment of 2 (mean values of 8 explants of one donor with intra-assay variation (SEM) are given). Right panels show the average of 4 experiments each with cartilage of another donor (mean values with inter-asay variation (SEM) are given). Open symbol (left) and open bar (right) represent control condition in the absence of IL-1β plus TNFα. Filled symbols and gray bars represent the condition in the presence of IL-1β plus TNFα (200 and 800 pg/ml, respectively) without and with celecoxib. Celecoxib is able to improve proteoglycan synthesis and to normalize newly formed proteoglycan release, GAG release and content in a dose dependent way (left panel) and on average (right panel).

catabolic properties of these cells (112). Also the inflamed synovial tissue produces significant amounts of the cartilage destructive enzymes (111, 113). Negative involvement 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 nonsteroidal anti-inflammatory drugs (NSAID). Mostly adverse, but also no- or positive effects have been reported. NSAIDs have been demonstrated to inhibit the synthesis of cartilage proteoglycans (60, 65, 66) and to increase the release of proteoglycans (67). Also recovery from adverse effects of pro-inflammatory cytokines such as IL-1β has been reported to be diminished (68-70). These are direct effects on cartilage and should be seen in the context of the significant anti-inflammatory effects of these NSAIDs and with that the indirect beneficial effect on cartilage integrity.
In animal models of arthritis, selective COX-2 inhibitors not only prevent characteristics of inflammation but also prevent cartilage destruction (114-118). This however does not exclude adverse direct effects on cartilage as the above may have been achieved by inhibition of cartilage destructive inflammation. In the case of prolonged treatment such adverse effects might on the long term dominate the anti-inflammation mediated cartilage protection.
Although in an *in vitro* model, the present study 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 (67, 69, 99). This absence of an adverse effect of celecoxib was to be expected, in the light of selective COX-2 inhibition, the isoform 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 are via inhibition of COX-1, which could be essential for normal chondrocyte function, or if other mechanisms are involved as well.
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 adversely influenced by inflammation. Inflammatory cell induced loss of GAGs could be reversed, normalizing proteoglycan content despite the absence of a noticeable effect on proteoglycan synthesis. The latter suggests that inhibition of new formation contribute significantly less to a decrease in proteoglycan content than the enhanced loss of resident proteoglycans. These effects of celecoxib could entirely depend on an inhibition of the catabolic properties of the inflammatory cells. However, it appeared that pro-inflammatory cytokines levels produced by the inflammatory cells were not significantly influenced by celecoxib. Although not conclusive, this suggested that celecoxib had a direct effect on chondrocytes when being under the influence of MC. This was proven by the effect of celecoxib on cartilage exposed to IL-1β plus TNFα. Assuming celecoxib not to be able to neutralize both cytokines in their activity directly by e.g. 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 and diminished retention of newly formed proteoglycans as well as enhanced proteoglycan release, under the influence of IL-1β plus TNFα is for the larger part COX-2 mediated. With respect to the synthesis, not being normalized completely, additional pathways must be involved. For the gastric-intestinal tract COX-2 is suggested to be involved in tissue repair (119-123). The present data show that with respect to cartilage under the influence of inflammation, COX-2 is involved in tissue breakdown.

The induction of COX-2 by IL-1β plus TNFα might be directly linked to excessive prostaglandin-E_2 (PGE_2) production providing an explanation for disturbed proteoglycan turnover. Only a small body of data on the effects of prostaglandins on cartilage has been published. The effect of PGE_2 on cartilage metabolism is still controversial and in addition depends on the type (81) and differentiation stage of the target cells (124-127). Detection of high concentrations of PGE_2 in rheumatoid synovial fluid (128-130) and synovial tissue (131), as well as stimulation of PGE_2 synthesis by articular cartilage chondrocytes cultivated in conditioned media of rheumatoid and non-rheumatoid synovial tissue cells (132) (and present observations), suggests the involvement of PGE_2 in the catabolic processes of articular cartilage in rheumatoid arthritis. An adverse effect of excessive PGE_2 on cartilage is indirectly supported by our experiments.

In conclusion, the present study suggests that inflammation induced adverse effects on cartilage are COX-2 mediated and that celecoxib as a selective COX-2 inhibitor has cartilage protective capacities as it can restore cartilage matrix integrity under inflammatory conditions. Although in the present experimental set-up elevated PGE_2 levels have been found, additional studies with osteoarthritc cartilage and cartilage from rheumatoid arthritis 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 treatment of chronic destructive joint diseases where anti-inflammatory drugs need to be used for a prolonged period of time.

Chapter 3

Selective COX-2 inhibition is favorable to human early and late-stage osteoarthritic cartilage: a human in vitro study

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Abstract

Objective: NSAIDs are widely used in the treatment of OA. For the outcome of treatment the direct effects of NSAIDs on cartilage may be more important than indirect effects on inflammation, considered being secondary in OA. For clinical practice it is relevant to study effects of NSAIDs on early stages of OA. Therefore we studied the direct effects of celecoxib on human degenerated OA cartilage and compared the effects with those on human healthy cartilage and human end stage OA cartilage.

Methods: Degenerated, late-stage OA, and healthy human articular cartilage were exposed (7-days of culture) to celecoxib (0.1–10 μM). Changes in cartilage proteoglycan turnover (synthesis, retention, and release), proteoglycan content, PGE_2 and NO production were determined.

Results: Both degenerated and established OA cartilage showed its characteristic changes in proteoglycan turnover (all $p<0.05$). Celecoxib at 1 μM was able to increase synthesis of degenerated cartilage and normalize both releases of newly formed and resident proteoglycans. Importantly, 1 μM celecoxib influenced matrix integrity by enhancing proteoglycan content. Similar results were found for end-stage OA cartilage. Enhanced PGE_2 production in degenerative and OA cartilage could be decreased by celecoxib, whereas no effect on enhanced NO production was found. No significant effects of celecoxib on normal cartilage were found.

Discussion: Celecoxib, in a clinical relevant concentration, showed *in vitro* a significant beneficial effect, not only on late-stage OA but also on more early stages of OA, whereas healthy cartilage remained unaffected, suggesting chondroprotective properties of celecoxib in treatment of degenerative joint disorders.

Introduction

In osteoarthritis (OA) damage to cartilage is characterized by loss of matrix proteoglycans and damage of the collagen structure. Although osteoarthritis is primarily an intrinsic process of the cartilage, secondary synovitis may trigger clinical symptoms and add to the final damage of the cartilage tissue and with that to joint damage in general (2, 17, 133).

Nonsteroidal anti-inflammatory drugs (NSAIDs) diminish inflammation, therefore NSAIDs may indirectly be beneficial to cartilage under inflammatory conditions although this has never been proven in clinical trials. Based on *in vitro* and animal studies, direct effects of NSAIDs on cartilage have frequently been reported (65, 99, 100, 134). Although different experimental set-ups have been used in different studies these studies clearly show that some of the used NSAIDs have direct adverse effects on cartilage, though beneficial and neutral effects have been reported as well (66, 99, 100, 134). These direct effects cannot be studied easily in clinical trials and therefore they are generally ignored in clinical practice. This is because these direct effects on cartilage are shaded by the effects of NSAIDs on

inflammation and because (intrinsic) cartilage changes, catabolic and anabolic are generally very slow processes in OA.
Direct effects of NSAIDs on cartilage may be important specifically in long-term treatment of joint disease in which inflammation is only mild and secondary as in OA. Thus, although NSAIDs may be very useful in reducing pain and inflammation in OA, when their direct effects are adverse, they may contribute to the process of cartilage degeneration by interfering with the intrinsic repair activity.
The anti-inflammatory effects of NSAIDs are mainly due to their ability to inhibit prostaglandin production by COX in a non-specific manner, thereby demonstrating efficacy but also toxicity (95, 135, 136). The discovery of two isoforms of cyclooxygenase (COX), COX-1 and COX-2, provided insight into the mechanism of action and toxicity of NSAIDs. Much has been learned about these isoenzymes since their discovery.
The discovery of COX-2 has spurred the development of drugs that could selectively inhibit COX-2, lacking the adverse effects of traditional NSAIDs. Celecoxib, as one of the first selective COX-2 inhibitors, has been shown to be an effective anti-inflammatory and analgesic drug in patients with rheumatoid arthritis and OA, comparable to that of traditional NSAIDs, such as naproxen, diclofenac and ibuprofen (57, 96, 97). A significant reduction of gastrointestinal adverse events with selective COX-2 inhibitors compared to non-selective NSAIDs has been demonstrated frequently.
In treatment of OA, as explained above, it remains at least as important to know the direct effects of these drugs on cartilage. This is specifically important because COX-2 is found to be expressed in OA tissues (78, 137). Both in chondrocytes and synovial cells of OA joints elevated levels of COX-2 have been found. Also elevated levels of prostaglandin-E_2 (PGE_2) have been found in OA cartilage, indirectly demonstrating a role for COX-2 in diseased cartilage (138). Most recently, the expression of COX-2 and PGE_2 in OA meniscus, synovial membrane, osteophytic fibrocartilage and in the articular OA cartilage has been described (137). Furthermore, the latter study showed an increased proteoglycan degradation that correlated with COX-2 protein expression and PGE_2 production by the synovial membrane. In a previous study of our group we showed a beneficial effect of COX-2 inhibition in IL-1β and TNFα treated cartilage (139). Because these mediators play a role in cartilage damage in OA, this finding indirectly indicates a possible role of COX-2, in the process of cartilage degeneration and the attempt to repair, as seen in OA. Recent findings by El Hajjaji *et al*. showed that celecoxib was able to increase proteoglycan synthesis and to diminish proteoglycan release of OA cartilage obtained at joint replacement surgery (140). However, this cartilage represents an end-stage of OA, the result of degeneration and repair that has taken place over many years. For treatment of OA in clinical practice, it is more relevant to evaluate more early stages of cartilage degeneration. Moreover, in addition to knowledge on proteoglycan turnover (synthesis and release) it is also important whether this beneficial effect on turnover results in actual improvement of matrix integrity as

indicated by an increase in proteoglycan content that is significantly diminished in case of OA.
For this reason, the present study evaluates the *in vitro* effect of celecoxib on human articular cartilage. Effects on degenerated (pre-clinical) OA cartilage were compared to those on established (end stage) OA and healthy cartilage.

Material and Methods

Cartilage culture technique

Normal healthy cartilage based on a smooth glossy appearance was obtained *post-mortem* from patients without history of joint disorders (mean age 69±5 years (±SEM), 2 male, 4 female). Degenerated articular cartilage tissue that was obtained *post mortem* as well (mean age 80±3 years (±SEM), 2 male, 2 female). Degenerated cartilage was identified macroscopically on the bases of a fibrillated surface as previously reported (101). Also these donors had no clinical history on joint disorders. This stresses the difference between degenerative changes in cartilage that could have been visualized by radiography and actual clinical manifestations of the disease, which may result in the diagnosis of OA. In general, all changes observed in degenerated cartilage are, although less pronounced, observed in osteoarthritic (OA) cartilage and are significantly different from normal healthy cartilage. For that reason degenerated cartilage obtained *post-mortem* can be considered as a pre-clinical phase of osteoarthritis (101). OA cartilage obtained from patients at knee replacement surgery with diagnosed OA (mean age 69±2 years (±SEM), 1 male, 5 female) was obtained post-operatively. NSAID medication is stopped seven days before surgery, thus no interference of previous medication use is to be expected. It should be kept in mind that only the cartilage that could be cut from the deteriorated joints (after replacement surgery) was used. Cartilage that appeared full thickness with significant fibrillation was selected. Thus in fact the entire joint had a worse appearance than represented by the cartilage used for evaluation. Mostly significant parts of the joint were only covered with thin or sometimes no cartilage. Osteoarthritic cartilage obtained at joint replacement surgery represents an end stage of OA that reflects degenerated as well as reparative processes and in that respect differs from degenerated cartilage although the histological classification may be comparable. Cartilage obtained post-mortem was taken within 24 hours of death from human knee condyles, cartilage obtained post-surgery always within 4 hours after dissection. Collecting cartilage was according to the medical ethical regulations of the University Medical Center Utrecht.
Slices of cartilage were cut aseptically as thick as possible from the articular surface, excluding the underlying bone, and kept in phosphate buffered saline (PBS). Within one hour 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 microtiter plates (200 μl culture medium, 5% CO_2 in air, 37°C). The culture medium consisted of Dulbecco's modified Eagle's medium (D-MEM), supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin

sulfate (100 μg/ml), ascorbic acid (0,085 mM), and 10 % heat inactivated pooled human male AB^+ serum. Cartilage was always pre-cultured for 24 hours (wash-out period), where after culture medium was refreshed before start of the experiment.
In addition, 3 tissue samples of each donor were fixed in 4% phosphate buffered formalin for standard light microscopy. Sections were stained with safranin-O fast green-iron haematoxylin and graded for features of OA according to the slightly modified criteria (141) described by Mankin. The tidemark between cartilage and bone was not present in our cartilage samples since bone was not included. Also as a result of our dissection method, cartilage samples were not covered with pannus. Therefore, the maximum score that could be obtained was 11 instead of the original 14, when all criteria described by Mankin (142) (including pannus, clefts to calcified zone, and tidemark crossed by blood vessels) could have been included.

Experimental setup
Healthy, early and late osteoarthritic human articular cartilage tissue were cultured for 7 days. Celecoxib (supplied by Pfizer USA) was added at the start of the culture in concentrations of 0.1, 1 and 10 μM. The mean pharmacological plasma concentration being 5 μM (104). After 4 days medium was refreshed and cartilage cultured for a successive 3 days with the same additions. Changes in cartilage-matrix turnover (proteoglycan synthesis, -retention, and -release) and matrix integrity (proteoglycan content) were determined. Experiments were repeated at least 4 times, for each experiment cartilage of another donor was used.

Proteoglycan analyses
Sulphate incorporation rate was determined, as a measure of the proteoglycan synthesis rate, during the last 4 hours of the first 4-day culture period, as described previously (105). Before addition of $^{35}SO_4^{2-}$ ($Na_2{}^{35}SO_4$, 14.8 kBq/200 μl, DuPont NEX-041-H, carrier-free), culture medium was replaced by equilibrated (CO_2 and temperature) fresh medium. After 4 hour labeling, the cartilage explants were rinsed 3 times for 45 minutes in culture medium under culture conditions and incubated for the 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 before (143). Papain digests were diluted to the appropriate concentrations and glycosaminoglycans (GAGs) were stained and precipitated with Alcian Blue dye solution (144). The pellet obtained after centrifugation (9000g, 10 min.) was washed once (NaAc-buffer containing 0.1 M $MgCl_2$) and subsequently dissolved in sodium dodecyl sulphate (SDS). The $^{35}SO_4^{2-}$ radioactivity of the samples was measured by liquid scintillation analysis. $^{35}SO_4^{2-}$ incorporation was normalized to the specific activity of the medium, labeling time and wet weight of the cartilage

samples, and proteoglycan synthesis rate expressed as nmoles sulfate incorporated per hour per gram wet weight of the cartilage (nmol/h.g).
Release of newly formed proteoglycans as a measure of retention of these proteoglycans was determined similarly. GAGs were precipitated from the medium obtained from day 4 to 7 and stained with Alcian Blue dye solution (144). The radio-labeled GAGs were measured by liquid scintillation analysis and normalized to the proteoglycan synthesis rate and expressed as percentage release of newly formed proteoglycans.
For the total release of proteoglycans, the GAG in the medium obtained from day 4 to 7 were precipitated and stained with Alcian Blue as described above (105). The GAG content in the papain digest of cartilage samples, as a measure of proteoglycan content, was analyzed the same way. Blue staining was quantified photometrically by the change in absorbence at 620 nm. Chondroitin sulphate (Sigma C4383) was used as a reference. Values for content were normalized to the wet weight of the cartilage and expressed as mg of GAG/gram wet weight of cartilage (mg/g). Values for release were normalized to the GAG content of the explants and expressed as percentage release of GAGs.

Prostaglandin-E_2 and Nitric Oxide determination
Prostaglandin-E_2 (PGE_2) was determined in culture medium at day 4 by Enzyme Immuno Assay (EIA, Caymann Chemical) and expressed in pg/ml. Nitrite and nitrate concentration (as a measure for nitric oxide; NO) in the same culture medium of the first 4 days of culture was quantified by colorimetric assay based on the Griess reaction and expressed in μM.

Calculations and statistical analysis
Because of focal differences in composition and bioactivity of the cartilage on the femur condyles, the results of 10 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 each cartilage of a different donor (at least n=4) were performed. Statistical evaluation of differences between healthy, degenerated and OA cartilage was performed with a non-parametric test for unpaired data (Mann-Whitney). Statistical evaluation of the effects of treatment was performed with a non-parametric test for paired data (Wilcoxon). *P* values less than or equal to 0.05 were considered statistically significant.

Results

The effect of celecoxib on normal cartilage
Average Mankin grade of the normal cartilage, as expected, was low; 1 ± 0.1 on a scale of 11. The cartilage had always an intact articular surface; safranin-O staining was equally distributed throughout the tissue and chondrocyte localization showed a normal appearance (figure 1A and table 1).

Table 1. *The histological and biochemical characteristics of human healthy cartilage, degenerative and osteoarthritic cartilage.*

	normal cartilage (average ±SEM; n=4)	degenerative cartilage (average ±SEM; n=4)	P <	OA cartilage (average ±SEM; n=6)	P <
Histological cartilage damage	1 ± 0.1	4 ± 0.5	0.05	5 ± 0.5	0.05
PG synthesis (nmol/h.g)	10 ± 1	8 ± 3	ns	5 ± 2	0.05
% new release (%)	6 ± 1	8 ± 1	0.01	11 ± 1	0.01
% total release (%)	3 ± 1	5 ± 1	0.03	6 ± 1	0.02
PG-content (mg/g)	35 ± 2	23 ± 5	0.03	18 ± 3	0.01
Prostaglandin-E_2 (pg/ml)	64 ± 9	129 ± 12	0.03	215 ± 34	0.03
Nitric Oxide (μM)	2.9 ± 0.3	4.3 ± 1.1	ns	8.0 ± 1.6	0.05

Proteoglycan synthesis rate as measure of cartilage matrix synthesis, percentage release of newly formed proteoglycans as a measure of retention for the newly formed proteoglycans (normalized to the synthesis of these proteoglycans), percentages total release of proteoglycans, calculated by the percentage release of glycosaminoglycans (GAG; normalized to the GAG content), proteoglycan content, prostaglandin E_2 and nitric oxide. The results are presented as means of at least 4 experiments (viz. 4 cartilage donors) ±SEM. Statistical evaluation of differences between healthy human articular cartilage and degenerated or osteoarthritic cartilage was performed with a non-parametric test for unpaired data. P values less than or equal to 0.05 were considered statistically significant.

Celecoxib, at a concentration of 10 μM, did not change proteoglycan turnover of normal healthy human articular cartilage (dotted lines in figure 2A-D); proteoglycan synthesis rate (figure 2A), percentage release of newly formed proteoglycans (figure 2B), as a measure of retention of these newly formed proteoglycans, the percentage release of proteoglycans (resident ones plus newly formed, figure 2C) did not change. Also GAG content (figure 2D) was not influenced significantly by celecoxib. When release of newly formed and total amount of proteoglycans were calculated as absolute values, not normalized to synthesis rate and content, respectively, also no effects of celecoxib on normal cartilage were found (data not shown). Also at a concentration of 0.1 and 1 μM no effects were found as reported previously (139).

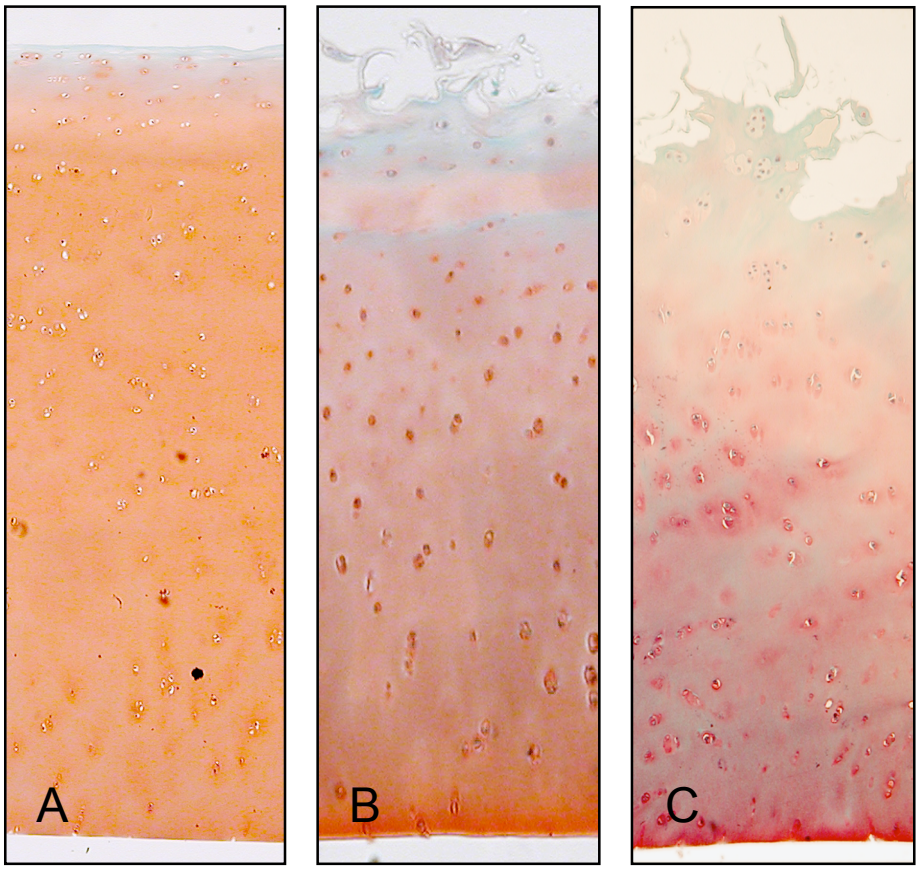

Figure 1. *Normal healthy, degenerative and osteoarthritic cartilage histology.*
Representive light micrographs of condylar cartilage obtained *post-mortem* from joints with normal healthy cartilage (A), or joints with degenerated cartilage (B) and cartilage obtained at joint replacement surgery (C). Sections are stained with safranin-O fast green-iron haematoxylin and graded for features of osteoarthritis according to the slightly modified criteria described by Mankin *et al.*, scores for the depicted samples are 0, 4 and 6, respectively (see appendix).

Effects of celecoxib on degenerated (pre-clinical OA) cartilage

Mostly treatment with NSAIDs will take place on early stages of joint degeneration. Therefore, we tested celecoxib on degenerated (pre-clinical OA) cartilage. The degenerated cartilage had on average modified Mankin score of 4 ± 0.5 (table 1). In the degenerated tissue, surface deterioration was visible. The safranin O staining was lost from the surface layer of the degenerated samples and chondrocyte distribution was disturbed (figure 1B). The early OA cartilage showed the typical intermediate biochemical features with respect to proteoglycan turnover when compared to healthy human cartilage and osteoarthritic cartilage (table 1) (101).

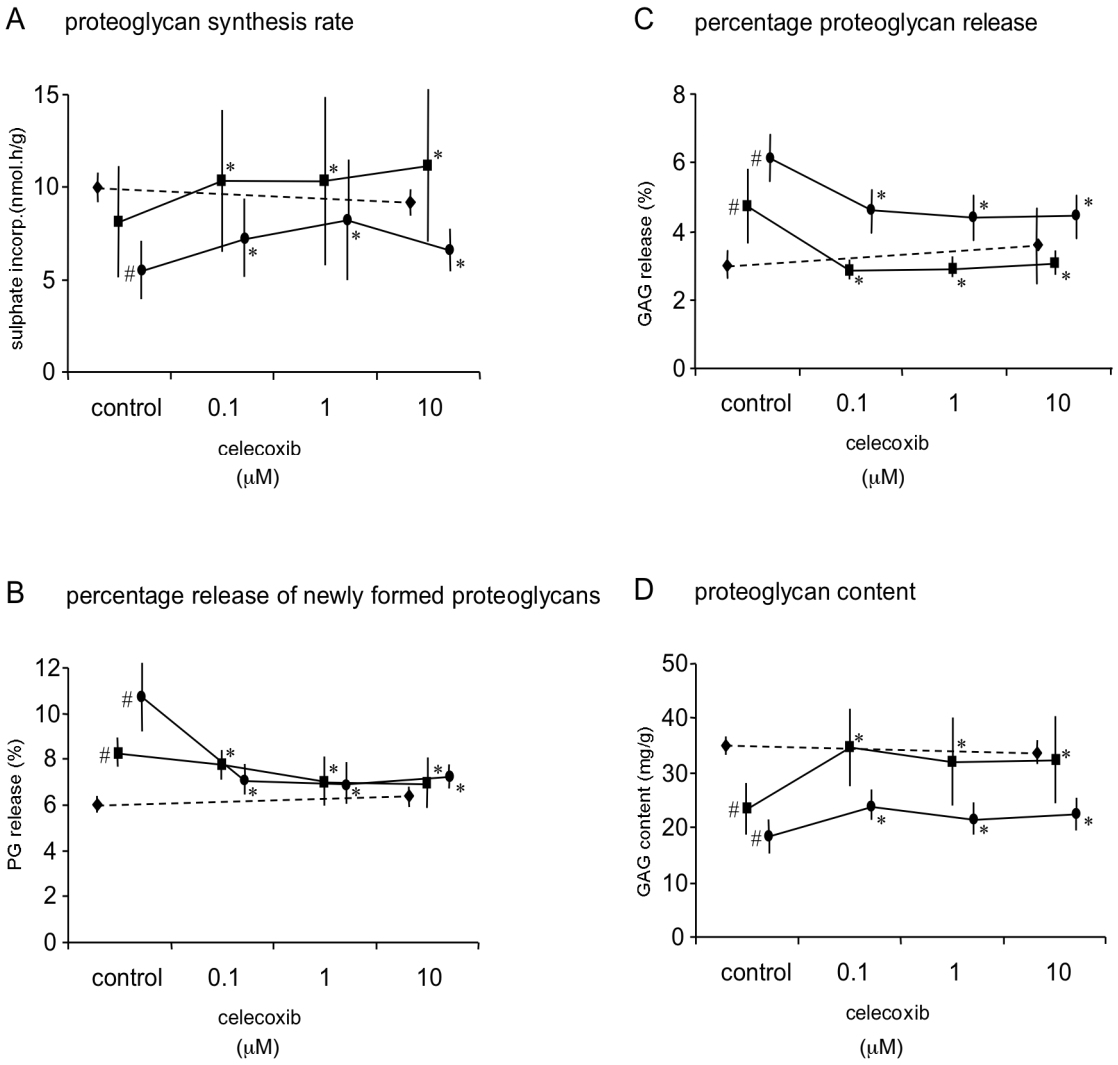

Figure 2. *The effect of celecoxib on degenerated and osteoarthritic human articular cartilage explants.*

Proteoglycan synthesis rate as measure of cartilage matrix synthesis (A), percentage release of newly formed proteoglycans (B) as a measure of retention for the newly formed proteoglycans (normalized to the synthesis of these proteoglycans), percentages total release of proteoglycans (C), measured by the percentage release of glycosaminoglycans (GAG; normalized to the GAG content), and proteoglycan content (D) are depicted. Diamonds represent effects of celecoxib (only 10 μM) on normal cartilage, squares represent effects of celecoxib on degenerated cartilage, and dots represent effects of celecoxib on OA cartilage. The results are presented as means of at least 4 experiments with each cartilage of a different donor ± SEM. Statistically differences between degenerated and OA cartilage compared to normal cartilage, calculated by non-parametric unpaired analysis (p-value <0.05) at baseline are marked with an #. Statistically differences of the effects of celecoxib compared to controls, calculated by non-parametric paired analysis (p-value <0.05) are marked with an asterisk.

When degenerated cartilage was incubated with celecoxib, in a concentration of 0.1- 10 μM, a slight but dose dependent increase of the cartilage matrix synthesis was observed (figure 2A squares), at all concentrations tested statistically significant compared to untreated control degenerated cartilage. The actual synthesis rate reached even higher levels than normal cartilage although not statistically significant.
Release of newly formed proteoglycans, as absolute values (data not shown) and as percentage (normalized to the proteoglycan synthesis rate) was significantly reduced up to nearly complete normalization by addition of celecoxib (at all concentrations tested statistically significant; figure 2B squares). Although there appeared to be a slight dose dependency, statistically, normalization was reached already at the lowest concentration tested.
Similar results were found for the release of all proteoglycans, newly formed plus resident proteoglycans, when calculated as absolute (data not shown) and normalized to the proteoglycan content representing percentage total proteoglycan release (figure 2C squares). Again we found a reduction up to complete normalization under the influence of celecoxib. Already at the lowest concentration used, the addition of celecoxib resulted in a retention of newly formed proteoglycans not different from normal healthy cartilage anymore.
With respect to matrix integrity celecoxib was able to induce normalization in proteoglycan content (figure 2D squares) that was statistically significant at all concentrations tested.

Effects of celecoxib on osteoarthritic cartilage.
The osteoarthritic cartilage had on average a modified Mankin score of 5 ± 0.5, slightly higher than that of degenerative cartilage (table 1) and significant higher than that of normal cartilage. It should be kept in mind that only the cartilage that could be cut from the joint surfaces after replacement surgery was used. Thus in fact the entire joint had a worse appearance than represented by the modified Mankin score of the cartilage used. The OA cartilage surface deterioration was clearly visible by light microscopy. The safranin O staining was lost from the surface layer of the degenerated samples and chondrocyte distribution was disturbed (clusters of chondrocytes in the surface layer of the cartilage were visible; figure 1C). The osteoarthritic cartilage showed the typical biochemical features with respect to proteoglycan turnover when compared to healthy human cartilage (table 1) and these changes always exceeded the changes as observed in degenerated cartilage. The effects of celecoxib, observed for late-stage OA cartilage, were similar as for degenerative OA cartilage (figure 2A-D).
For all proteoglycan parameters there was a statistically significant improvement found upon addition of celecoxib. However, both proteoglycan synthesis rate and proteoglycan content remained statistically significant below healthy cartilage control values. None of the effects of celecoxib on OA cartilage were statistically significant different from the effects on degenerated cartilage.

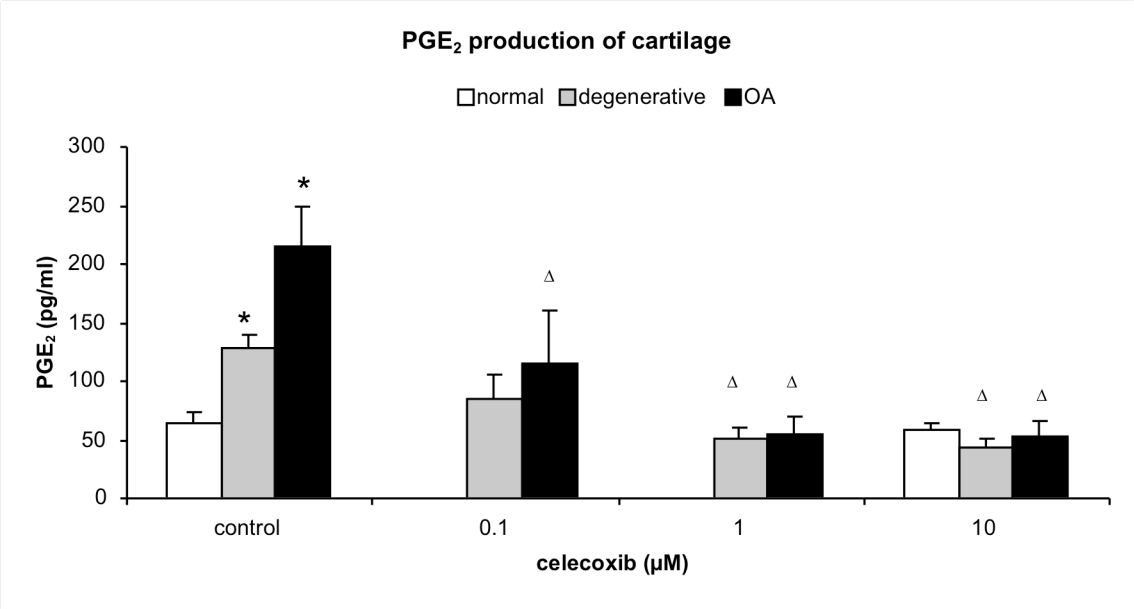

Figure 3. *The effect of celecoxib on prostaglandin-E_2 of normal, degenerated and osteoarthritic human articular cartilage explants.*
Prostaglandin-E_2 production of normal, degenerated, and osteoarthritic cartilage with or without celecoxib are depicted. Open bars represent effects of celecoxib on normal cartilage, gray bars represent effects of celecoxib on degenerated cartilage, and black bars represent effects of celecoxib on OA cartilage. The results are presented as means of at least 4 experiments with each cartilage of a different donor ± SEM. Statistically differences between degenerated and OA cartilage compared to normal cartilage, calculated by non-parametric unpaired analysis (p-value <0.05) at baseline are marked with an asterisk. Statistically differences of the effects of celecoxib compared to untreated controls, calculated by non-parametric paired analysis (p-value <0.05) are marked with a triangle.

Effect of celecoxib on PGE_2 and NO production.
In addition to proteoglycan turnover and content, we have determined the effect of celecoxib on prostaglandin-E_2 (PGE_2) production as a indirect measurement of COX-2 activity. To demonstrate that the effects of celecoxib were COX-2 (viz PGE_2) related we also quantified the nitric oxide (NO) production of the different types of cartilage.
In both degenerated and OA cartilage the amount of PGE_2 formed and measured in the supernatant of the cartilage cultures was significantly elevated compared to normal cartilage (table 1). OA cartilage showed the highest amounts of PGE_2, most likely due to the strongest COX-2 activity, where the basal level of normal cartilage is most likely due to non or low COX-2 activity.
Similar result where found for the nitric oxide levels (table 1). In both types of diseased cartilage nitric oxide was elevated (on average 2.9 ± 0.3 μM, 4.3 ± 1.1 μM, and 8.0 ± 1.6 μM for normal, degenerated, and clinical OA cartilage, respectively), again with the highest levels found in the OA cartilage.

The basal PGE_2 production of normal cartilage was not influenced by celecoxib at a concentration of 10 μM (figure 3, open bars). In contrast, when degenerated cartilage was incubated with celecoxib, normalization of the elevated PGE_2 levels occurred in a dose dependent manner (figure 3 gray bars), Also the elevated PGE_2 levels of OA cartilage could be normalized, in a dose-dependent manner, when incubated with celecoxib (figure 3, black bars). The normalization of PGE_2 production in both degenerated and OA cartilage shows indirect that celecoxib inhibits COX-2 activity in degenerative and OA cartilage.
In contrast to PGE_2, the NO production of normal, degenerated and OA cartilage was not influenced by celecoxib (on average 3.1 ± 0.9 μM, 4.3 ± 2.5 μM and 7.0 ± 1.5 μM for normal, degenerated and clinical OA cartilage incubated with 10 μM celecoxib).

Discussion

Our study shows that the selective COX-2 inhibitor celecoxib has a favorable effect on proteoglycan synthesis, retention, release and content of both degenerated (pre-clinical) and (late-stage) osteoarthritic cartilage. On normal healthy cartilage no effects were observed, confirming our previous results (139) where we showed in a complete dose response curve that normal cartilage remained unaffected. Our results with respect to proteoglycan synthesis and retention of late-stage osteoarthritic cartilage corroborate recent findings by El Hajjaji *et al*. (140) using a slightly different approach. In their study they also demonstrated a beneficial effect on hyaluronan synthesis in human late-stage osteoarthritic cartilage obtained at joint replacement surgery. The present study adds in this respect that also release of resident proteoglycans and more importantly proteoglycan content are beneficially influenced by celecoxib. In addition our study shows that also for degenerated cartilage a chondroprotective effect of celecoxib can be observed.
All beneficial effects on cartilage proteoglycan turnover and content were similar for OA cartilage obtained at joint replacement surgery and degenerated (pre-clinical) cartilage. However especially degenerated cartilage seems to profit from COX-2 inhibition as all four parameters normalized whereas for OA cartilage COX-2 inhibition normalized only retention and release. This, and the fact that normal healthy cartilage stays unaffected is of major importance in treatment of patients with OA. This could mean that treatment of OA patients with celecoxib specifically at an early stage of their disease could slowdown further damage and with that postpone further invasive chirurgical treatment (joint replacement surgery).
These effects contrast the results of several other studies obtained with NSAIDs tested under comparable conditions. Frequently used NSAIDs such as naproxen, and ibuprofen have been demonstrated to inhibit the synthesis of cartilage proteoglycans (65-67, 134) and to increase the release of proteoglycans (67). However, although mostly effects have been reported to be adverse, also no- or positive effects of NSAIDs have been described (60, 65-67, 134).

These are all direct effects on cartilage and should be seen in the context of the significant anti-inflammatory effects of these NSAIDs. By inhibiting joint inflammation they may indirectly be beneficial to cartilage, specifically when inflammation is primary in the cause of cartilage damage as e.g. in rheumatoid arthritis. In case of osteoarthritis, where inflammation may contribute but is not primarily responsible for cartilage damage, adverse direct effects of NSAIDs on cartilage during long-term treatment may have important impact on long-term outcome.

This study describes a beneficial effect of a selective COX-2 inhibitor on osteoarthritic cartilage proteoglycan turnover and content, whereas no effects are observed on normal healthy cartilage. This could be due to better accessibility of celecoxib in (pre-clinical) OA cartilage, due to its fibrillated surface. However, in a previous study (139) we showed that healthy cartilage with an intact surface, when co-cultured with peripheral blood mononuclear cells of rheumatoid arthritis patients or with a combination of IL-1β and TNFα demonstrated the same cellular characteristics, as OA chondrocytes in the present study, which could be normalized by addition of celecoxib. This suggests that the difference between normal and (pre-clinical) OA cartilage is not due to accessibility. Furthermore, the elevated PGE_2 levels in degenerative and even more elevated in OA cartilage (according to literature (78, 137)), that could be normalized by addition of celecoxib suggest COX-2 regulation to be involved, rather than just accessibility.

An other important mediator of cartilage damage is nitric oxide (NO). The production of NO in joint pathology could contribute to disease pathogenesis in variety of ways (145). These effects of NO on synovial cells and chondrocytes include: inhibition of collagen and proteoglycan synthesis; activation of metalloproteinases; and apoptosis (146). It appeared that celecoxib was not able to influence the elevated NO production of degenerative and OA cartilage in our study. This implies that the favorable effect of celecoxib on (pre-clinical) OA cartilage is mainly due to inhibiting COX-2 activity. The remaining elevated levels of NO could explain why COX-2 inhibition does not normalize for both types of diseased cartilage all proteoglycan parameters. Especially proteoglycan synthesis seems to favor only partially from COX-2 inhibition. This fits with the knowledge that high levels of NO decrease PG synthesis (146).

The presence of COX-2 in OA cartilage, directly measured (78, 137) or indirectly via PGE_2 (138), is evident. It could be, although speculative, that the adverse effects on cartilage of some of the conventional NSAIDs results from inhibition of COX-1, which could be essential for normal chondrocyte function. However, other effects of these NSAIDs, COX independent, might be involved as well (135). The other way around, whether selective COX-2 inhibitors other than celecoxib are as beneficial for (pre-clinical) OA cartilage remains to be established as well.

All the effects found in the present study are beneficial to cartilage. Moreover, the response is obtained with a clinical relevant concentration of the drug because the mean pharmacological plasma concentration is 5 μM (104). The response is also

quick, within 7 days chondrocytes appear to be able to start restoration of the extracellular matrix. Whether these promising effects take also place *in vivo* remains of course to be seen. *In vivo* animal studies and clinical trials have to be performed to prove the suggestive chondroprotection of COX-2 inhibition found *in vitro*. The present results imply an important role for COX-2 in the disturbed proteoglycan turnover in osteoarthritis, making COX-2 inhibitors a relevant choice of treatment in the elderly. On the other hand, questions have been raised about cardiovascular side effects, and about possible adverse effects on bone healing. These effects appear to be shared with non-selective NSAIDs (147-149).
In conclusion, although *in vitro* findings, the present study suggests that besides the anti-inflammatory and analgesic characteristics of selective COX-2 inhibitors, celecoxib has cartilage protective capacities, contributing directly to improvement of cartilage matrix integrity in osteoarthritis already in an early phase, which expectedly depends on its selective COX-2 inhibition.

Chapter 4

Synthesis and release of cartilage matrix proteoglycans are differently regulated by nitric oxide and prostaglandine-E_2.

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submitted for publication

Abstract

Objective: Recent studies showed beneficial effects of COX-2 inhibition on proteoglycan turnover of both IL-1β+TNFα induced cartilage damage as well as of osteoarthritic cartilage. Although proteoglycan release and content normalized, proteoglycan synthesis was only partially influenced. Prostaglandin-E_2 is the main product formed by COX-2. This suggests an important role for prostaglandin-E_2 in disturbance of cartilage proteoglycan release but not synthesis, which was subject of the present study.

Methods: Human healthy cartilage, alone or in the presence of IL-1β+TNFα, was cultured for 7 days with or without prostaglandin-E_2 or the selective COX-2 inhibitor (celecoxib 10 μM). Changes in cartilage matrix proteoglycan turnover, prostaglandin-E_2 and nitric oxide were determined.

Results: Proteoglycan synthesis and release of the cartilage were not affected by prostaglandin-E_2 alone. Addition of IL-1β+TNFα to the healthy cartilage resulted in inhibition of proteoglycan synthesis and increased proteoglycan release. When in addition to IL-1β+TNFα, prostaglandin-E_2 was added, proteoglycan release further increased, but proteoglycan synthesis was not further influenced. Addition of a selective COX-2 inhibitor to the IL-1β+TNFα treated cartilage inhibited the enhanced prostaglandin-E_2 production and almost complete normalized proteoglycan release, whereas synthesis remained unaffected. Also the enhanced NO-levels remained elevated. Prostaglandin-E_2 levels correlated significantly with proteoglycan release whereas NO levels were significantly negative correlated with proteoglycan synthesis.

Conclusion: The present results suggest involvement of prostaglandin-E_2 in enhanced cartilage proteoglycan release but not synthesis, although healthy cartilage has to be sensitized by IL-1β+TNFα. IL-1β+TNFα induced NO seems to be involved in inhibition of proteoglycan synthesis, independent of prostaglandin-E_2 and with that insensitive to regulation by (selective) COX-2 inhibitors.

Introduction

Osteoarthritis (OA), a chronic disease with slowly progressive destruction of the articular cartilage, results from the failure of chondrocytes to maintain the balance between synthesis and degradation of the extracellular matrix (150). Although osteoarthritis was characterized originally as a noninflammatory arthropathy (osteoarthrosis), inflammatory responses in the synovial membrane caused by direct biomechanical perturbation and/or caused by reaction to cartilage matrix degradation products will contribute to disease progression (151, 152). However, the major events in osteoarthritis pathogenesis are expectedly localized within the cartilage itself. There appears to be a central role for the cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNFα) produced by the chondrocytes and synovial tissue (20, 153). These cytokines stimulate the synthesis of prostaglandin-E_2 (PGE_2) and nitric oxide (NO) (154, 155). More recent studies showed that the *in vivo* selective inhibition of iNOS, responsible for the production of NO, reduces the symptoms of inflammation and the biochemical abnormalities of osteoarthritic

joints (156-158). Various roles of NO as mediator of other IL-1 induced responses, including the inhibition of aggrecan and collagen synthesis (159-161), enhancement of MMP-activity and chondrocyte apoptosis (162-164) and reduction of the production of IL-1 receptor antagonist (IL-1RA) (158, 165) have also been suggested.

While literature is quite clear that excessive production of NO exerts predominantly catabolic effects being harmful to cartilage, observations with respect to prostaglandin overproduction are less clear. In articular chondrocytes IL-1β and TNFα synergistically induce COX-2, whereas COX-1 expression remains unchanged (166). Prostaglandin-E_2 is elevated in the osteoarthritic joint in cartilage as well as synovium (78, 137). Prostaglandin-E_2, a predominant product of COX-2, can exert anabolic effects in synovium and cartilage. For example, prostaglandin-E_2 reversers proteoglycan degradation induced by IL-1β in bovine and human cartilage explants (167). Prostaglandin-E_2 also inhibits IL-1β induced MMP (collagenase, stromelysine) expression in human of synovial fibroblasts (168, 169). At nanomolar concentrations, prostaglandin-E_2 enhances collagen type II and proteoglycan synthesis (170, 171). In contrast, in studies using growth plate chondrocytes prostaglandin-E_2 inhibited collagen synthesis, but did not alter non-collagen protein synthesis (170). Prostaglandin-E_2 is also capable to inhibit chondrocyte differentiation: prostaglandin-E_2 suppresses maturation of growth plate chondrocytes and dose-dependently inhibits the expression of several maturation genes, such as type X collagen and metalloproteinase-13 (172).

Indirectly, catabolic effects of prostaglandin-E_2 are demonstrated by the use of selective COX-2 inhibition. Selective COX-2 inhibition and with that prostaglandin-E_2 production was beneficial for cartilage under the influence of IL-1β and TNFα (139). Findings by El Hajjaji *et al*. showed that selective COX-2 inhibition *in vitro* was able to increase proteoglycan synthesis and to diminish proteoglycan release of osteoarthritic cartilage obtained at joint replacement surgery (140). Recent findings by our group confirmed these data and additionally demonstrated that selective COX-2 inhibition, had *in vitro* a favorable effect on proteoglycan synthesis, retention, release and content of both degenerated (pre-clinical) and (late-stage) human osteoarthritic cartilage (173). As prostaglandin-E_2 is a predominant product of COX-2 these data favor the catabolic role of prostaglandin-E_2.

Because the effects of prostaglandin-E_2 in cartilage degeneration are not conclusive, in the present study we further evaluated the effect of prostaglandin-E_2 in relation to NO in human articular cartilage explants with a focus on the differential effects on proteoglycan synthesis and release.

Material and Methods

Cartilage culture technique

Human articular cartilage tissue was obtained *post mortem* from knee condyles within 24 hours of death. The donors were without known history of joint disorders. Slices of cartilage were cut aseptically from the articular surface, excluding the underlying bone, and kept in phosphate buffered (pH 7.4) saline (PBS). Within one hour 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 microtiter plates (200 μl culture medium, 5% CO_2 in air, 37°C). The culture medium consisted of Dulbecco's modified Eagle's medium (D-MEM), supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin sulfate (100 μg/ml), ascorbic acid (0,085 mM), and 10 % heat inactivated pooled human male AB^+ serum.

Experimental setup

Healthy cartilage tissue was cultured for 7 days alone, in the presence of different concentrations prostaglandin-E_2 (PGE_2; Caymann Chemical), in the presence of IL-1β plus TNFα (200 pg/ml, Biosource, PHC0814, and 800 pg/ml, Pharmingen, 19761T, respectively), in the presence of IL-1β plus TNFα together with prostaglandin-E_2, and in the presence of IL-1β plus TNFα with a selective COX-2 inhibitor (celecoxib; supplied by Pfizer USA) inhibiting prostaglandin-E_2 production. After 4 days medium was refreshed and cartilage cultured for a successive 3 days with the same additions. Changes in cartilage matrix turnover (proteoglycan-synthesis, -retention, -release and -content) and, when relevant, prostaglandin-E_2 and Nitric Oxide (NO) production were determined.

Proteoglycan analyses

Sulphate incorporation rate was determined, as a measure of the proteoglycan synthesis rate, during the last 4 hours of the first 4-day culture period, as described previously (105) Before addition of $^{35}SO_4^{2-}$ ($Na_2{}^{35}SO_4$, 14.8 kBq/200 μl, DuPont NEX-041-H, carrier-free), culture medium was replaced by equilibrated (CO_2 and temperature) fresh medium. Medium of the 4 days of culture was used for prostaglandin-E_2 and NO analyses. After 4 hour labeling, the cartilage explants were rinsed 3 times for 45 minutes in culture medium under culture conditions and incubated for the additional period of 3 days. After this second culture period, medium was removed and stored at -20°C for analysis of proteoglycans release. Cartilage tissue samples were digested (2 h, 65°C) in papain buffer. Papain digests were diluted to the appropriate concentrations and glycosaminoglycans (GAGs) were stained and precipitated with Alcian Blue dye solution (144). The pellet obtained after centrifugation (9000g, 10 min.) was washed once and subsequently dissolved. The $^{35}SO_4^{2-}$ radioactivity of the samples was measured by liquid scintillation analysis. $^{35}SO_4^{2-}$ incorporation was normalized to the specific activity of the medium, labeling time and wet weight of the cartilage samples.

Proteoglycan synthesis rate is expressed as percentage change compared to the untreated control values.
Release of newly formed proteoglycans as a measure of retention of these proteoglycans was determined similarly. GAGs were precipitated from the medium obtained from day 4 to 7 and stained with Alcian Blue dye solution (144). The radio-labeled GAGs were measured by liquid scintillation analysis and normalized to the proteoglycan synthesis rate (percentage release). Release of newly formed proteoglycans is expressed as percentage change compared to the untreated control values.
For the total release of all proteoglycans (newly formed and resident ones), the GAG in the medium obtained from day 4 to 7 were precipitated and stained with Alcian Blue as described above. Blue staining was quantified photometrically by the change in absorbence at 620 nm. Chondroitin sulphate (Sigma C4383) was used as a reference. The GAG content in the papain digest of cartilage tissue samples was analyzed the same way. Values for content were normalized to the wet weight of the cartilage and expressed as percentage change compared to the untreated control values. Values for total release were normalized to the GAG content of the explants (percentage total release). Values for total release of GAGs are expressed as percentage change compared to the untreated control values.

Prostaglandin-E_2 and Nitric Oxide determination
Prostaglandin-E_2 (PGE_2) and nitric oxide (NO) were determined in the 4 day culture medium after the first 4 days of culture. Prostaglandin-E_2 was determined by Enzyme Immuno Assay (EIA, Caymann Chemical) and expressed as μg per ml per gram (wet weight) cartilage tissue. Nitric Oxide was determined in culture medium by standard Griess reaction and expressed as mM per gram (wet weight) of cartilage tissue.

Calculations and statistical analysis
Because of focal differences in composition and bioactivity of the cartilage in the knee joint, the results of 10 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 each cartilage of a different donor were performed. Statistical evaluation of the effects of a single intervention compared to the untreated cartilage of the same donors was performed with a non-parametric test for paired data (Wilcoxon). For statistical evaluation of differences between different interventions the percentage change compared to untreated cartilage of the same donors was calculated and compared statistically by use of a non-parametric test for unpaired data (Mann-Whitney). *P* values less than or equal to 0.05 were considered statistically significant.

Results

The effect of prostaglandin-E_2 on normal human articular cartilage

Prostaglandin-E_2, in a concentration ranging from 3 to 500 pg/ml, did not change proteoglycan turnover of normal healthy human articular cartilage (table 1). Proteoglycan synthesis rate, release of newly formed proteoglycans, as a measure of retention of these newly formed proteoglycans, total release of proteoglycans, newly formed plus resident ones, as well as proteoglycan content were not influenced by exogenous prostaglandin-E_2.

The effect of prostaglandin-E_2 on human articular cartilage explants under the influence of IL-1β plus TNFα

To evaluate whether prostaglandin-E_2 needed stimulated chondrocytes to display an effect, the cartilage was sensitized. Cartilage was exposed to two major mediators of cartilage degeneration, IL-1β and TNFα. Under the influence of IL-1β plus TNFα proteoglycan synthesis rate was significantly inhibited (on average almost 75% inhibition, $p<0.05$; figure 1). Addition of prostaglandin-E_2 showed no effect on this inhibited proteoglycan synthesis rate (still about 70% inhibition, $p<0.05$; gray bars in figure 1). Percentage release of newly formed proteoglycans (normalized to the proteoglycan synthesis rate) was significantly enhanced by the addition of IL-1β plus TNFα; (about 25%, open bar; figure 1), indicating an impaired retention of the newly formed proteoglycans. Remarkably, when prostaglandin-E_2 was added this release of newly formed proteoglycans was more than doubled (close to 100%, gray bar in figure 1; $p<0.05$), significantly different from the IL-1β/TNFα condition without prostaglandin-E_2 ($p\leq0.04$). Also the total proteoglycan release, consisting of both newly formed proteoglycan as well as resident proteoglycans was increased by the addition of prostaglandin-E_2 in the same way. Addition of IL-1β plus TNFα alone displayed a total release of about 80% ($p<0.05$), which more than doubled when prostaglandin-E_2 was added (more than 200%, $p<0.01$). IL-1β plus TNFα also induced a statistically significant decreased proteoglycan content of the cartilage of a few percent ($p<0.05$, figure 1). Prostaglandin-E_2 also for this parameter amplified the effect significantly to an almost 15% decrease in proteoglycan content ($p\leq0.02$).

Table 1. ***Proteoglycan turnover and content of human cartilage treated with prostaglandin-E_2***

	PGE_2 (3 pg/ml)	PGE_2 (5 pg/ml)	PGE_2 (10 pg/ml)	PGE_2 (30 pg/ml)	PGE_2 (50 pg/ml)	PGE_2 (100 pg/ml)	PGE_2 (500 pg/ml)
PG synthesis rate (% change)	95 ± 10	101 ± 13	99 ± 14	102 ± 9	101 ± 11	100 ± 10	103 ± 11
% new PG release (% change)	100 ± 5	99 ± 6	101 ± 3	100 ± 5	101 ± 5	100 ± 4	101 ± 4
% total release (% change)	102 ± 6	98 ± 7	100 ± 5	99 ± 6	101 ± 7	101 ± 6	99 ± 3
PG content (% change)	100 ± 11	103 ± 9	99 ± 15	100 ± 14	101 ± 8	104 ± 10	97 ± 13

Percentage change compared to controls (cartilage without addition) of proteoglycan synthesis rate, percentage release of newly formed proteoglycans, percentage total release of proteoglycans, and proteoglycan content of human healthy cartilage under the influence of different concentrations of prostaglandin-E_2 (PGE_2). The results are presented as means of 4 experiments (viz. 4 cartilage donors) ± SEM. No statistical differences were found between the different concentrations groups.

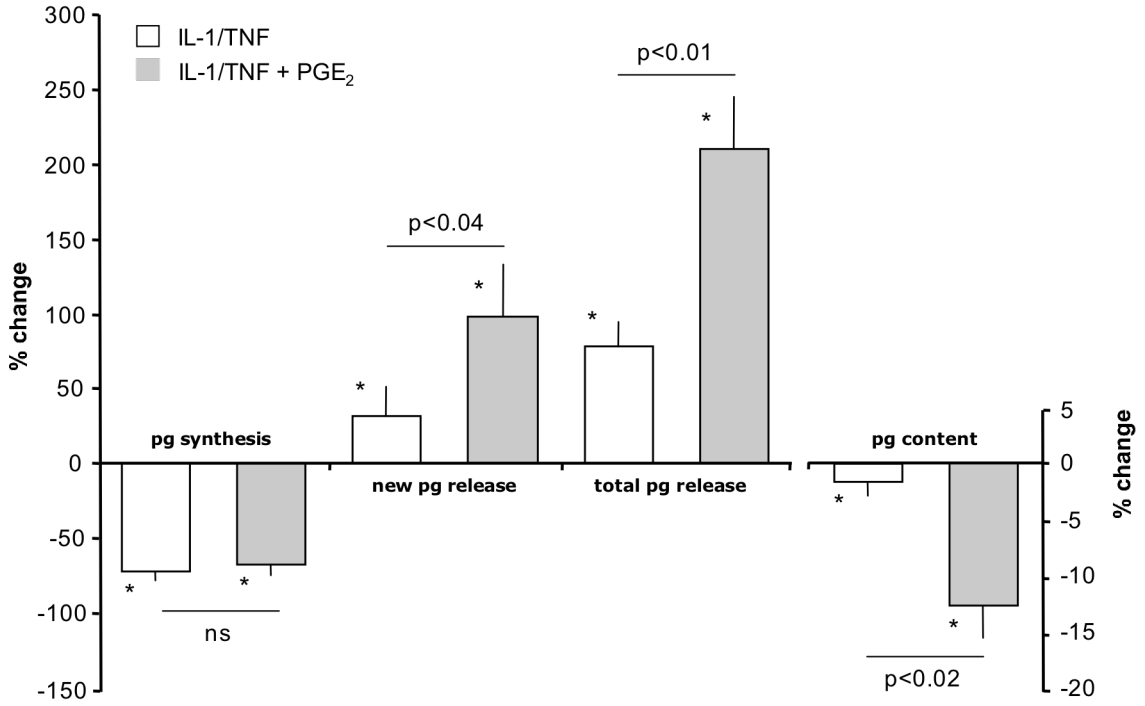

Figure 1. *Effect of prostaglandin-E_2 on IL-1β plus TNFα induced cartilage damage*
Percentage change compared to healthy cartilage of IL-1β plus TNFα treated cartilage in the absence (open bars) or presence (gray bars) of prostaglandin-E_2. Effects on proteoglycan (pg) synthesis rate, percentage release of newly formed proteoglycans, percentages total release of proteoglycans, and proteoglycan content are depicted. Open bars represent effects of IL-1β plus TNFα (200/800 pg/ml, respectively) on human healthy articular knee cartilage. Grey bars represent effects of prostaglandin-E_2 (200 pg/ml) when added in addition to IL-1β plus TNFα. The results are presented as means of at least 4 experiments with each cartilage of a different donor ± SEM. Statistically significant differences with untreated control cartilage are indicated by asterisks. Statistically significant effects of prostaglandin-E_2 are indicated by p values (ns = not significant).

The effect of COX-2 inhibition on proteoglycan synthesis, - release, prostaglandin-E_2, and nitric oxide production of human articular cartilage under influence of IL-1β plus TNFα

The addition of prostaglandin-E_2 to cartilage under the influence of IL-1β plus TNFα specifically affected proteoglycan release whereas proteoglycan synthesis rate was not changed by prostaglandin-E_2. The other way around, recent studies showed that the beneficial effect of COX-2 inhibition (indirect prostaglandin-E_2 inhibition) in cartilage was mainly reflected by a normalization of the proteoglycan release, for both cartilage under influence of pro-inflammatory cytokines as well as osteoarthritic cartilage (139, 173). Therefore, it was studied whether proteoglycan synthesis and release were independently regulated, release being prostaglandin-E_2 dependent, but synthesis not. Under the influence of IL-1β plus TNFα proteoglycan synthesis was significantly inhibited (on average 75% inhibition, $p \leq 0.02$; grey bar, figure 2A) compared to healthy control cartilage (white bar). Celecoxib was not able to reverse this inhibition of proteoglycan synthesis (black bar). Release of proteoglycans was increased upon addition of IL-1β plus TNFα

(on average 40%, p≤0.02; figure 2B). Celecoxib was able to reverse this increased release almost completely.

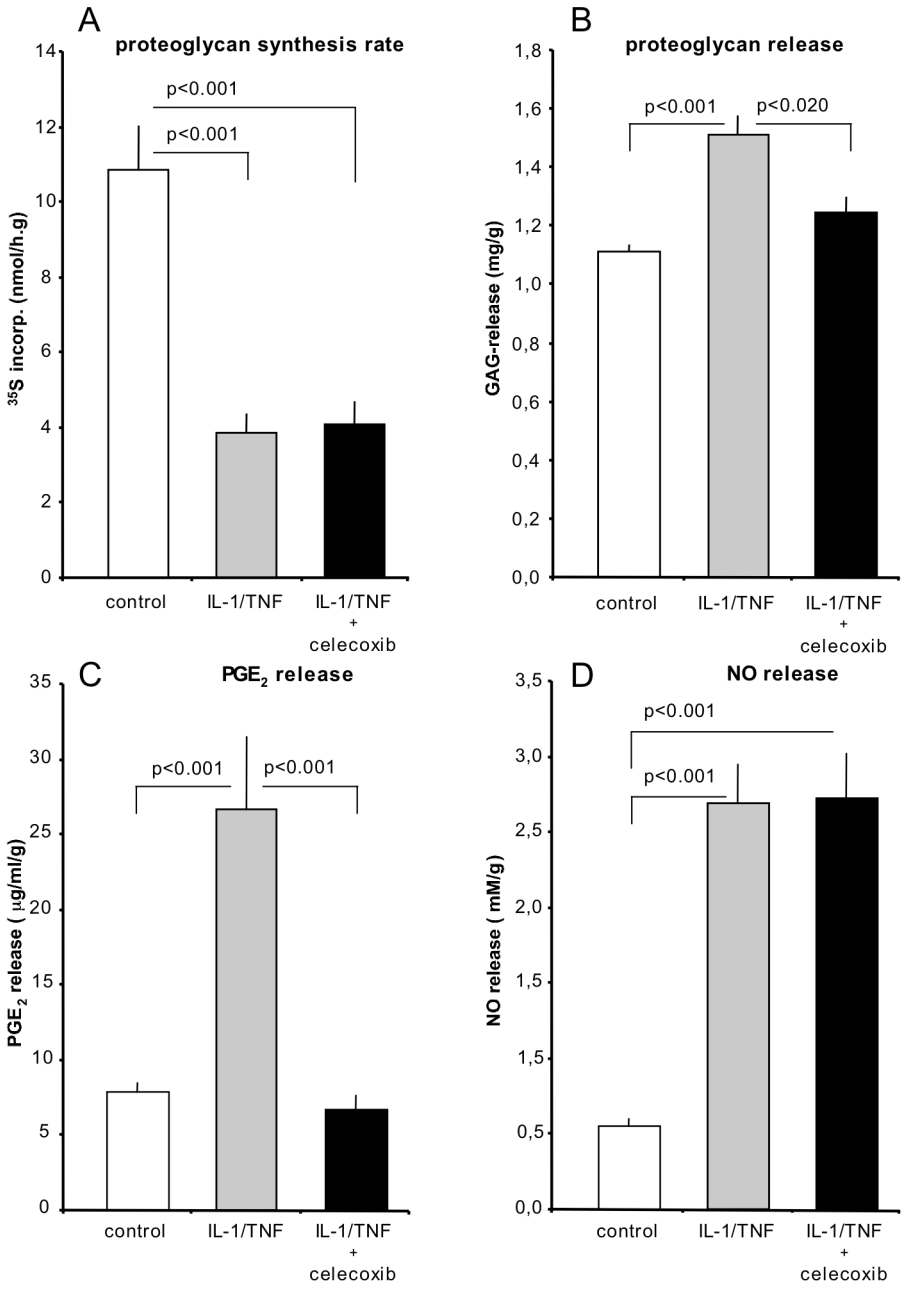

Figure 2. *The effect of COX-2 inhibition on proteoglycan synthesis, proteoglycan release, prostaglandin-E_2, and nitric oxide production of human articular cartilage under influence of IL-1β plus TNFα*

Proteoglycan synthesis rate (upper left panel), total release of proteoglycans (upper right panel), prostaglandin-E_2 production (PGE_2, lower left panel) production, and nitric oxide production (NO, lower right panel) are depicted. White bars represent the parameters of human healthy cartilage alone, grey bars represent the parameters of the articular cartilage under influence of IL-1β plus TNFα, and the black bars represent the parameters of the cartilage under influence of IL-1β plus TNFα in the presence of celecoxib (10 μM) as a selective COX-2 (and with that PGE_2) inhibitor. The results are presented as means of at least 8 experiments (viz. 8 cartilage donors) ± SEM. Statistical significant differences compared to untreated control cartilage is depicted by p values.

Prostaglandin-E_2 production (figure 2C) and nitric oxide production (figure 2D) of cartilage were both significantly elevated under influence of IL-1β plus TNFα (gray bars) compared to control (white bars). Addition of celecoxib resulted only in a normalization of prostaglandin-E_2 levels (black bar). Surprisingly, nitric oxide levels were not influenced by celecoxib.
Interestingly, when the proteoglycan release was compared to prostaglandin-E_2 production we found a strong positive correlation (figure 3A): the higher the prostaglandin-E_2 concentration the higher the proteoglycan release. There was no correlation found between prostaglandin-E_2 production and proteoglycan synthesis rate (table 2). However, there was a negative correlation found between proteoglycan synthesis rate and nitric oxide production (figure 3B): the higher the nitric oxide concentration the lower the proteoglycan synthesis. In addition, there was also a positive correlation between nitric oxide production and proteoglycan release (table 2).

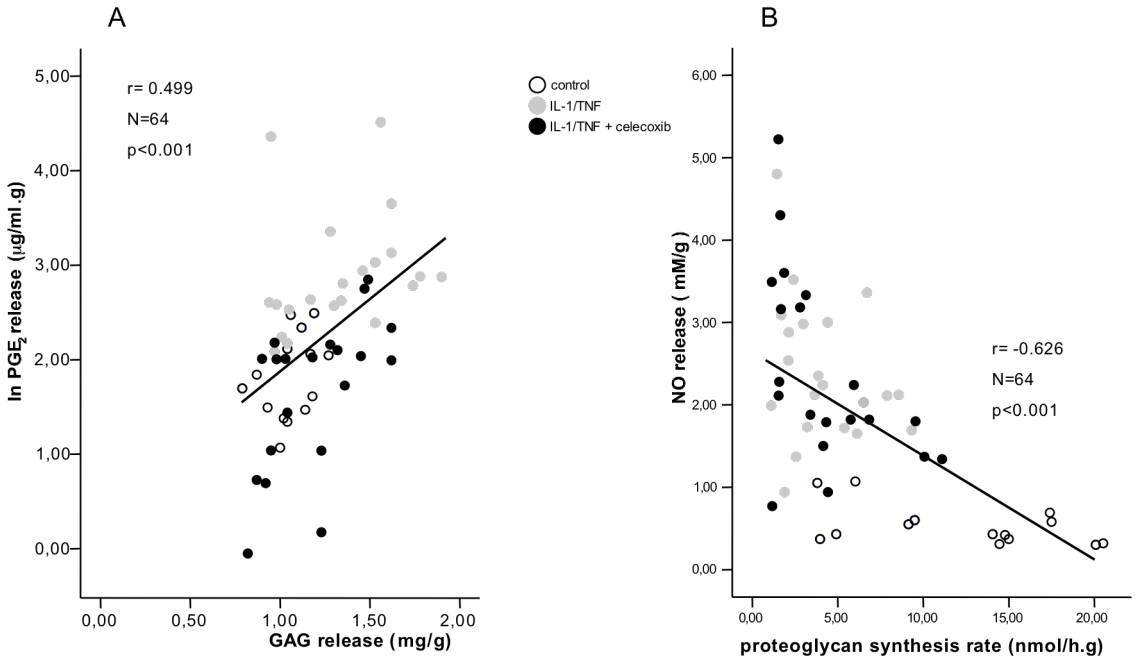

Figure 3. *Correlations of proteoglycan release with prostaglandin-E_2 and proteoglycan synthesis rate with nitric oxide production.*
Proteoglycan release and proteoglycan synthesis of human articular cartilage correlated with prostaglandin-E_2 and NO production, respectively. Prostaglandin-E_2 is converted to a natural logarithm (ln) to obtain normal distribution of values. Each data point is the mean of 4 samples. Open circles represent data of human articular cartilage alone, grey dots represent human articular cartilage under influence of IL-1β plus TNFα, and the black dots represent human articular cartilage under influence of IL-1β plus TNFα in presence of celecoxib (10 μM).

Table 2. *Correlations of proteoglycan release and synthesis rate with prostaglandin-E_2 and nitric oxide production.*

	prostaglandin-E_2 (ln µg/ml.g)	NO (mM/g)
PG release (mg/g)	0.499 p≤0.000	0.446 p≤0.000
PG synthesis rate (nmol/h.g)	-0.146 ns	-0.626 p≤0.000

Proteoglycan synthesis did not correlate with prostaglandin-E_2 production. Pearson correlation coefficients and corresponding p values are given.

Discussion

In the present study, it is demonstrated that prostaglandin-E_2 alone has no effect on the proteoglycan turnover of healthy human articular cartilage. When cartilage is cultured in presence of IL-1β plus TNFα adverse changes in proteoglycan synthesis rate and release are induced. When prostaglandin-E_2 is added to this sensitized cartilage, prostaglandin-E_2 amplifies only the adverse changes in proteoglycan release. The other way around, when prostaglandin-E_2 production is inhibited (by selective COX-2 inhibition) the adverse effects of IL-1β and TNFα are only reduced for proteoglycan release. Most interestingly, for the first time it is shown that prostaglandin-E_2 sorts its effect mainly (exclusively) on proteoglycan release, supported by a clear correlation between the two, and not on proteoglycans synthesis. Proteoglycan synthesis seems to depend much more on nitric oxide production.

Recent work by our group and others showed that inhibition of COX-2, and thus inhibition of prostaglandin-E_2 production, resulted in improvement of proteoglycan turnover and hyaluronan synthesis in degenerated cartilage (139, 173). These studies indicate that significant prostaglandin E_2 production (up to 200 pg/ml) is involved in disturbing the proteoglycan turnover of degenerated cartilage. However, in the present experiments no direct catabolic effects of prostaglandin-E_2 on healthy cartilage were found, although we used concentrations resembling those found in degenerated cartilage (173). Even higher concentrations (500 pg/ml) did not exert any effect.

A possible explanation for this lack of effect of protaglandin-E_2 on healthy cartilage could be the prostaglandin-E_2 receptor profile on healthy chondrocytes by which the biological actions of prostaglandin-E_2 are mediated. Prostaglandin-E_2 can act through at least four different receptors (EP receptors), and it is not clear which of these contributes to cartilage degeneration. These EP receptors, termed EP1-4, are G-protein coupled heptahelical proteins. Each receptor subtype has a distinct pharmacological signature based on its prostaglandin-E_2-evoked signal transduction (174, 175).

Literature is sparse with respect to the role of the different EP-receptors on chondrocytes. It is known that TNFα and IL-1β can upregulate different EP receptors on cultured cells (176, 177). EP1 and 2 receptors have been suggested to be present on rat growth plate chondrocytes (178, 179) and EP4 receptors have been identified in bovine articular chondrocytes (180). Another study showed that prostaglandin-E_2 contributes to progression of cartilage damage, using an arthritis animal model, at least in part by binding to the EP4 receptor (181). Homozygous deletion of the EP1, EP2, or EP3 receptor did not affect the development of arthritis, whereas EP4 receptor deficient mice showed decreased joint damage including decreased proteoglycan loss, and type II collagen breakdown in cartilage. These data suggest an important role of the EP4 receptor in cartilage degeneration. Nevertheless, a full evaluation of al subtypes of receptors and their role in articular cartilage hasn't been done and certainly not for human chondrocytes.
In our study it was found that adding prostaglandin-E_2 to IL-1β plus TNFα sensitized cartilage resulted in a significant amplification of the adverse effects induced by IL-1β plus TNFα. Whether this effect is exerted by upregulated EP4 receptor expression needs further study.
Remarkably, the addition of prostaglandin-E_2 to cytokine induced cartilage degeneration has a major effect on the release of proteoglycans, both newly formed and resident, whereas proteoglycan synthesis rate was hardly influenced by prostaglandin-E_2 addition. The other way around, when prostaglandin-E_2 production is inhibited (by selective COX-2 inhibition) the adverse effects of IL-1β and TNFα were diminished. Again the most outspoken effects were found on release of proteoglycans and hardly any effect on proteoglycans synthesis was observed. We found a clear correlation between prostaglandin-E_2 production and proteoglycan release whereas the proteoglycan synthesis did not display such a correlation with prostaglandin-E_2 production. The importance of prostaglandin-E_2 in regulation of proteoglycans release is in concordance with recent studies using a selective COX-2 inhibitor (139, 173) where inhibition of COX-2 resulted in normalization of proteoglycan release. It is known that prostaglandin E_2 is capable to inhibit the synthesis of collagens (182). There is also evidence that prostaglandin-E_2 has the capacity to activate matrix metalloproteinasess (MMPs) (183). Prostaglandin-E_2 has been reported to stimulate collagenase gene expression in human synoviocytes (184) and to increase MMP-3 production of osteoarthritic cartilage explants (146). These effects might play a role in the increased proteoglycan release and decreased proteoglycan content as found in the present study. Apparently, under IL-1β/TNFα conditions, prostaglandin-E_2 (regulation) has not a direct effect on proteoglycans synthesis. There have to be other prostaglandin-E_2 independent pathways that regulate chondrocytes proteoglycan synthesis.
Previous studies showed that nitric oxide, being a product of affected chondrocytes as well, exerts a number of effects on synovial cell and chondrocyte functions

which would be expected to promote degradation of cartilage including inhibition of collagen and proteoglycan synthesis (161), activation of MMPs (185), and apoptosis (186, 187). The present study showed that there is a clear negative correlation between the nitric oxide production and proteoglycan synthesis and a postive corrleatin with proteoglycan release. Inhibition of prostaglandin-E_2 by using selective COX-2 inhibition had no effect on the nitric oxide production. There are a number of reports, corroborating this observation, indicating that inhibition of prostaglandin-E_2 production has no influence on nitric oxide production. However, inhibition of nitric oxide (not studied in our set-up) leads to a change in prostaglandin-E_2 production (145, 146, 188). This may explain the correlation of nitric oxide production with proteoglycans release in addition to its correlation with synthesis.

Interestingly, in clinically defined osteoarthritic cartilage, the correlations between prostaglandin-E_2 and proteoglycan release and nitric oxide production and proteoglycan synthesis as found in IL-1β/TNFα modulated cartilage, were not as clear (data not shown). This indicates that the more complex cartilage degeneration (and repair activity in the mean time) becomes, the more intermingled the different pathways of regulation of synthesis and release become.

In summary, this study demonstrates the involvement of prostaglandin-E_2 in disturbing human articular cartilage proteoglycan release. Additional pathways such as nitric oxide production seem needed to modulate proteoglycan synthesis. Further studies are needed to unravel these distinct pathways to provide tools that can modulate cartilage degeneration specifically with respect to synthesis. Although selective COX-2 inhibition displayed chondro-reparative activity (139, 173) this is restricted to inhibition of cartilage matrix proteoglycan release, not to enhancement of proteoglycan synthesis.

Chapter 5

Differential direct effect of COX-1/COX-2 inhibition in human osteoarthritic cartilage.

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Abstract

Objective: NSAIDs in treatment of osteoarthritis (OA) diminish inflammation, and with that mediators involved in cartilage destruction. However, possible adverse direct effects of NSAIDs on cartilage are to be considered, specifically in case treatment is given for a prolonged period of time. Therefore we compared the direct effects of indomethacin, naproxen, aceclofenac, and celecoxib on matrix turnover of human OA cartilage tissue.

Methods: Human clinically defined OA cartilage of 5 different donors was exposed for 7 days in culture to indomethacin, naproxen, aceclofenac, celecoxib, a selection based on COX-2 selectivity. As control, SC-560, a selective COX-1 inhibitor, was used. Changes in cartilage proteoglycan turnover and prostaglandin-E_2 (PGE_2) production were determined.

Results: OA cartilage showed the characteristic proteoglycan turnover. Indomethacin further inhibited proteoglycan synthesis. No significant effect on the proteoglycan release was found; proteoglycan content tended to decrease. Naproxen did not show changes for all parameters. In contrast, aceclofenac and, most outspoken, celecoxib were beneficial for OA cartilage. Both showed an increase in proteoglycan synthesis, and a normalized release. Importantly, both NSAIDs improved proteoglycan content. Inhibition of PGE_2 production indirectly showed that all NSAIDs inhibited cyclooxygenase, the more COX-2 specific, the more pronounced. Selective COX-1 inhibition resulted in adverse effects for all parameters and PGE_2 production was only mildly inhibited.

Conclusions: NSAIDs with low COX-2/COX-1 selectivity show adverse direct effects on OA cartilage, whereas high COX-2/COX-1 selective NSAIDs did not show such effects and even might induce cartilage reparative effects.

Introduction

Osteoarthritis (OA), the most prevalent disease in our society (189), is a slowly progressive degenerative disease characterized by gradual loss of articular cartilage (20). It results in joint stiffness and dysfunction, but the main problem for most patients is pain, loss of function, and social restrictions. Conventional approaches for the treatment of OA range from conservative measures to surgical intervention and eventually joint replacement. Currently there is no curative treatment for the disease. Nonsteroidal antiinflamatory drugs (NSAIDs) are widely used to alleviate the symptoms (190). Clinical efficacy and side-effects with respect to gastro-intestinal problems are mostly well understood (191). Cardiovascular side-effects of the second generation NSAIDs, the selective COX-2 inhibitors, became evident (84, 192). But also for the conventional NSAIDs this always has been a concern (193). It also is controversial as to what (side) effects NSAIDs and selective COX-2 inhibitors may have on articular (osteoarthritic) cartilage tissue.

Direct effects of NSAIDs on cartilage cannot be studied easily in clinical trials and therefore they are generally ignored in clinical practice. Relatively quick beneficial symptomatic effects of NSAIDs on inflammation shade the direct effects, on

cartilage, because (intrinsic) cartilage changes, catabolic and anabolic are rather slow processes.
Direct effects of NSAIDs on cartilage may be important specifically in treatment of joint disease in which inflammation is only mild and secondary as in OA, and when treatment is chronic. Thus, although NSAIDs may be very useful in reducing pain and inflammation in OA, they may enhance the process of cartilage degeneration by interfering with the intrinsic repair activity in case their direct effects are adverse. In that situation these direct effects should be considered in addition to the gastro-intestinal effects and cardio-vascular effects, when prescribing NSAIDs in treatment of OA.
In vitro studies showed that several types of conventional NSAIDs, such as sodium salicylate and indomethacin, inhibited the synthesis of cartilage matrix components, while others, such as aceclofenac and meloxicam, increased matrix synthesis and protected the chondrocytes against apoptosis (67, 134, 194-196). Other NSAIDs, for instance piroxicam, had no effect. Studies in animal models of OA verified that NSAIDs had detrimental or favorable action on OA progression (197-200), although the same NSAID had diverse effects on articular cartilage in different studies depending on the animal model used (199, 200).
With respect to the second generation NSAIDs less data are available. We recently showed a beneficial effect of celecoxib (Celebrex) in normal cartilage explants under the influence of IL-1β and TNFα; on normal healthy cartilage no effects were observed (139). Findings by El Hajjaji *et al.* showed that celecoxib was able to increase proteoglycan synthesis and to diminish proteoglycan release of OA cartilage obtained at joint replacement surgery (140). Recent findings by our group confirmed these data and additionally demonstrated that celecoxib, had a favorable effect on proteoglycan synthesis, retention, release and content of both degenerated (pre-clinical) and (late-stage) osteoarthritic cartilage (173).
NSAIDs inhibit both COX-1 and COX-2 (201). This appears to be associated with the well-known gastrointestinal toxicity: the more COX-1-selective drugs appear to have the tendency to cause more gastrointestinal damage (202). The other way around, a more recent debate has started whether the more COX-2 selective, the more risk for cardio-vascular side effects (84, 192). In the present study it was questioned whether also the direct effects on cartilage by NSAIDs are dependent on their COX-2 (un)selectivity. It could well be that the adverse effects on cartilage of some of the conventional NSAIDs results from inhibition of COX-1.
For this reason, the present study evaluates the *in vitro* effect of several frequently used NSAIDs on human osteoarthritic articular cartilage. Effects of indomethacin and naproxen as non-selective NSAIDs with moderately COX-1 selectivity (201) are compared with effects of aceclofenac (as moderately selective for COX-2 (202)) and the selective COX-2 inhibitor celecoxib, covering a range from COX-1 to COX-2 selectivity.

Material and Methods

Cartilage culture technique

OA cartilage obtained from patients at knee replacement surgery with diagnosed OA was obtained post-operatively. NSAID medication was stopped seven days before surgery, thus no interference of previous medication use is to be expected. Cartilage that appeared full thickness with significant fibrillation was selected (143). Thus in fact the entire joint had a more worse appearance than represented by the cartilage used for evaluation. Cartilage-bone samples were stored at phosphate buffered saline for no longer than 4 hours. Collecting of cartilage was according to the medical ethical regulations of the University Medical Center Utrecht.

Slices of cartilage were cut aseptically as thick as possible from the articular bone surface, excluding the underlying bone, cut into square pieces, weighed aseptically (range 5-15 mg, accuracy ± 0.1 mg) and cultured individually in 96-well round-bottomed microtiter plates (200 μl culture medium, 5% CO_2 in air, 37°C). The culture medium consisted of Dulbecco's modified Eagle's medium (D-MEM), supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin sulfate (100 μg/ml), ascorbic acid (0,085 mM), and 10% heat inactivated pooled human male AB^+ serum. Cartilage was always pre-cultured for 24 hours (wash-out period), after which culture medium was refreshed before start of the experiment.

In addition, 3 tissue samples of each donor were fixed in 4% phosphate buffered formalin for standard light microscopy. Sections were stained with safranin-O fast green-iron haematoxylin and graded for features of OA according to the slightly modified criteria of Mankin (141). The tide mark between cartilage and bone was not present in our cartilage samples and cartilage samples were not covered with pannus. Therefore, the maximum score that could be obtained was 11 instead of the original 14, when all criteria described by Mankin (142) (including pannus, clefts to calcified zone, and tidemark crossed by blood vessels) could have been included.

Experimental setup

Osteoarthritic human articular cartilage tissue was cultured for 7 days in the absence or presence of the following additions: indomethacin (10 μM; Sigma), naproxen (300 μM; Sigma), aceclofenac (0.03 μM; supplied by UCB Pharma Spain) or celecoxib (1 μM; supplied by Pfizer USA). Final concentrations resembled the mean pharmacological plasma concentration of each of the NSAIDs (104, 203, 204). In addition SC-560 (0.1 μM; Sigma), which is an experimental COX-1 inhibitor was added. 0.1 μM guaranties COX-1 selectivity, higher concentrations also inhibit COX-2. After 4 days medium was refreshed and cartilage cultured for a successive 3 days with the same additions. Changes in cartilage matrix turnover (proteoglycan synthesis, -retention, and -release) and matrix integrity (proteoglycan content) were determined. Experiments were repeated 5 times, for each experiment cartilage of another donor was used (n=5).

Proteoglycan analyses

Sulphate incorporation rate was determined, as a measure of the proteoglycan synthesis rate, during the last 4 hours of the first 4-day culture period, as described previously (105). Before addition of $^{35}SO_4^{2-}$ ($Na_2{}^{35}SO_4$, 14.8 kBq/200 μl, DuPont NEX-041-H, carrier-free), culture medium was replaced by equilibrated (CO_2 and temperature) fresh medium. After 4 hour labeling, the cartilage explants were rinsed 3 times for 45 minutes in culture medium under culture conditions and incubated for the 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 before (143). Papain digests were diluted to the appropriate concentrations and glycosaminoglycans (GAGs) were stained and precipitated with Alcian Blue dye solution (144). The pellet obtained after centrifugation (9000g, 10 min.) was washed once (NaAc-buffer containing 0.1 M $MgCl_2$) and subsequently dissolved in sodium dodecyl sulphate (SDS). The $^{35}SO_4^{2-}$ radioactivity of the samples was measured by liquid scintillation analysis. $^{35}SO_4^{2-}$ incorporation was normalized to the specific activity of the medium, labeling time and wet weight of the cartilage samples. Proteoglycan synthesis rate is expressed as percentage change compared to untreated control values.

Release of newly formed proteoglycans as a measure of retention of these proteoglycans was determined similarly. GAGs were precipitated from the medium obtained from day 4 to 7 with Alcian Blue (144). The radio-labeled GAGs were measured by liquid scintillation analysis and normalized to the proteoglycan synthesis rate. Percentage release of newly formed proteoglycans is expressed as percentage change compared to untreated control values.

For the total release of proteoglycans, the GAG in the medium obtained from day 4 to 7 were precipitated and stained with Alcian Blue as described above. The GAG content in the papain digest of cartilage samples, as a measure of proteoglycan content, was analyzed the same way. Blue staining was quantified photometrically by the change in absorbence at 620 nm. Chondroitin sulphate (Sigma C4383) was used as a reference. Values for content were normalized to the wet weight of the cartilage and expressed as percentage change compared to untreated control values. Values for release were normalized to the GAG content of the explants. Percentage release of GAGs is expressed as percentage change compared to untreated control values.

Prostaglandin-E_2 determination

Prostaglandin-E_2 (PGE_2) was determined in culture medium at day 4 by Enzyme Immuno Assay (EIA, Caymann Chemical) and expressed as percentage change compared to control.

Calculations and statistical analysis

Because of focal differences in composition and bioactivity of the cartilage in the knee joint, the results of 10 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 each cartilage of a different donor (n=5) were performed. Statistical evaluation of the effects of a single intervention (*viz.* NSAIDs) compared to the untreated cartilage of the same donors was performed with a non-parametric test for paired data (Wilcoxon). For statistical evaluation of differences between different interventions the percentage change compared to untreated cartilage of the same donors was calculated. The effects of different treatments were compared with a non-parametric test for unpaired data (Mann-Whitney). *P* values less than or equal to 0.05 were considered statistically significant.

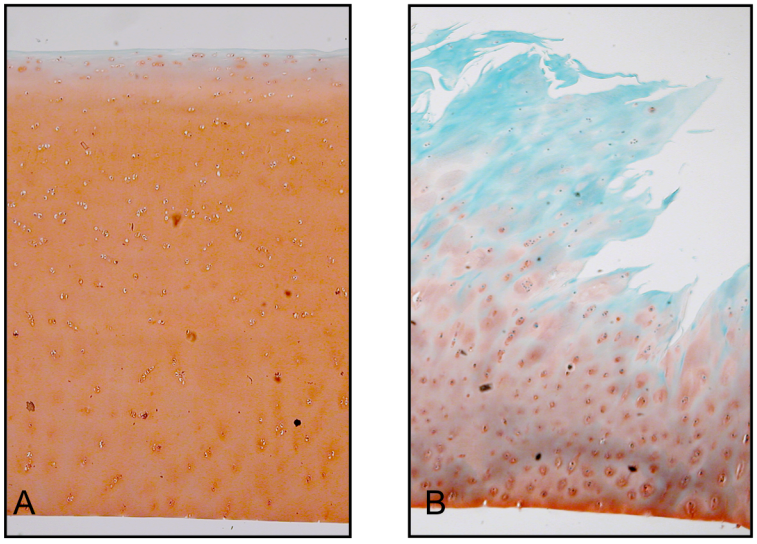

Figure 1. *Normal healthy and osteoarthritic cartilage histology.*
Representive light micrographs of condylar cartilage obtained *post-mortem* from joints with normal healthy cartilage (A) and cartilage obtained at joint replacement surgery (B). Sections are stained with safranin-O fast green-iron haematoxylin and graded for features of OA according to the slightly modified criteria described by Mankin et al., scores for the depicted samples are 0 and 7, respectively (see appendix).

Results

Effects of selective vs. non-selective NSAIDs on osteoarthritic cartilage.

The osteoarthritic cartilage of the different donors used had on average a modified Mankin score of 5 ± 1. It should be kept in mind that only the cartilage that could be cut from the joint surfaces after replacement surgery was used. Thus in fact the entire joint had a worse appearance than represented by the modified Mankin score of the cartilage used. The OA cartilage surface deterioration was clearly visible by light microscopy. An example of a severely affected cartilage tissue explant is depicted in figure 1B, to be compared with normal healthy cartilage as depicted in figure 1A. The latter was obtained from a healthy joint (post mortem) not used for the present study. The safranin O staining was lost from the surface

layer of the osteoarthritic samples and chondrocyte distribution was disturbed (clusters of chondrocytes in the surface layer of the cartilage were visible; figure 1B).

Table 1. *The histological and biochemical characteristics of baseline values of human osteoarthritic cartilage for the different treatment groups.*

	indomethacin (10 μM)	naproxen (300 μM)	aceclofenac (0.03 μM)	celecoxib (1 μM)
age (years)	70 ± 2	67 ± 3	63 ± 2	70 ± 3
sex (female/male)	4/1	3/2	4/1	4/1
histological cartilage damage (Mankin grade)	3 ± 1	4 ± 1	6 ± 2	5 ± 1
PG synthesis rate (nmol/h.g)	6.8 ± 2.3	3.4 ± 1.9	4.8 ± 0.9	5.5 ± 1.8
% new PG release (%)	11 ± 2	13 ± 2	12 ± 2	11 ± 2
% total release (%)	9 ± 2	7 ± 2	5 ± 1	6 ± 1
PG content (mg/g)	22 ± 2	19 ± 1	17 ± 6	19 ± 4

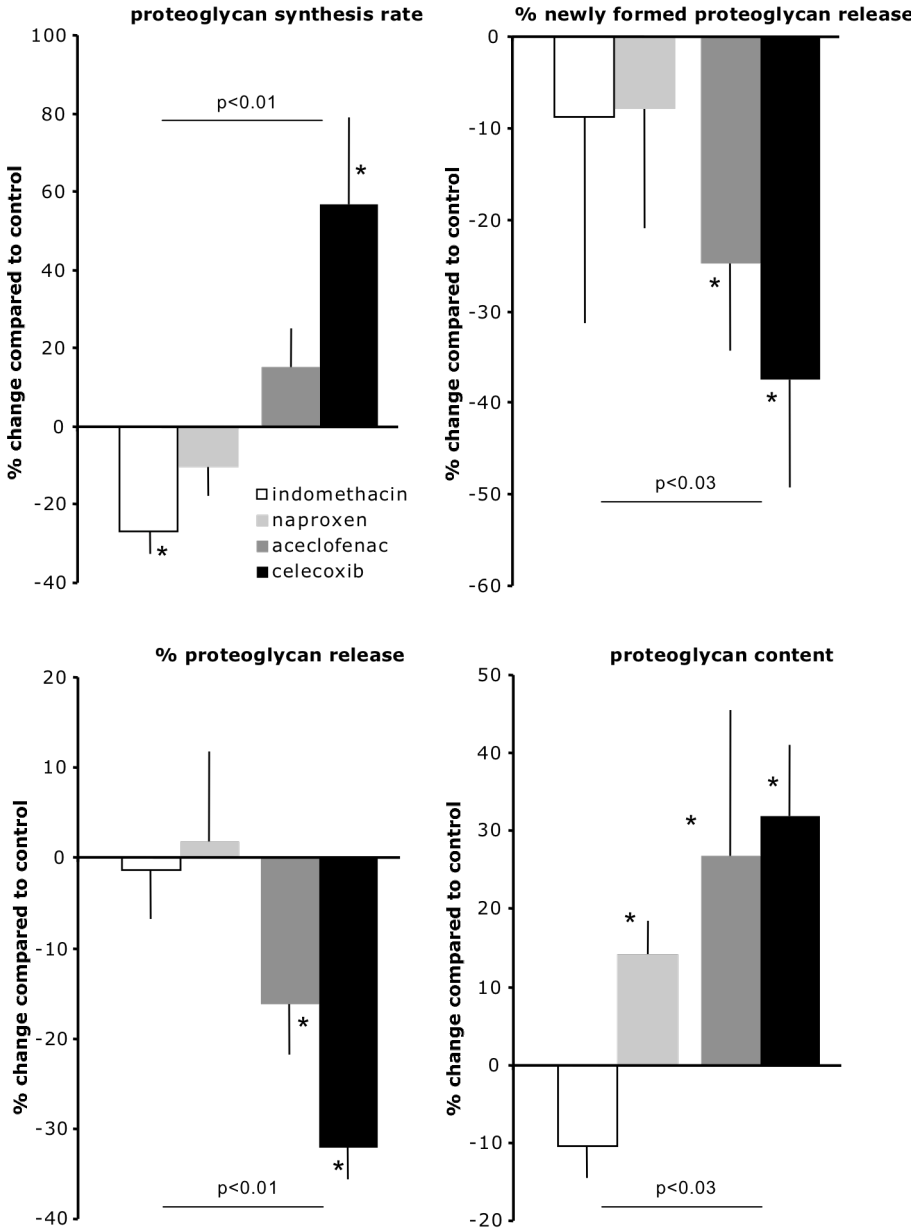

Figure 2a. *Effects of four NSAIDs on PG turnover and content of OA cartilage.*
Percentage change, compared to untreated osteoarthritic cartilage of the same donor, of proteoglycan synthesis rate as measure of cartilage matrix synthesis (pg synthesis), percentage release of newly formed proteoglycans (new pg release) as a measure of retention for the newly formed proteoglycans (normalized to the synthesis of these proteoglycans), percentages total release of proteoglycans (total pg release), measured by the percentage release of glycosaminoglycans (GAG; normalized to the GAG content), and proteoglycan content (pg content) are depicted. White bars represent effects of indomethacin (10 μM); light grey bars represent naproxen (300 μM), dark grey bars represent aceclofenac (0.03 μM) and black bars represent celecoxib (1 μM). The results are presented as means of 5 experiments (with each cartilage of a different donor) ± SEM. Statistically differences for the effect of an NSAID compared to OA cartilage of the same donors is calculated by non-parametric paired analysis (p-value <0.05; asterisks indicate statistical difference). Difference between percentage changes for the two non-selective NSAIDs compared to the (more) selective NSAIDs (n=10 *vs.* n=10) is calculated by non-parametric non-paired analysis (p-value are given for each of the parameters).

The osteoarthritic cartilage showed the typical basal biochemical features with respect to proteoglycan turnover (table 1), a low proteoglycan synthesis, a high proteoglycan release, both newly formed proteoglycans and resident proteoglycans, and a diminished proteoglycan content. Baseline data between the four groups were not statistically significant different. Data obtained from healthy cartilage (n=5; age 68 ± 5 years) from femoral condyles given as a reference are: histological grade, 0.7 ± 0.1; PG synthesis rate, 12.5 ± 1.1 nmols/h/g; % new PG release, 7.1 ± 0.5 %; total PG release, 3.9 ± 0.5 %; and PG content, 29.2 ± 3.4 mg/g. This cartilage was obtained post mortem from donors without any history of joint disorders and treated the same way during the similar time period.

Indomethacin decreased proteoglycan synthesis of the OA cartilage (-27 ± 6 % compared to untreated control OA cartilage; $p<0.05$; figure 2a, white bars). No significant effect was found on the proteoglycan release, both newly formed and resident proteoglycans. There was a tendency towards a decrease of proteoglycan content (on average -11% ± 4 %). Naproxen had no significant effects on the proteoglycan turnover of OA cartilage, although there was a tendency to behave like indomethacin. Remarkably, naproxen showed a slight but statistically significant increase in proteoglycan content (14 ± 4 %; $p<0.05$; figure 2a light grey bars).

In contrast to indomethacin and naproxen, aceclofenac, more selective for COX-2 inhibition (202), showed improvement of all parameters (figure 2a dark grey bars). The proteoglycan synthesis was on average increased by 15 ± 10 %, although not statistically significant. However, this increased synthesis was combined with an improved retention of these newly formed proteoglycans, as reflected by the diminished newly formed proteoglycan release (-25 ± 10 %; $p<0.05$). This was also the case for the total proteoglycan release, which was reduced (-16 ± 6 %; $p<0.05$). More importantly, aceclofenac improved the proteoglycan content on average by 27 ± 19 % ($p<0.05$).

The most selective COX-2 inhibitor of the four tested, celecoxib, showed even a stronger improvement of the proteoglycan parameters compared to untreated controls (figure 2a black bars). Proteoglycan synthesis increased 57 ± 22 % ($p<0.05$), while the release of those newly formed proteoglycans reduced with 38 ± 12 % ($p<0.05$). Comparable reduction was found for the total proteoglycan release (-32 ± 4 %; $p<0.05$). With respect to matrix integrity celecoxib was able to improve the proteoglycan content by 32 ± 9 % ($p<0.05$).

When the effects of aceclofenac and celecoxib were compared to those of indomethacin and naproxen, the beneficial effects were statistically significant different from the adverse effects for proteoglycan synthesis, - retention, - release and - content (figure 2a).

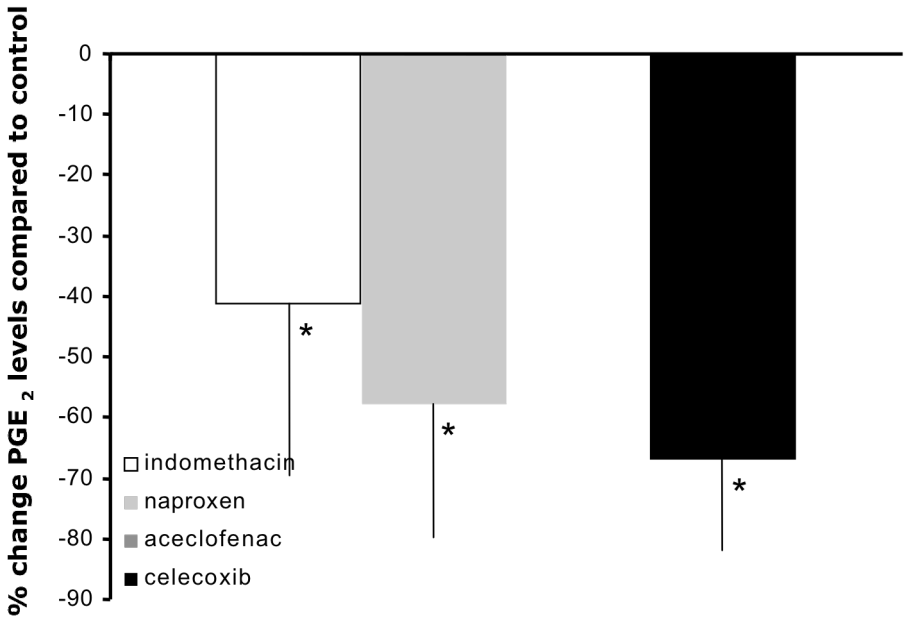

Figure 2b. *Effects of three of the four NSAIDs on PGE_2 production of OA cartilage*
Percentage changes in prostaglandin E_2 levels in culture supernatants of osteoarthritic cartilage treated with different NSAIDs are depicted. Mean values (n=5 ± SEM) are presented for indomethacin (open bar), naproxen (light grey bar), and celecoxib (black bar). Effects of aceclofenac were not measured. Statistically differences of the effects of the different NSAIDs compared to untreated controls were calculated by non-parametric paired analysis (p-value <0.05) and are marked with a asterisk.

All NSAIDs inhibited prostaglandin-E_2, as indirect measurement of COX inhibiting activity (on average more than 60% inhibition for all compounds compared to untreated controls; $p<0.05$; figure 2b). However, there was a tendency that the more selective the NSAID for COX-2 the more pronounced the inhibition of PGE_2. Unfortunately, the culture media from the aceclofenac samples could not be analysed.

The effects of SC-560 on osteoarthritic cartilage
Because it appeared that the absence of selectivity for COX-2 inhibition of the NSAID resulted in no or even adverse direct effects on cartilage we studied the effect of an experimental selective COX-1 inhibitor as well. In these experiments the average age of donors used was 73 ± 3 years and they were all female. The average modified Mankin grade of these donors was 6 ± 1. These donors did not differ statistically significant from the other OA donors for any of the parameters depicted in table 1.
When SC-560 was added to the OA cartilage cultures an inhibition of proteoglycan synthesis (-10 ± 9 % compared to untreated control; $p<0.05$, figure 3) was found. This was accompanied with an enhanced release of newly formed proteoglycans (10 ± 10 % compared to untreated control; $p<0.05$). There was no

statistically significant change in total proteoglycan release. The inhibition in synthesis and retention did not lead to a further statistically significant reduction in proteoglycan content, although there was a tendency towards a decrease in PG content. Inhibition of prostaglandin E_2 production was relatively mild compared to the NSAIDs, on average by 30 ± 15 % ($p<0.05$). The effects from this selective COX-1 inhibitor were not statistically different from those of the non-selective NSAIDs but were for all parameters statistically different from the (more) COX-2 selective NSAIDs (data not shown).

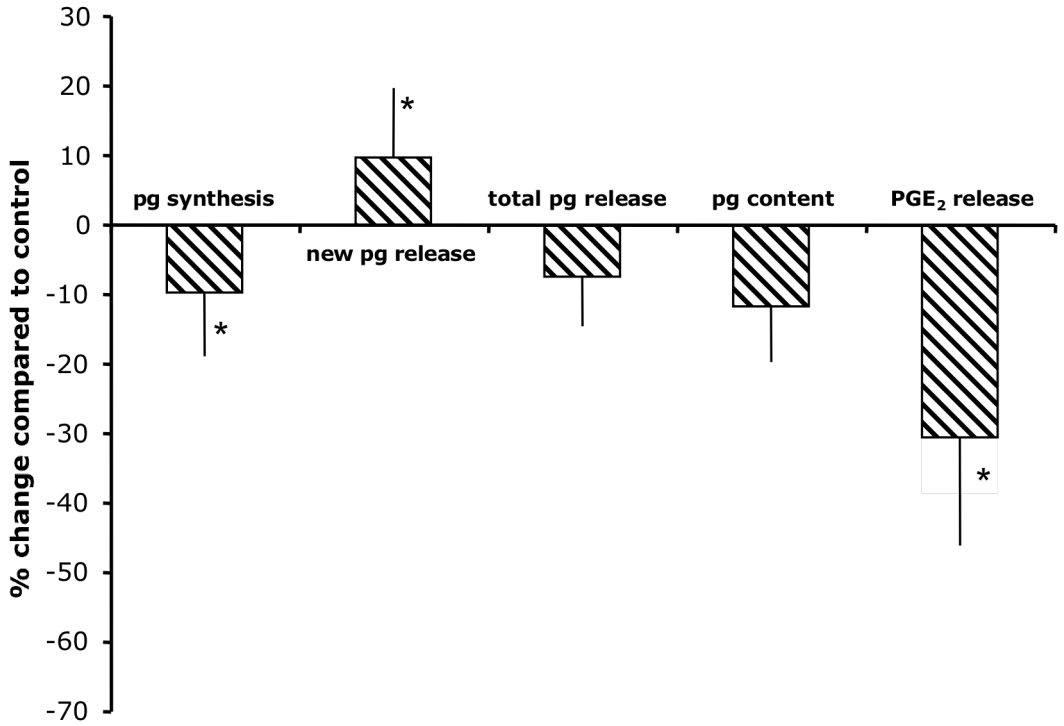

Figure 3. *Effect of a selective COX-1 inhibition in OA cartilage*
Percentage change compared to healthy cartilage of proteoglycan synthesis rate as measure of cartilage matrix synthesis (pg synthesis), percentage release of newly formed proteoglycans (new pg release) as a measure of retention for the newly formed proteoglycans (normalized to the synthesis of these proteoglycans), percentages total release of proteoglycans (total pg release), measured by the percentage release of glycosaminoglycans (GAG; normalized to the GAG content), proteoglycan content (pg content), and prostaglandin E_2 release (PGE_2 release) under the influence of 0.1 μM SC-560, a selective COX-1 inhibitor, are depicted. The results are presented as means of 5 experiments with each cartilage of a different donor ± SEM. The absolute values of untreated controls are: PG synthesis rate, 3.4 ± 1.3 nmols/h.g; % new PG release, 11.4 ± 0.6 %; total PG release, 6.4 ± 1.1 %; and PG content, 17.9 ± 0.8 mg/g. Statistically differences SC-560 treated and untreated cartilage calculated by non-parametric paired analysis (p-value <0.05; asterisks) are given.

Discussion

The purpose of this study was to evaluate the effect of frequently used NSAIDs on human osteoarthritic articular cartilage *in vitro*. There was an emphasis on possible differences between conventional non-selective COX-2 inhibitors and the more

selective COX-2 inhibitors, as recently classified by Warner *et al*. (201). It appeared that COX-2 selectivity resulted in cartilage reparative properties, whereas the absence of COX-2 selectivity even resulted in negative effects. The adverse effects of an experimental COX-1 selective compound underscore the latter.

The direct negative effects of indomethacin are mainly reflected by an inhibition of proteoglycan synthesis and diminished retention of these newly formed proteoglycans. This is in accordance with previous reports that studied frequently used NSAIDs. Indomethacin, naproxen, and ibuprofen, tested under comparable *in vitro* conditions, are known to inhibit the synthesis of cartilage proteoglycans (65-67, 134) and to increase the release of proteoglycans (67, 134). In addition, indomethacin was found to have deleterious effects on articular cartilage of both left and right knees in OA rats induced by injections of sodium iodoacetate in the right knee (134). Also naproxen showed adverse effects in an *in vivo* study using the ACLT canine model of OA (200). This study showed increased water content of cartilage due to naproxen. However, a different ACLT canine study showed that naproxen was able to significantly suppress the decrease in proteoglycan content and metalloproteinase activities in knee articular cartilage (199). In our hands naproxen did not show a pronounced adverse effect on osteoarthritic cartilage *in vitro* as found before, although there was a tendency.

With respect to the direct effects of selective COX-2 inhibitors we recently showed a beneficial effect of celecoxib in normal cartilage under the influence of IL-1β and TNFα, inducing degenerative changes; on normal healthy cartilage no effects were observed (139). Findings by El Hajjaji *et al*. showed that celecoxib was able to increase proteoglycan synthesis and to diminish proteoglycan release of OA cartilage obtained at joint replacement surgery (140). Recent findings by our group confirmed these data and additionally demonstrated that celecoxib had a favorable effect on proteoglycan synthesis, retention, release and content of both degenerated (pre-clinical) and (late-stage) osteoarthritic cartilage (173). Remarkably, the effects of aceclofenac, a derivate of diclofenac, were similar to celecoxib, suggesting a similar mechanism of action. Based on the classification of Warner *et al*. (202) diclofenac has a preference for COX-2, from this and our results we assume that aceclofenac has a comparable selectivity. The metabolism of aceclofenac differs from that of diclofenac and is human-specific(205). The main metabolite of aceclofenac is 4-hydroxy-aceclofenac. The other metabolites, namely diclofenac and 4-hydroxy-diclofenac, correspond to only 5% of the administered dose (203). Aceclofenac, either by acting directly on the production of cytokines that induce cyclooxygenase in the inflamed tissues (206), or by its preferential intracellular conversions into COX(-2) active metabolites (207, 208), or most likely by both processes at the same time, acts as a functional inhibitor of prostaglandin-E_2 (PGE_2) production (208).

The experimental selective COX-1 inhibitor SC-560 showed similar effects as indomethacin, indicating that inhibition of COX-1 results in an adverse effect on

proteoglycan synthesis and retention. In contrast, when COX-2 is selectively inhibited in OA cartilage we found a beneficial effect with respect to proteoglycan turnover. These results imply an important role for COX-2 in the disturbed proteoglycan turnover in OA whereas COX-1 is involved in a more physiological role in the chondrocytes. This is in concordance with the general accepted thought that COX-1 is regarded as the "housekeeping" isoform of cyclooxygenase and has clear physiological functions. Its activation leads, for instance, to the production of prostacyclin, which when released by the endothelium, is antithrombogenic (209), and when released by the gastric mucosa is cytoprotective (209). In contrast, COX-2 is excessively induced under inflammatory and detrimental conditions, such as OA. This established concept has been modified by recent investigations demonstrating a significant participation of prostaglandins derived via the COX-1 pathway in some inflammatory processes (210-212), especially pain. Also the recent development with respect to cardiovascular side effect in relation to selective COX-2 inhibitors, and NSAIDs in general, forces us to reconsider the current concept. Nevertheless, in the case of proteoglycan turnover in osteoarthritic cartilage the concept apparently still holds true.

COX-2 is found to be expressed in OA tissues. The expression of COX-2 and PGE_2 in OA meniscus, synovial membrane, osteophytic fibrocartilage and in the articular OA cartilage has been described (137). However, when we selectively inhibited COX-1, thereby inhibiting only a relatively small amount of PGE_2, the proteoglycan turnover get worse, especially proteoglycan synthesis. This indicates that COX-1 inhibition results in alteration of products formed by COX-1, but independent of PGE_2 that influence the proteoglycan turnover negatively. Notwithstanding, the upregulation of PGE_2 in OA cartilage, together with the beneficial effects of COX-2 inhibition implies an important role for COX-2 in osteoarthritic cartilage, support the use of selective COX-2 inhibitors in treatment of OA.

However, other effects of NSAIDs, COX independent, might be involved as well (213). Several studies have demonstrated that certain NSAIDs, such as ibuprofen, cause anti-inflammatory effects independent of cyclooxygenase activity and prostaglandin synthesis inhibition (214-216). These effects are mediated through inhibition of certain transcription factors such as NF-κB and AP-1 (217-219). The respective NSAIDs might interfere directly with the transcription factors, but their effects are probably mediated predominantly through alterations of the activity of cellular kinases such as IKKβ, Erk, p38, or MAPK (220). These effects are not shared by all NSAIDs, since indomethacin failed to inhibit NF-κB and AP-1 activation as well Erk activity (218, 221, 222). In contrast, indomethacin is able to activate PPARγ, which is not sensitive for sodium salicate or aspirin (223).

Importantly, we discussed solely the direct effects of NSAIDs on cartilage. These effects should be seen in the context of the significant anti-inflammatory effects of these NSAIDs. By inhibiting joint inflammation they may indirectly be beneficial to cartilage, specifically when inflammation is primary in the cause of cartilage damage as e.g., in rheumatoid arthritis. However, in case of OA, where

inflammation may contribute but is not primarily responsible for cartilage damage, adverse direct effects of NSAIDs on cartilage during long-term treatment may have important impact on long-term outcome.
In conclusion, although *in vitro* findings, the present study suggests that besides the anti-inflammatory and analgesic characteristics of selective COX-2 inhibitors, their gastro-intestinal as well as their cardiovascular side effects, direct (side) effects of these NSAIDs on cartilage should also be considered in the choice of NSAID during chronic treatment of joint diseases such as OA.

Chapter 6

The canine 'groove' model of osteoarthritis is more than simply the expression of surgically applied damage.

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Abstract

Objective: Recently a new canine model of osteoarthritis (OA; the 'groove' model) has been described. This model is based on surgically applied mechanical damage of the articular cartilage followed by transient forced loading of the affected joint. Ten weeks after surgery this model shows characteristics of OA, mimicking human OA. To establish whether the observed characteristics of degeneration in this model represent the surgically applied damage, or are the results of progressive features of OA, we evaluated this 'groove' model shortly after surgery.

Methods: In 20 female Beagle dogs, articular cartilage of the weight bearing areas of the femoral condyles in the right knee was damaged without affecting the underlying bone. After surgery dogs were let out on a patio 5 days/week for 4 hrs/day. Three days/week, the dogs were forced to load the experimental joint by fixing the contralateral control limb to the trunk. The severity of OA was evaluated at 3 (n=10) or 10 weeks (n=10) after surgery. Synovial inflammation, cartilage damage and cartilage matrix turnover were determined.

Results: Ten weeks after surgery osteoarthritic features were found, as described previously. Proteoglycan synthesis, percentage release of newly formed proteoglycans, and that of total amount of proteoglycans were enhanced, whereas proteoglycan content was significantly diminished (all $p<0.05$). Importantly, three weeks after surgery these characteristics of OA were not yet evident.

Conclusions: The present results clearly show that the characteristics observed 10 weeks after induction of joint degeneration in the groove model are not just the expression of the surgically applied damage but are the result of progressive features of (experimental) OA.

Introduction

Osteoarthritis (OA) is a degenerative joint disease, which is characterized by damage of articular cartilage and changes in the subchondral bone and is frequently accompanied by secondary synovitis (2, 14). The disease progresses slowly and develops over many years (224). Age has been identified as one of the main risk factors (26). Most often, OA is diagnosed at a relatively late stage of the disease since, apart from symptoms, no early markers are available. Ideally, therapies to prevent progression of the disease should be applied early in the course of OA. For this reason studies on early changes in OA are important. During further development of markers to detect the early changes in the process of human OA, animal models may be helpful. Several animal models of OA have been developed to study biochemical and histological changes associated with joint degeneration in early stages of OA *in vivo* (225-227).

Recently a new canine model of OA (the 'groove' model), has been described (228, 229). In this canine model, damage to the articular cartilage of the weight-bearing areas of the femoral condyles in one knee, not damaging the subchondral bone and not causing joint instability, is the trigger for development of OA. To strengthen this trigger for development of OA, loading of the affected joint is

forced/intensified, by fixing the contralateral control limb to the trunk of the dog temporarily. Biochemical and histological evaluation showed degenerative changes in the joint, which closely resembles those in the anterior cruciate ligament transection (ACLT) model, which is one of the most frequently used (canine) models of OA (230-235), both models mimicking human early OA very well (228, 229).
In the groove model (228, 229) at 10 weeks, collagen was damaged and proteoglycan turnover was disturbed: an ineffective synthesis combined with an enhanced release resulted in a diminished proteoglycan content. Matrix metalloproteinase activity measured in synovial fluid was enhanced. Histologically, moderate cartilage destruction, characterized by loss of safranin-O staining, fibrillation of the articular surface and chondrocyte clustering was evident. These characteristics of OA were also clearly visible in the tibial plateau, although this cartilage was not harmed during surgery. All changes were very consistent between animals. Most important, only mild signs of inflammation were present as demonstrated histologically.
In the canine groove model the degenerative changes in the cartilage matrix integrity are slowly progressive over time in the first year after induction, while synovial inflammation decreases (229). Whether the slowly progressive phase in the first year after the initial induction phase will proceed into full blown osteoarthritis in several years, as demonstrated for the ACLT model (236), needs still to be demonstrated. Although the changes in the groove model are slightly progressive over time in the first year of follow-up, it might be disputed whether the observed changes are actual characteristics of osteoarthritis or merely the result of the surgery applied cartilage damage. Therefore, in the present study we evaluated cartilage damage in this 'groove' model shortly after surgical procedures, and compared it to the changes observed 10 weeks after induction.

Materials and Methods

Animals

Female Beagle dogs, mean age 2.4 ± 0.3 years, weighing 10-15 kg, were obtained from the animal laboratory of Utrecht University, the Netherlands. They were housed in groups of 2 to 3 dogs per pen, and were let out on a patio in large groups for at least 2 hours daily. They were fed a standard diet and had water *ad libitum*. The dogs were divided in 4 groups of 5 animals each. The study was approved by the Utrecht University Medical Ethical Committee for animal studies.

Anaesthesia, general surgery, and post-surgical treatment

After induction with Nesdonal, the dogs (in groups of 5) were anaesthetized with halothane in a mixture of oxygen and nitrous oxide. Surgery was carried out through a 2 to 2.5 cm medial incision close to the *ligamentum patellae* in one knee. Care was taken to prevent bleeding and soft tissue damage as much as possible. After surgery, synovium, fasciae and skin were sutured. The contra-lateral

unoperated knee served as a control. The animals received analgesics (Buprenorphine 0.01 mg/kg) and antibiotics (Amoxicyclin 400 mg/kg) during the first 3 days after surgery. Starting 2 days after surgery, the dogs were let out daily on the patio, again. At the end of the experiment, the dogs were euthanized with an intravenous injection of Euthesate (Na-Pentobarbital). Both hind limbs were amputated and synovium and cartilage were collected and processed within 2 hours. Procedures were carried out under laminar flow conditions.

Groove model

In all dogs (n=20), the cartilage of the right lateral and medial condyles was damaged with a Kirschner-wire (1.5 mm diameter) that was bent 90° at 0.5 mm from the tip. This ensures that depth of the grooves was restricted to around 0.5 mm. In utmost flexion, ten longitudinal and diagonal grooves were made on the weight-bearing parts of the femoral condyles without damaging the subchondral bone (228, 236). The latter was checked by histology at the end of the experiment. There was no absolute visual control over the procedure, but post mortem macroscopic evaluation of the knee joints showed similar patterns in all affected knees. To ensure (intensified) mechanical loading of the affected knee, the dogs were forced to load the experimental joint intermittently by fixing the contralateral limb to the trunk for approximately 4 hours per day, 3 days per week, for 3 weeks (two groups of 5 animals) and 10 weeks (2x5 animals), respectively. This is less than 10% of the total loading time of the affected joint. Thus, the control limb was not weight bearing for less than 10% of the time. We demonstrated that the control limbs of these animals did not differ from those of animals from which the limbs were not fixed to the trunk (228). The severity of OA was evaluated at 3 (n=10) or 10 weeks (n=10) after surgery.

Synovial tissue analysis

Three infra-patellar synovial tissue samples per joint (medial middle and lateral) were fixed in 4% phosphate-buffered formalin (pH 7.0) and embedded in paraffin. Deparaffined sections were stained with hematoxylin-eosin. The histological sections were examined separately in random order and independently by two observers who were not aware of the source of the synovium. Each specimen was analysed to determine the degree of inflammation, using the slightly modified (237) criteria described by Goldenberg and Cohen (238). For assessing the overall grade, three specimens from each knee were considered as a unit.

Cartilage analysis

Cartilage samples for histological and biochemical analyses were obtained from predetermined locations on the weight-bearing areas of the femoral condyles and the tibial plateau of both experimental and control joints. The locations were identically paired with the same location in the contralateral joint (228). Cartilage was cut as thick as possible, while excluding the underlying bone. Samples were

cut into full-thickness cubes, kept in 200 μl culture medium (Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.085 mM ascorbic acid, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated Beagle serum), and weighed (3-10 mg; accuracy 0.1 mg).
For histology, four samples from tibial plateau and four from femoral condyles from each knee were fixed in 4% phosphate-buffered formalin containing 2% sucrose (pH 7.0). Cartilage degeneration was evaluated in safranin-O-fast-green iron hematoxylin-stained sections by light microscopy according to the slightly modified (143) criteria of Mankin (142). The tide mark between cartilage and bone was not present in our cartilage samples since bone was not included. Also as a result of our dissection method, cartilage samples were not covered with pannus. Therefore, the maximum score that could be obtained was 11 instead of the original 14, when all criteria described by Mankin (including pannus, clefts to calcified zone, and tidemark crossed by blood vessels) could have been included. With respect to staining, paired control and experimental samples (of the same animal) were stained in the same safranin-O baths. The samples of the 5 animals of each of the four groups, were stained in the same assay.
Specimens were graded in random order by two observers unaware of the source of the cartilage. The average of the four specimens was used for statistical evaluation.
For biochemical analysis, the cartilage samples were cultured individually in 96-well culture plates (NUNCLON®, Denmark) in 200 μl culture medium. Cartilage explants were cultured according to standard procedures as described previously (143, 228). For femoral condyles and tibial plateau, cartilage proteoglycan (PG) content, PG synthesis, PG retention, and PG release were determined and averaged for 6 explants per parameter (239).

Proteoglycan synthesis.
As a measure of PG synthesis, the rate of sulphate incorporation was determined ex vivo (105). After 1 hour of pre-culture, 148 kBq $Na_2{}^{35}SO_4$ (Dupont, NEX-041-H, carrier free) in 10 μl DMEM was added to each sample. After 4 hours, the cartilage samples were washed with cold PBS and digested with papain for 2 hours at 65°C. Glycosaminoglycans (GAGs) were precipitated by addition of cetylpyridium chloride (CPC), and $^{35}SO_4^{2-}$ -labelled GAGs were measured by liquid scintillation analysis. The total sulphate incorporation rate of each cartilage sample was calculated using the specific activity of the medium and was normalized to time and wet weight of the explants. Synthetic activity is expressed as nmoles of sulphate incorporated per hour per gram wet weight of the cartilage (nmol/h.g).

Proteoglycan release
For determination of the release of the newly synthesized PGs as a measure for retention of these PGs, the release of $^{35}SO_4^{2-}$ -labelled PGs in the medium was determined. After labelling (see above) the cartilage samples were rinsed 3 times

for 45 minutes in 1.5 ml culture medium and then incubated in 200 μl fresh culture medium without sulphate label for 3 days. Thereafter the samples were washed with cold PBS and GAGs were precipitated from the medium and were stained with Alcian Blue dye solution, as described previously (105). The $^{35}SO_4^{2-}$-labelled GAGs were measured by liquid scintillation analysis and the release was normalized to the specific activity of the medium and the wet weight of the explants. The release of newly formed PGs is normalized to the total amount of newly synthesized PGs and expressed as percentage release of newly formed PGs in the three days (% new PG release).
For the total release of PGs, Alcian blue staining of the medium was quantified photometrically with chondroitin sulphate (Sigma C4384) as a reference. The total amount of GAGs released (blue staining) is expressed as a percentage of the original tissue content (% GAG release)

Proteoglycan content
As a measure of PG content of the cartilage samples the amount of GAG was determined as described previously (106). The GAGs in the papain digest of cartilage samples were precipitated and stained with Alcian Blue as described above. Blue staining was quantified photometrically by the change in absorbence at 620 nm. Chondroitin sulphate (Sigma C4383) was used as a reference. Values were normalized to the wet weight of the cartilage explants (mg/g).

Calculations and statistics
Delta (histology) or percentage (proteoglycan turnover) change in mean values ± SEM (n=10 animals) of femoral and tibial cartilage of experimental knees compared to control knees respectively, are presented. The paired Student's t-test was used to compare data of the experimental and contralateral control joints within each group and the unpaired t-test was used to analyse differences between the two groups of animals with different follow-up. P values less than 0.05 were considered statistically significant.

Results

Cartilage damage
Three weeks after induction of experimental osteoarthritis, the affected knees clearly showed macroscopic damage of the articular cartilage of femoral condyles. No damage was found on the condylar cartilage of the control knees. Damage, although less pronounced, was also found on the tibial plateau. The macroscopic changes at 3 weeks appeared (not objectivated) less severe when compared to those seen 10 weeks post-surgery, both for femoral condyles and tibial plateau.
These macroscopic observations were confirmed by histological analysis (figure 1, table 1 and table 2). In the 3 weeks post-surgery group the average modified Mankin score of the cartilage degeneration in the experimental femoral condyles was mild but significantly different from that of the contra-lateral control joints

(table 1). The grooves were clearly visible as depicted by a representative micrograph in figure 1 top-left. On average minor but statistically significant cartilage degradation of the experimental tibial plateau cartilage was seen in the 3 weeks post-surgery group (compared to the contra-lateral control; figure 1

Table 1. *Histological and biochemical characteristics of experimental and contra-lateral control joints in the groove model, 3 and 10 weeks post-surgery*

	3 weeks post-surgery			10 weeks post-surgery		
	control	experimental	*p*<	control	experimental	*p*<
Histological cartilage damage (modified Mankin grade)						
condyles	0.4 ±0.1	2.1 ±0.3	*0.005*	0.3 ±0.1	3.4 ±0.3	*0.005*
plateau	0.8 ±0.2	1.4 ±0.2	*0.005*	1.3 ±0.02	2.5 ±0.4	*0.02*
Histological synovial inflammation (Goldenberg and Cohen grade)						
joint	1.2 ±0.3	2.5 ±0.2	*0.01*	0.7 ± 0.2	2.0 ±0.3	*0.01*
Proteoglycan synthesis (nmol/h.g)						
condyles	5.7 ±0.4	8.8 ±1.6	*0.03*	9.3 ±0.9	17.1 ±1.8	*0.003*
plateau	3.8 ±0.3	5.9 ±0.9	*0.01*	7.2 ±0.5	11.0 ±1.1	*0.03*
Percentage release new proteoglycans (%)						
condyles	9 ± 1	9 ± 1	*ns*	11 ± 1	19 ± 3	*0.005*
plateau	18 ± 2	23 ± 2	*0.005*	16 ± 1	20 ± 1	*0.005*
Percentage release of total proteoglycans (%)						
condyles	4 ± 1	4 ± 1	*ns*	7 ± 1	13 ± 1	*0.005*
plateau	14 ± 1	13 ± 1	*ns*	12 ± 1	16 ± 1	*0.005*
Proteoglycan content (mg/g)						
condyles	38 ± 3	37 ± 2	*ns*	49 ± 3	39 ± 1	*0.005*
plateau	37 ± 3	37 ± 2	*ns*	49 ± 3	42 ± 2	*0.005*

The absolute values of both condyles and tibial plateau are given for contra-lateral control knees and experimental knees (mean ± SEM). Histological cartilage damage was graded according to modified criteria of Mankin (maximum score of 11). Synovial inflammation had a maximum score of 10 and was graded according to the modified criteria of Goldenberg and Cohen. Proteoglycan synthesis was measured by ^{35}S-sulfate incorporation (nmol/h.g). % New PG release: release of the newly formed PGs normalized to PG-synthesis (%) as a measure of retention of the newly formed PGs. % total release: release of total amount of PGs in 3 days normalized to PG-content of the cartilage (%). Glycosaminoglycan content was determined as measure of PG-content (mg/g)

Figure 1.
Representative light micrographs of condylar cartilage (top), tibial cartilage (middle;safranin-O stained) and synovial tissue (bottom; Hematoxylin-Eosine stained) 3weeks (left) and 10 weeks (right) post-surgery. Control joint (controls, left side) at 3 weeks and controls, right side at 10 weeks, had for cartilage a modified Mankin grade 0 and for synovial tissue a modified Goldenbeg and Cohen grade of 0. The experimental joint had at 3 weeks a modified Mankin grade 4 and 1 for condylar and plateau cartilage, respectively and for synovial inflammation a score of 3. The experimental joint at 10 weeks had a modified Mankin grade 5 and 4 for condylar and plateau cartilage, respectively and for synovial inflammation a score of 3 (see appendix).

middle-left and table 1). At 10 weeks post-surgery histological cartilage damage was more severe as observed in both femoral condyles (figure 1, top-right) and the tibial plateau (figure 1, middle-right). On average this was statistically significant for both cartilage surfaces of the experimental joints (table 1). Most important paired analysis, as depicted in figure 2, left panel, the delta change in histological cartilage damage (experimental compared to the contra-lateral joints), of both femoral and tibial cartilage, was statistically significantly less severe in the 3 weeks post-surgery group than in the 10 weeks post-surgery group. The structural changes and the cellularity of the cartilage, contributed the most to this histological difference between 3 and 10 weeks (table 2).

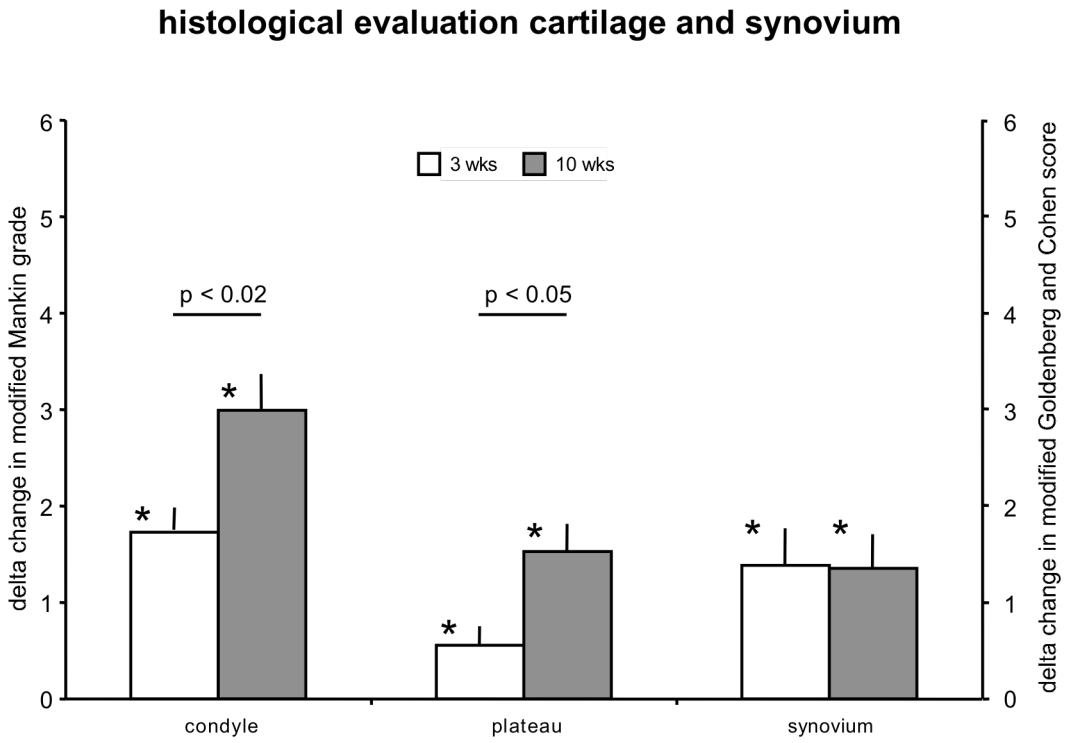

Figure 2.
Average change in histologically determined cartilage damage (modified Mankin score) for femoral condyles and tibial plateau (left y-axis) and average change in synovial tissue inflammation (modified Goldenbeg and Cohen score; right y-axis), both calculated as delta change between experimental joint compared to contra-lateral control joint for each animal. Open bars represent the mean values ± SEM of cartilage damage 3 weeks post-surgery (n=10) and solid bars represent the mean values ± SEM of cartilage damage 10 weeks post-surgery (n=10). Asterisks indicate statistically significant ($p<0.05$) change compared to contra lateral controls. Statistically significant differences between groups are indicated.

Synovial inflammation

Macroscopic evaluation of the synovial tissue showed in both, the 3 weeks and the 10 weeks post-surgery group very mild signs of inflammation in the experimental joints, which could be confirmed by light-microscopic examination (table 1, figure 1, lower panels and figure 2, right bars).

On average the delta change in synovial inflammation between the experimental joints and the contra-lateral control joints was not statistically significant different for the 10 weeks post-surgery group compared to the 3 weeks post-surgery group (figure 2, right bars).

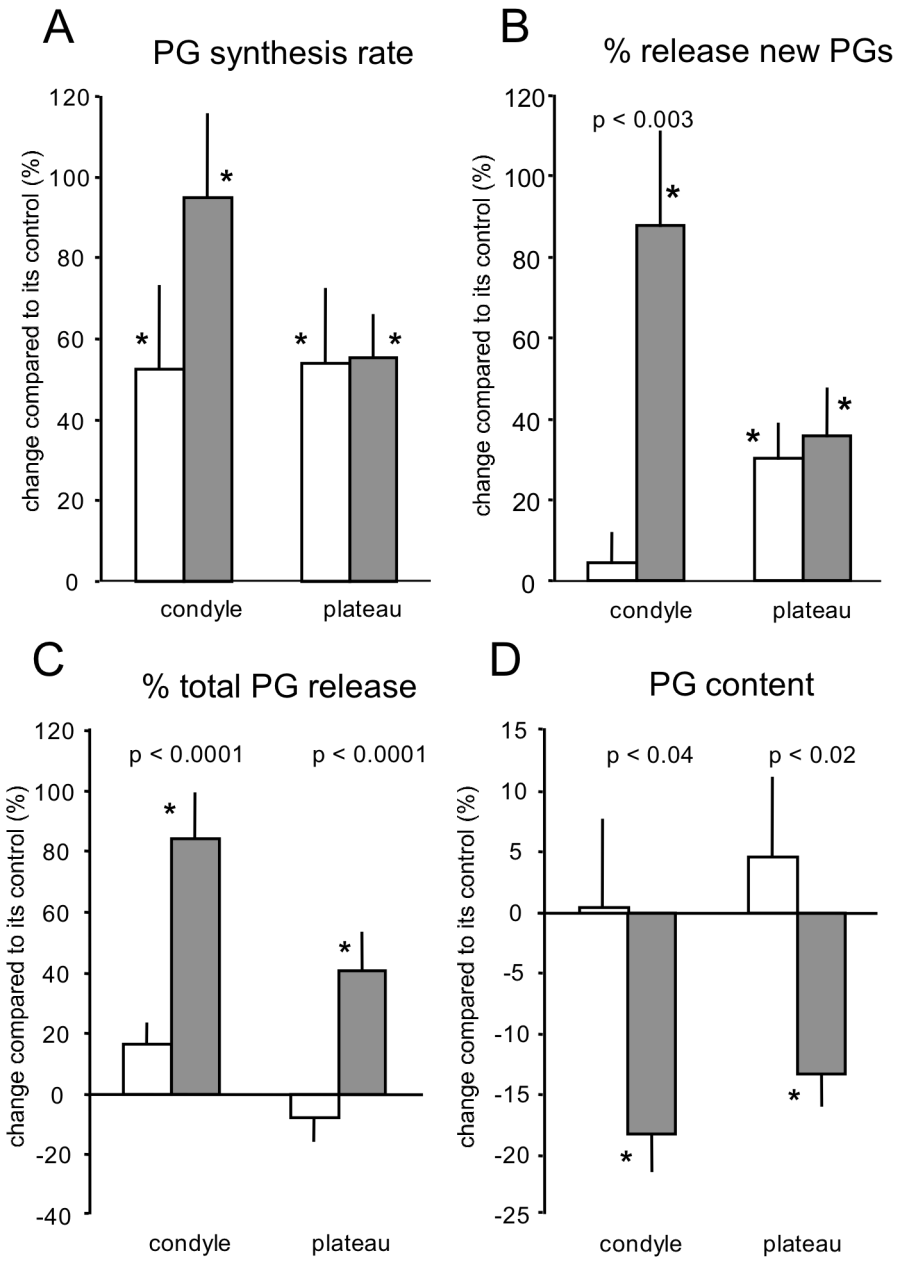

Figure 3.
Changes in proteoglycan synthesis rate (figure 3A), percentage release of newly formed proteoglycans (figure 3B), percentage total release of proteoglycans (figure 3C) and proteoglycan content (3D) of femoral and tibial cartilage calculated as percent from the control femoral and tibial cartilage controls for each animal. Mean values (n=10 ± SEM) are presented for 3 weeks post-surgery group (open bars) and 10 weeks post-surgery group (solid bars). Asterisks indicate statistically significant ($p<0.05$) change compared to contra lateral controls. Statistically significant differences between groups are indicated.

Proteoglycan turnover

Synthesis of proteoglycans (PGs) was increased in the experimental joints compared to the contra-lateral control joints at femoral condyles as well as at tibial plateaus both 3 weeks and 10 weeks post surgery (table 1). Although for femoral condyles the increase at 10 weeks was higher than at 3 weeks post-surgery (figure 3A) this apparent difference was not statistically significant.

The release of newly formed PGs, normalized to the synthesis of PGs (percentage new release), as a measure of retention of the newly formed PGs was not increased in the experimental femoral cartilage 3 weeks post-surgery (table 1). For tibial plateaus at 3 weeks and for both femoral condyles and tibial plateaus at 10 weeks post surgery the percentage release of newly formed proteoglycans was significantly increased (table 1). The increased release of newly formed proteoglycans in condylar cartilage was significantly higher at 10 weeks than at 3 weeks (figure 3B). The total amount of PGs released, normalized to the content and expressed as percentage release of PGs, of the experimental joints was enhanced in the 10 weeks post-surgery group but not in the 3 weeks post-surgery group (table 1). This difference between 3 and 10 weeks post-surgery was observed for the tibial plateau cartilage as well as condylar cartilage. For both cartilage surfaces this difference between 3 and 10 weeks was statistically significant (figure 3C).

As result of the ineffective PG synthesis and enhanced PG release in the 10 weeks post-surgery group the content of proteoglycans was decreased significantly in the femoral cartilage as well in the tibial cartilage of the experimental joints compared to the contra-lateral joints. In contrast, PG content was not changed significantly in the 3 weeks post-surgery group for both the femoral and tibial cartilage (figure 3D). The almost 20% decrease of the PG content of the femoral cartilage at 10 weeks post-surgery, and 13% decrease in tibial cartilage was significantly different from the changes observed at 3 weeks (figure 3D).

Discussion

Many different *in vivo* animal models of osteoarthritis (OA) have been described from mice models to equine models, from spontaneous to chemically induced model (225-227). The goal of studies using these models is extrapolation of results obtained in these models to the human situation, with respect to the pathogenic processes or effects of treatment. Each model has in this perspective its characteristics, advantages, and disadvantages. In the canine groove model chondral damage combined with forced loading of the experimental joint induces osteoarthritic features. This model appeared in short-term follow-up to induce very similar changes, with slightly more cartilage damage and slightly less inflammation, than the ACLT model (228). This sequence of events is similar to that

Tabel 2. *Specification of histological characteristics of experimental and contra-lateral control cartilage in the groove model, 3 and 10 weeks post-surgery*

	3 weeks post-surgery				10 weeks post-surgery				
	control	experimental	*p*<	delta change	control	experimental	*p*<	delta change	*p*< *(3 vs. 10 weeks)*
Histological cartilage damage structure									
condyles	0.10 ±0.05	0.78 ±0.15	*0.011*	0.68 ±0.15	0.08 ±0.05	1.42 ±0.17	*0.005*	1.34 ±0.18	*0.019*
plateau	0.53 ±0.04	0.78 ±0.07	*ns*	0.25 ±0.10	0.73 ±0.10	1.13 ±0.18	*ns*	0.39 ±0.21	*ns*
Histological cartilage damage cells									
condyles	0.00 ±0.00	0.53 ±0.11	*0.011*	0.53 ±0.11	0.01 ±0.01	1.13 ±0.13	*0.005*	1.12 ±0.13	*0.009*
plateau	0.08 ±0.03	0.10 ±0.04	*ns*	0.03 ±0.06	0.19 ±0.07	0.70 ±0.17	*0.038*	0.52 ±0.20	*0.011*
Histological cartilage damage safranin-O staining									
condyles	0.25 ±0.06	0.78 ±0.07	*0.004*	0.53 ±0.03	0.21 ±0.07	0.80 ±0.10	*0.005*	0.59 ±0.11	*ns*
plateau	0.18 ±0.04	0.58 ±0.06	*0.004*	0.40 ±0.05	0.39 ±0.11	0.77 ±0.10	*0.012*	0.38 ±0.10	*ns*

Histological cartilage damage was according to modified criteria of Mankin (maximum score of 11, see materials & methods). Figures of each of the three components (structure, cellularity, and safranin-O staining) are depicted. The absolute values of both condyles and tibial plateau are given for contra-lateral control knees and experimental knees (mean ± SEM) of the 3 week and 10 weeks post-surgery groups. In addition delta changes (mean ± SEM; experimental compared to control) of each post-surgery group are given. Statistically differences in delta changes between the post-surgery groups are shown in the far

described for human OA. This demonstrates that completely different causes, i.e. chondral trauma and joint instability, respectively, can lead to very similar features of experimental secondary osteoarthritis. Whether these features remain similar during further progression over the years remains to be established and needs a 4 to 5 year follow-up group for the groove model as has been reported on for the ACLT model (236).

Although with respect to outcome the similarity between the ACLT model and the groove model is striking, it was never evaluated whether the observed characteristics were the results of experimental (secondary) osteoarthritis or whether they were rather the expression of surgically applied chondral damage. The present results clearly show that the changes observed in the groove model of OA at 10 weeks are not observed at 3 weeks post surgery. Thus, the characteristics observed 10 weeks after induction of joint degeneration according to the groove model are not just the expression of the surgically applied damage but are the result of progressive features of experimental secondary osteoarthritis.

It is noticeable from the table that the control values between the 3 and 10 weeks post-surgery group are not similar. This is explained by the fact that 4 different "batches" of animals were used. Although we realize that this is a potential bias, we feel that this is of less relevance for the present study because the data of the experimental joints were analysed in relation to their contralateral control values (paired observation, within each animal). This excludes any biological variation between animals (animal groups). This is also the reason why data, in addition to absolute values (table 1), have been expressed as percentage changes (figure 2 and 3).

As soon as 3 weeks following surgically applied chondral damage an increased synthesis of proteoglycans was observed, an apparent attempt to repair the damaged tissue (106). This increased synthesis was similar 3 and 10 weeks post surgery. However, in contrast, at 3 weeks this repair appeared, at least for the femoral condyles, to be effective. Retention of newly formed proteoglycans was normal 3 weeks post-surgery, whereas it was significantly impaired 7 weeks later. Thus, between 3 weeks and 10 weeks after the chondral trauma the enhanced synthesis becomes ineffective. As expected enhanced catabolism characterised by an increased breakdown and release of resident proteoglycans causes a shift from normal to impaired retention of newly formed proteoglycans. The breakdown and release of resident proteoglycans has frequently been reported to be dependent on matrix-metalloproteinases (MMPs) and aggrecanases (49, 240). Because, the groove model is associated with only very mild synovial inflammation, synovial fluid could seldom be aspirated. It was therefore impossible to measure accurately these proteinases/aggrecanases.

However it seems likely that this increase in proteinase/aggrecanase activity contributes to the damage in the tibial plateau, which has not been surgically

damaged. Hardly any histological damage (no damage of the articular surface and no changes in cellularity, table 2) or biochemically determined proteoglycan release (figure 3C) was seen 3 weeks post surgery, whereas 10 weeks after the experimentally induced trauma the tibial cartilage was significantly adversely affected. Incongruity, as a result of movement of the roughened condylar surface on a smooth tibial plateau, may in addition add to the development of the osteoarthritic features at the tibial plateau. The development of catabolic activity between 3 and 10 weeks post-surgery is clearly represented in the proteoglycan content of the cartilage when biochemically determined. This does not corroborate the histological findings because there it were the structural and cellular changes (table 2) rather than the loss in safranin-O staining that determined the difference between 3 and 10 weeks post-surgery. It is however, not unexpected that subtle change (such as a 20% change) in safranin-O staining cannot be detected by histochemistry. Biochemically determined changes in proteoglycan content are more reliable in that respect. The increased synthesis without a significant increase in catabolic activity even resulted in a slight increase in proteoglycan content (although not statistically significant; week 3), whereas the development of catabolic activity between 3 and 10 weeks results in a clearly diminished proteoglycan content 10 weeks post-surgery. Whether, during this change from a net anabolic to a net catabolic activity a point of no return is crossed, remains to be established.

Studies on the spontaneous healing of articular cartilage defects have been performed in many animal models, including the dog. Defects fully contained in articular cartilage (chondral defects) may persist with no sign of reparative tissue filling the lesion (241) or heal with limited filling by fibrous tissue, fibrocartilage, or hyaline cartilage. In some cases the reparative tissue that fills such defects displays the structure of articular cartilage (242) although complete regeneration of chondral effects have never been reported. In our model there seems to be a repair process at 3 weeks post-surgery whereas at 10 weeks post-surgery degeneration is evident. Apparently, possible reparative activities as observed in previous studies might nonetheless finally result in progressive damage later on. However, it might also be the intensified loading in our model that changes a potential repair process into progressive damage by forcing the process of joint degeneration to cross a point of no return. Future studies in this respect are needed to clarify the impact of chondral damage without forced loading on the development of osteoarthritis.

The present results show that the characteristics observed 10 weeks after induction of joint degeneration in the groove model are not just the expression of the surgically applied damage but are result of progressive features of experimental osteoarthritis. The groove model adds to existing animal models of osteoarthritis with features resembling of human osteoarthritis. One point of distinction is that the

degenerative changes are progressive while synovial inflammation is minimal and even diminish over time (229). Because of this, evaluation of cartilage protective effects of treatment is not hampered by inflammatory activity in the joint. This phenomenon makes the groove model especially suitable for evaluation of disease modifying osteoarthritic drugs (DMOADs). A second point of distinction is that there is no persistent trigger causing joint damage, which should render the model more sensitive to treatment. A persistent trigger for joint damage, such as joint instability used in the ACLT model, could counteract the possible beneficial effects of treatment. Moreover, assuming that cartilage repair is possible, the trigger, intrinsic to the cartilage damage itself, could be removed by treatment. Thus, the groove model might be suitable to monitor progression of osteoarthritis in the long-term after treatment is stopped; and may even be amenable to demonstrating a cure.

In conclusion, animal models are essential in research on osteoarthritis aimed at better understanding of the pathophysiology of osteoarthritis especially in its early phases and to study effects and mechanisms of treatment. The groove model of osteoarthritis may have an additional value in this respect.

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Chapter 7

Selective COX-2 inhibition in the canine groove model of osteoarthritis.

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Abstract

Objective: In vitro studies showed a beneficial effect of celecoxib on proteoglycans turnover and content of osteoarthritic (OA) cartilage. In the present study we evaluated whether these favorable effects of celecoxib could also be demonstrated in *in vivo*.

Methods: In 24 Beagle dogs OA was induced in one knee according to the groove model. The animals were divided in three groups and received orally placebo, 100 or 200 mg celecoxib daily, starting directly after surgery. After 15 weeks joint tissue of all dogs were analyzed.

Results: Induction of OA resulted in macroscopic and histological damage of cartilage, changes in cartilage proteoglycan turnover, loss of cartilage matrix proteoglycans, and slight synovial inflammation, all characteristic of early OA. Surprisingly, none of the parameters was significantly changed upon celecoxib treatment. Synovial fluid prostaglandin-E_2 levels were dose-dependently diminished by celecoxib demonstrating the celecoxib had reached the joint in sufficient amounts. Using an *in vitro* set-up, canine cartilage under degenerative conditions was favourably influenced by celecoxib demonstrating that canine cartilage is sensitive to celecoxib.

Conclusion: The present study showed no effect of celecoxib on the characteristics of experimentally induced OA *in vivo*, in contrast to the observed beneficial effect *in vitro*. It could be that celecoxib had been beneficial to degenerated cartilage *in vivo* but that these effects were counteracted by increased loading of the affected joint and with that progression of OA, because of the well-known analgesic effects of celecoxib.

Introduction

Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) are among the most widely prescribed drugs in treatment of osteoarthritis (OA) (190). The clinical efficacy of NSAIDs is primarily related to the inhibition of COX-2, whereas much of the toxicity, particularly in the gastrointestinal system, is related to COX-1 inhibition (191). This has lead to selective COX-2 inhibitors that specifically block COX-2 with similar efficacy and less toxicity (97). These COX-2 specific drugs have been clinically used for the past few years in treatment of (osteo)arthritis and other conditions (57, 96, 97). However, more recently it appeared that selective COX-2 inhibition also has its dark side and that it might increase the risk for cardiovascular diseases (84, 85, 192). In that respect caution and further study is needed.

Another problem of NSAIDs might be their direct (adverse) effects on cartilage. These effects cannot be studied easily in clinical trials and therefore they are generally ignored in clinical practice because (intrinsic) cartilage changes, catabolic and anabolic are in OA rather slow processes. In addition, beneficial

effects of NSAIDs on inflammation shade possible direct adverse effects on cartilage.
Direct effects of NSAIDs on cartilage may be important specifically in prolonged treatment of joint disease in which inflammation is only mild and secondary as in OA. Thus, although NSAIDs may be very useful in reducing pain and inflammation in OA, they may interfere directly with the process of cartilage degeneration/regeneration. These direct effects should be considered in addition to gastro-intestinal and cardio-vascular effects when prescribing NSAIDs in clinical practice.
With respect to the selective COX-2 inhibitors, data in this respect are limited. We recently showed *in vitro* a beneficial effect of celecoxib in normal cartilage under the influence of IL-1β and TNFα; on normal healthy cartilage no effects were observed (139). Findings by El Hajjaji *et al.* showed that celecoxib *in vitro* was able to increase proteoglycan synthesis and to diminish proteoglycan release of OA cartilage obtained at joint replacement surgery (140). Recent findings by our group confirmed these data and additionally demonstrated that celecoxib, had *in vitro* a favorable effect on proteoglycan synthesis, retention, release and content of both degenerated (pre-clinical) and (late-stage) human OA cartilage (173).
The lack of *in vivo* data from animal models of OA urged to study selective COX-2 inhibition in such a model. The groove model of OA has features representative of human OA (228). The model is distinctive in that the degenerative changes are progressive while synovial inflammation diminishes over time (229). Because of this, evaluation of direct effects of medication on cartilage is less hampered by a possible anti-inflammatory effect of treatment. Additionally the model is distinctive because there is no permanent trigger causing joint damage, making the model more sensitive to treatment. A permanent trigger for joint damage, such as joint instability used in the ACLT model, will counteract the possible beneficial effects of treatment. Assuming that cartilage repair is possible (64, 243, 244), the trigger, intrinsic to the cartilage damage itself, can be eliminated by treatment. Therefore, we chose the canine groove model of OA to study the effect of celecoxib on OA cartilage *in vivo*.

Materials and Methods

Animals

Female Beagle dogs, 24 in number, mean age 1.8 ± 0.1 years, weighing 10-15 kg, were obtained from the animal laboratory of the Utrecht University, the Netherlands. They were housed in groups of 2 to 3 dogs per pen, and were let out on a patio in large groups for at least 2 hours daily. They were fed a standard diet and had water *ad libitum*. The Utrecht University Medical Ethical Committee for animal studies approved the study.

The groove model

After induction with Nesdonal, the dogs were anaesthetized with halothane in a mixture of oxygen and nitrous oxide delivered endotracheally. Surgery was carried out through a 2 to 2.5 cm medial incision close to the *ligamentum patellae* in the right knee. Care was taken to prevent bleeding and soft tissue damage as much as possible. Cartilage of the lateral and medial condyle was damaged with a Kirschner-wire (1.5 mm diameter) that was bent 90° at 0.5 mm from the tip. This ensures that depth of the grooves was restricted to around 0.5 mm. In utmost flexion, ten longitudinal and diagonal grooves were made on the weight-bearing parts of the femoral condyles without damaging the subchondral bone (228, 229). The latter was confirmed by histology at the end of the experiment. Macroscopic evaluation after killing of the animals showed similar groove patterns in all affected knees. The tibial plateau was left untouched. After surgery, synovium, fasciae and skin were sutured. The contra-lateral unoperated knee served as a control. The animals received analgesics (Buprenorphine 0.01 mg/kg) and antibiotics (Amoxicyclin 400 mg/kg) during the first 3 days after surgery.
Starting 2 days after surgery, the dogs were let out daily on the patio. To ensure (intensified) mechanical loading of the affected knee, the dogs were forced to load the experimental joint intermittently by fixing the contra-lateral limb to the trunk for approximately 4 hours per day, 3 days per week, for 15 weeks. This is less than 10% of the total loading time of the affected joint. Thus, the control limb was not weight bearing for less than 10% of the time. We have demonstrated that the control limbs of these animals did not differ from those of animals from which the limbs were not fixed to the trunk (228).

Treatment

The dogs were divided in 3 groups of 8 animals each. Celecoxib (Celebrex, Pfizer, USA) was given once daily orally in two doses; one group received 100 mg/day, the second group received 200 mg/day, and the third group received a placebo. Treatment was started the day after surgery. The choice for these dosages was based on previous studies that focussed on the plasma kinetics of celecoxib in Beagle dogs (245, 246). The treatment compliance was 100%.

Evaluations
Severity of OA was evaluated 15 weeks after surgery. At the end of the experiment, the dogs were euthanized with an intravenous injection of Euthesate (Na-Pentobarbital). Both hind limbs were amputated and synovium and cartilage were collected and processed within 2 hours. Procedures were carried out under laminar flow conditions.

Synovial tissue analysis
Synovial inflammation was evaluated macroscopically on digital high-resolution photographs of synovium, by two observers unaware of the source of the photographs. Severity of inflammation was graded from 0-2 for colour, angiogenesis, and fibrillation: 0= none, 1= slightly, 2= strong. The sum of these three individual scores averaged for the two observers (a maximum of 6) was used as representative score of each joint and used for statistical analysis.
Three infra-patellar synovial tissue samples per joint (medial, middle, and lateral) were fixed in 4% phosphate-buffered formalin (pH 7.0) and embedded in paraffin. Deparaffined sections were stained with hematoxylin-eosin. Histological sections were examined separately in random order and independently by two observers unaware of the source of the synovium. To determine the degree of inflammation, the slightly modified (237) criteria described by Goldenberg and Cohen (238) was used. For assessing the overall grade, the scores of the three specimens from each knee and of the two observers were averaged (a maximum of 10). This score was used as representative score of each joint, and was used for statistical analysis.

Cartilage analysis
Cartilage damage was evaluated macroscopically on digital high-resolution photographs of tibia and femur, by two observers unaware of the source of the photographs. Severity of cartilage damage of the femoral condyle was graded from 0-4: 0= smooth surface, 1= slightly fibrillated, 2= fibrillated with shallow grooves, 3= deep sharp grooves, 4= deep sharp grooves with surrounding damage. Grading of cartilage damage of the tibial plateau was comparable: 0= smooth surface, 1= roughened, 2= slightly fibrillated, 3= fibrillated, 4= damaged. Scores of the two observers were averaged (a maximum of 4). This score was used as representative score of each photograph, and was used for statistical analysis.
Cartilage samples for histological and biochemical analyses were obtained from predetermined locations on the weight-bearing areas of the femoral condyles and the tibial plateau of both experimental and control joints. Locations were identically paired with the same location in the contra-lateral joint (228). Cartilage was cut as thick as possible, while excluding the underlying bone and

subsequently samples were cut into full-thickness cubes, weighing (3-10 mg; accuracy 0.1 mg).
For histology, four samples from tibial plateau and four from femoral condyles from each knee were fixed in 4% phosphate-buffered formalin containing 2% sucrose (pH 7.0). Cartilage degeneration was evaluated in safranin-O-fast-green iron hematoxylin-stained sections by light microscopy using the slightly modified (143) criteria of Mankin (142). Specimens were graded in random order by two observers unaware of the source of the cartilage. For assessing the overall grade, the scores of the four specimens from each knee surface and of the two observers were averaged (a maximum of 11). This score of each joint surface was used as representative score and used for statistical analysis.
For biochemical analysis, cartilage samples were cultured individually in 96-well culture plates (NUNCLON®, Denmark) in 200 μl culture medium (Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 0.085 mM ascorbic acid, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated Beagle serum) according to standard procedures (143). For femoral condyles and tibial plateau, cartilage proteoglycan (PG) content, -synthesis, -retention, and -release were determined of 6 explants per parameter of fixed locations with identical locations at the contralateral control joint. All 6 samples were handled individually. The average result of the six samples was taken as representative of that joint surface and was used for statistical analysis (239).
Proteoglycan synthesis. As a measure of PG synthesis, the rate of sulphate incorporation was determined *ex vivo* (105). After 4 hours labelling with $^{35}SO_4^{2-}$, the cartilage samples were washed with cold PBS and digested with papain for 2 hours at 65°C. Glycosaminoglycans (GAGs) were precipitated by addition of cetylpyridium chloride (CPC), and $^{35}SO_4^{2-}$ -labelled GAGs were measured by liquid scintillation analysis. Synthetic activity is expressed as nmoles of sulphate incorporated per hour per gram wet weight of the cartilage (nmol/h.g).
Proteoglycan retention and release. As a measure for retention of newly synthesized PGs, the release of $^{35}SO_4^{2-}$ -labelled PGs in the medium was determined. After labelling the cartilage samples were rinsed 3 times for 45 minutes in 1.5 ml culture medium and then incubated in 200 μl fresh culture medium without sulphate label for 3 days. GAGs were precipitated from the medium and were stained with Alcian Blue dye solution, as described previously (105). The $^{35}SO_4^{2-}$ -labelled GAGs were measured by liquid scintillation analysis and the release was normalized to the specific activity of the medium and the wet weight of the explants. The release of newly formed PGs is expressed as percentage release of newly formed PGs in the three days (% new PG release). For the total release of PGs, Alcian blue staining of the medium was quantified photometrically with chondroitin sulphate (Sigma C4384) as a reference. The total

amount of GAGs released (blue staining) is expressed as a percentage of the original tissue content (% GAG release).
Proteoglycan content. As a measure of PG content of the cartilage samples the amount of GAG was determined as described previously (106). The GAGs in the papain digest of cartilage samples were precipitated and stained with Alcian Blue as described above. Values were normalized to the wet weight of the cartilage explants (mg/g).

Prostaglandin-E_2 determination
Prostaglandin-E_2 (PGE_2) was determined in thawed (frozen at -80˚C) synovial fluid that had been taken after the joints had been opened, by Enzyme Immuno Assay (EIA, Amersham Biosciences) and expressed as pg/ml.

In vitro analyses
In addition, we performed *in vitro* experiments to see whether "human" celecoxib is able to modify degenerative changes of canine cartilage. Canine synovial tissue, obtained from knees with experimentally induced OA, was cultured for 3 days (247). Cell free supernatants were added to healthy canine hip cartilage in a concentration of 50% v/v, resulting in OA characteristic changes in PG turnover and content. These cultures were performed in the absence and presence of celecoxib (10 μM) to study whether celecoxib could reverse these effects as previously reported for human degenerated cartilage (139, 173).

Calculations and statistics
Mean values ± SEM (n=8 animals) of each joint for femoral and tibial cartilage separately are presented. Paired Student's t-test was used to compare data of the experimental and contra-lateral control joints within each group. Unpaired t-test was used to analyse differences between the three treatment groups (absolute values of both the control and experimental joints separately and the change in the experimental compared to the control joints). P values less than 0.05 were considered statistically significant.

Results

Induction of OA in the Groove model

Cartilage damage
Fifteen weeks after induction of experimental OA, the affected knees clearly showed macroscopically damage of the articular cartilage of femoral condyles in addition to the grooves that were surgically applied and still visible. No damage was found on the condylar cartilage of the control knees (representative photographs are depicted in figure 1B *vs.* 1A). Damage, although less pronounced, was also found on the tibial plateau of the experimental joints.

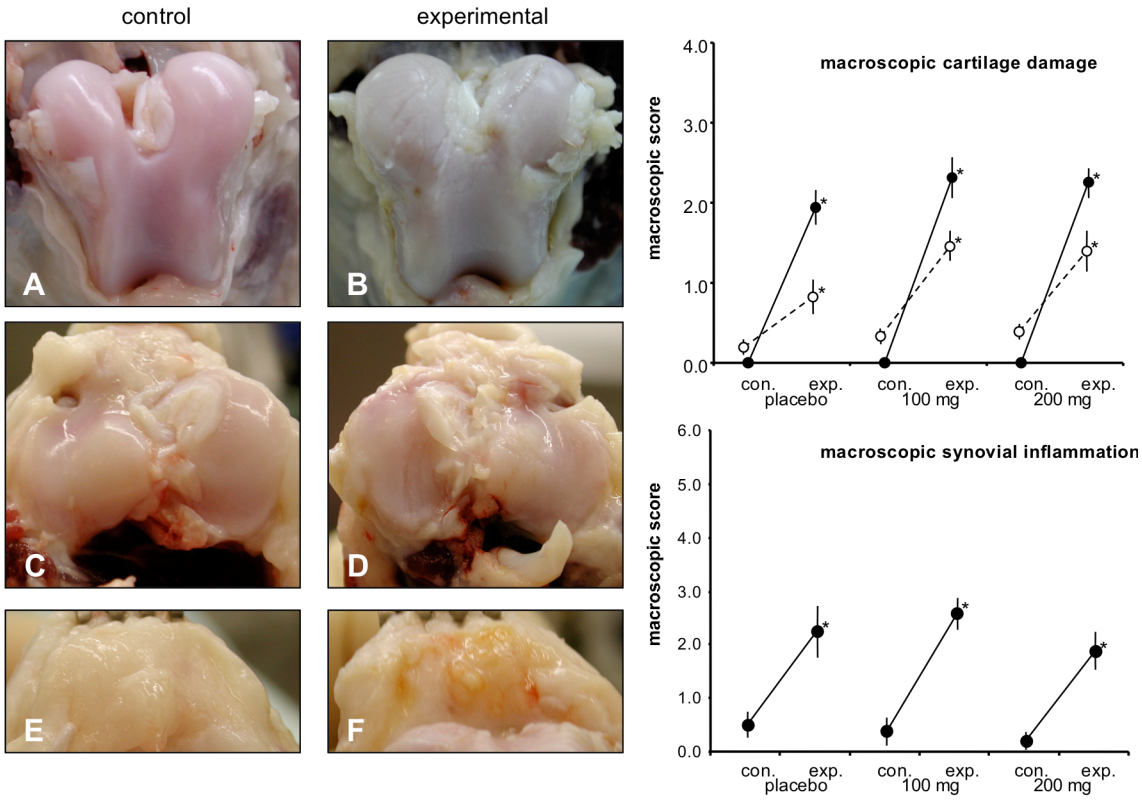

Figure 1. *Macroscopic changes as a result of experimentally induced OA and celecoxib treatment.*
Representative macroscopic view of articular cartilage surfaces and synovium. Femoral condyles (A + B), tibial plateaus (C + D) and synovium (E + F) of the control (left) and experimental (right) joint of a placebo treated dog are shown. Average macroscopic score of cartilage damage and synovial inflammation are depicted in graphs (G + H). For figure G: Closed symbols with solid lines represent macroscopic damage of femoral condyles and open symbols with dashed lines represent macroscopic damage of tibial plateaus. Mean ± SEM values (n=8) are presented for placebo, celecoxib 100 mg and celecoxib 200 mg treated animals. Asterisks indicate statistically significant ($p<0.05$) changes compared to contralateral controls. No statistically significant differences between groups were found (see appendix).

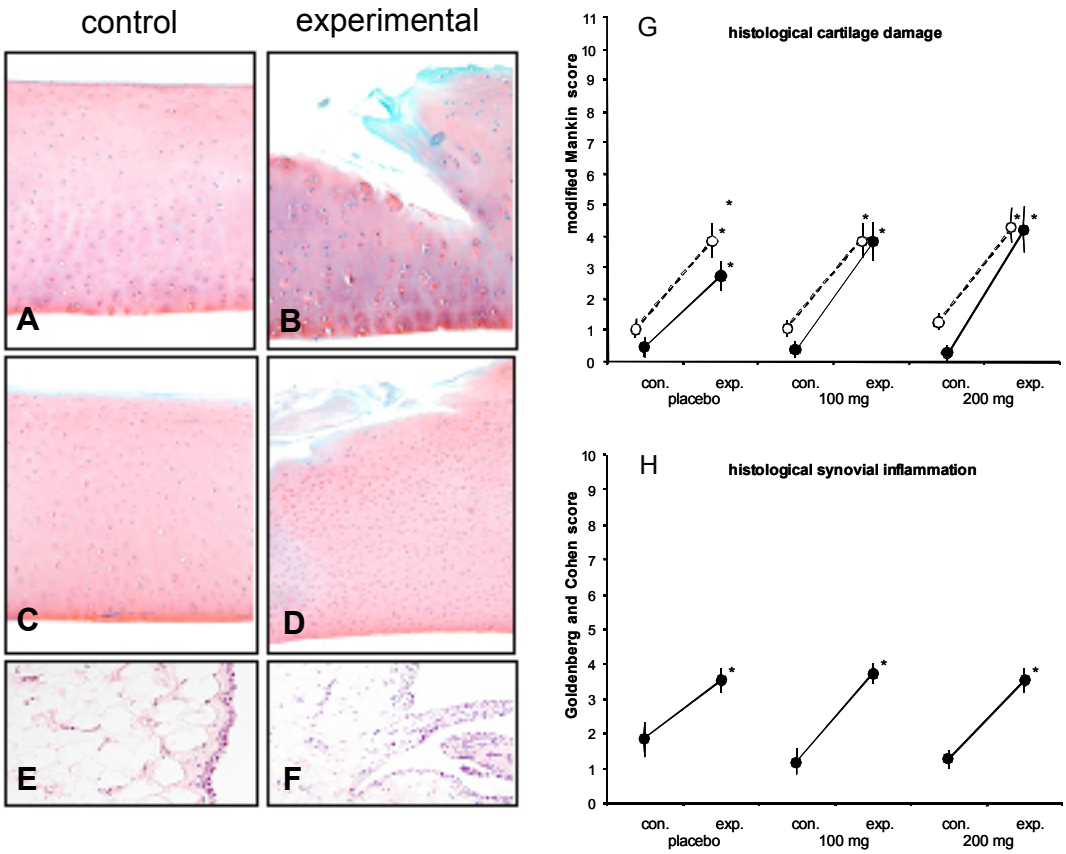

Figure 2. *Histological changes as a result of experimentally induced OA and celecoxib treatment.* Representative light micrographs of condylar cartilage (A + B), tibial cartilage (C + D) and synovial tissue (E + F) of the control (left) and experimental (right) joint of a placebo treated dog are shown. Average scores of histological determined cartilage damage (modified Mankin grade) for femoral condyles (closed circles, solid lines), tibial plateaus (open circles, dashed lines) and synovial inflammation (modified Goldenberg and Cohen score) are depicted in graphs (G + H). Mean ± SEM values (n=8) are presented for placebo, celecoxib 100 mg and celecoxib 200 mg treated animals. Asterisks indicate statistically significant ($p<0.05$) changes compared to contralateral controls. No statistically significant differences between groups were found (see appendix).

compared to the control joints (figure 1D *vs.* 1C). On average the macroscopic cartilage damage was significantly more severe in the experimental joints compared to that in the control joints (figure 1G, placebo; for femoral condyle solid lines and for tibial plateau dashed lines).
These macroscopic observations were confirmed by histological analysis. The average modified Mankin score of the cartilage degeneration in the experimental femoral condyles was mild but significantly higher compared to that of the contra-lateral control joints (figure 2G, placebo; solid line). The grooves were clearly visible as depicted by a representative micrograph in figure 2B *vs.* 2A. Although not surgically damaged, mild cartilage degradation of the experimental tibial plateau cartilage was also found in the placebo OA group when compared to the contra lateral control joints (representative micrographs are given in figure 2D *vs.* 2C). On average also for the tibial plateau histological damage was statistically significant different from control joints (figure 2 G, placebo, dashed line).

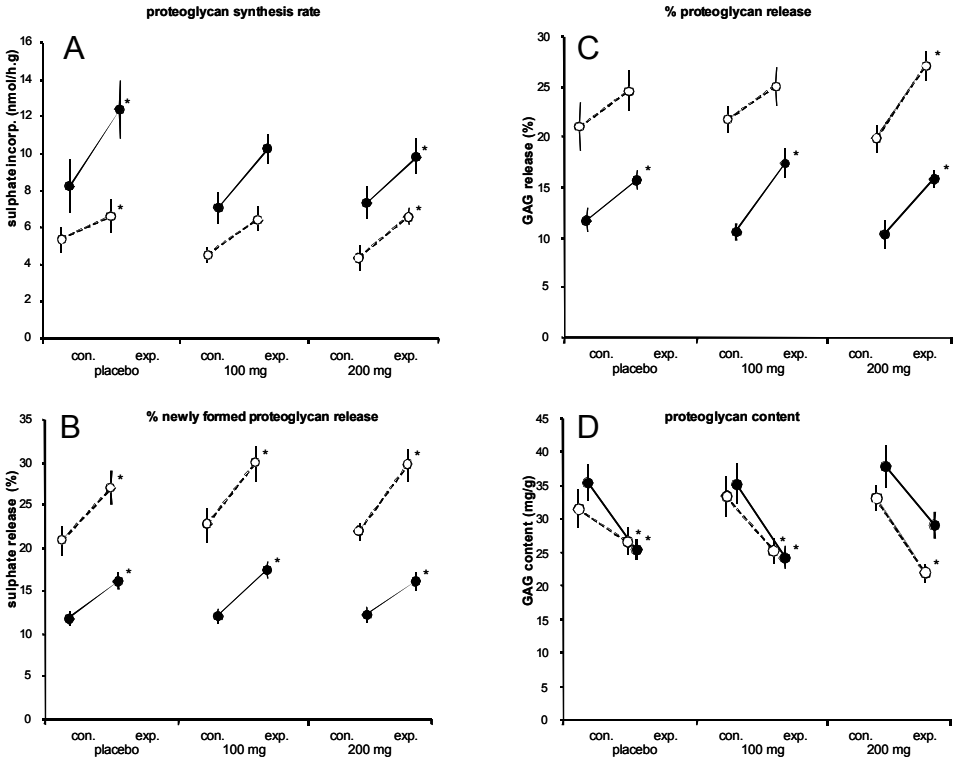

Figure 3. *Biochemical changes of experimentally induced OA and celecoxib treatment.* Proteoglycan synthesis rate (A), percentage release of newly formed proteoglycans (B), percentage total release of proteoglycans (C) and proteoglycan content (D) of femoral (closed symbols, solid lines) and tibial (open symbols, dashed lines) cartilage are depicted. Mean ± SEM values (n=8) are presented for placebo, celecoxib 100 mg and celecoxib 200 mg treated animals. Asterisks indicate statistically significant ($p<0.05$) changes compared to contra-lateral controls. No statistically significant differences between treatment groups were found

Synovial inflammation
Macroscopically the synovial tissue showed mild signs of inflammation in the experimental joints compared to the contra-lateral control joints. Figure 1E and 1F depict representative photographs; figure 1H left, depicts the averages for the placebo group. This was confirmed by light microscopic examination of the synovial tissue (respectively figure 2E *vs.* 2F, and 2H).

Proteoglycan turnover
Fifteen weeks post-surgery, synthesis of proteoglycans (PGs) of both femoral condyles as well as tibial plateaus were increased in the experimental joints compared to the contra-lateral control joints (figure 3A). This for OA characteristic increased proteoglycan synthesis is ineffective as the release of these newly formed PGs was also increased in the experimental femoral condyle and tibial plateau cartilage when compared to the control joints (figure 3B). When depicted as a percentage of the synthesis it demonstrates that there is a decreased retention of newly formed proteoglycans in the OA joints. Also the release of the total amount of proteoglycans was enhanced as a result of the experimentally induced OA in both femoral condyle and tibial plateau cartilage (figure 3C). As result of the ineffective PG synthesis and enhanced PG release the content of proteoglycans was significantly decreased in cartilage of femoral condyles and tibial plateau (figure 3D). There was no statistical significant change in cartilage DNA content due to induction of OA (OA: 0.12 ± 0.01 mg/g *vs.* control: 0.15 ± 0.01 mg/g and 0.13 ± 0.00 mg/g *vs.* 0.12 ± 0.01 mg/g, for femoral condyles and tibial plateau).

Effect of celecoxib treatment
One day after OA induction celecoxib treatment was started. Although all dogs were active after the surgery, the celecoxib treated dogs (both 100 mg and 200 mg) seemed to walk better and more when compared to the placebo group. Although this difference in walking pattern was independently noted by three different observers (SM, MV and FL) and discussed, this was not further quantified.

Cartilage damage
In all animals treated with celecoxib, both 100 and 200 mg, macroscopic cartilage damage was not different from that of the OA placebo groups, neither for the femoral condyles nor for the tibial plateau, not on absolute values and not when compared as a change (figure 1G).

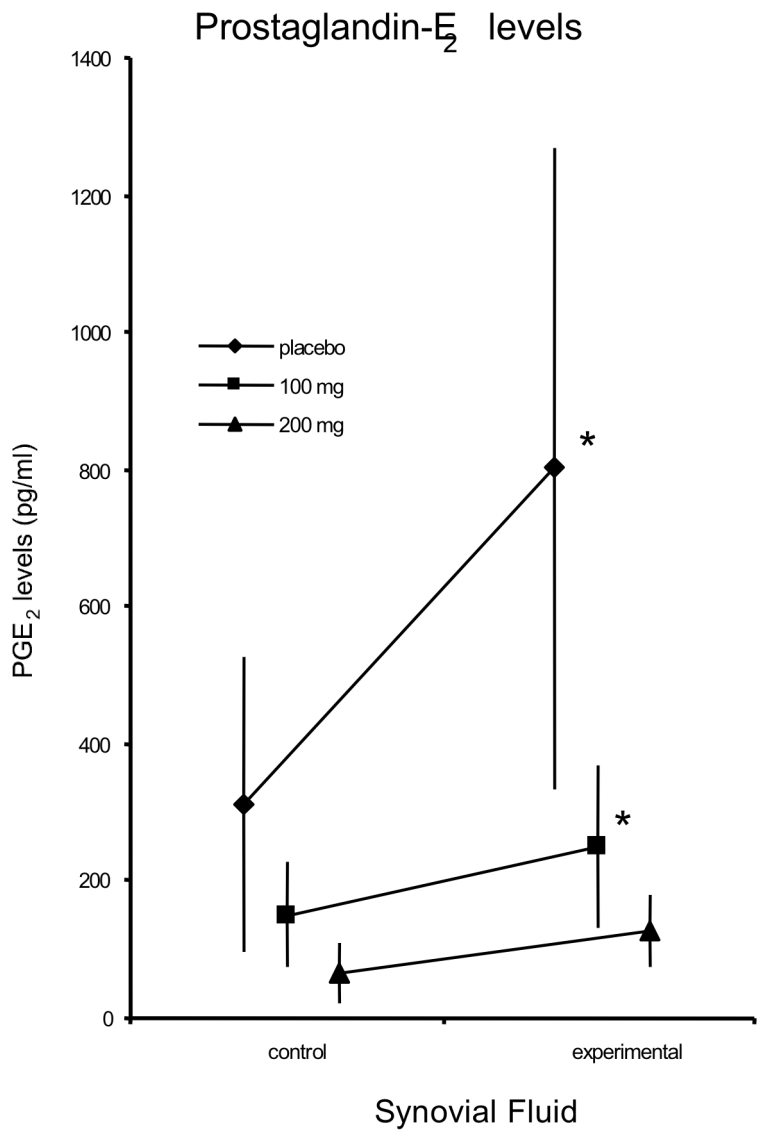

Figure 4. *Synovial fluid prostaglandin E_2 levels in experimental osteoarthritis with or without celecoxib treatment*

Synovial fluid prostaglandin E_2 (PGE_2) levels of control and experimental joints treated with either placebo (diamonds), celecoxib 100 mg (squares), or celecoxib 200 mg (triangles) are depicted. Mean ± SEM values (n=8) are presented for placebo, celecoxib 100 mg and celecoxib 200 mg treated animals. Statistically significant differences of the effects of celecoxib compared to untreated controls, calculated by non-parametric paired analysis (p-value <0.05) are marked with an asterisk.

Histological examination of the different groups confirmed the macroscopic impression and showed similar results for the celecoxib treated groups and the placebo group (figure 2G).

Synovial inflammation
Synovial inflammation in the celecoxib treated group when compared to the OA placebo group was not different based on macroscopic evaluation (figure 1H) and microscopic evaluation (figure 2H).

Proteoglycan turnover under influence of celecoxib
Biochemical evaluation of proteoglycan turnover and content demonstrated that celecoxib at both concentrations had not resulted in a difference in proteoglycan parameters of condylar and tibial plateau cartilage (figure 3 A-D). Also DNA content was not changed by celecoxib (data not shown).

Prostaglandin E_2 levels in synovial fluid
Because of lack of any significant difference in the celecoxib treated groups compared to the placebo OA group, it was doubtful whether celecoxib had entered the joint in sufficient amounts. Therefore the synovial fluid samples were analysed for PGE_2. In the synovial fluid of the placebo treated group, we found increased levels of PGE_2 (figure 4). In the 100 mg celecoxib treated group we found much lower levels of PGE_2 although still elevated compared to controls. In the 200 mg treated animals PGE_2 levels were even lower and not statistically different from control joints any more. Interestingly, also in the contra-lateral control joints a decrease in the synovial fluid PGE_2 levels was observed, although not statistically significant.

The in vitro effect of celecoxib on canine cartilage
Because celecoxib did not demonstrated an effect on the articular cartilage, although apparently present in sufficient amounts, it was questionable whether celecoxib was able to influence canine cartilage. This was evaluated *in vitro*. Canine synovium tissue supernatants were used to induce degenerative changes in healthy canine cartilage in the presence or absence of celecoxib. Celecoxib was able to influence the (degenerated) cartilage beneficially (figure 5). The enhanced PG release was decreased to levels even below that of the control cartilage (not treated with synovium culture supernatants; figure 5A). Also PG content of canine cartilage was increased by celecoxib to levels of controls (figure 5B). PG synthesis was only mildly increased which is in accordance with the effects of celecoxib on PG synthesis of human degenerated cartilage (139, 173).

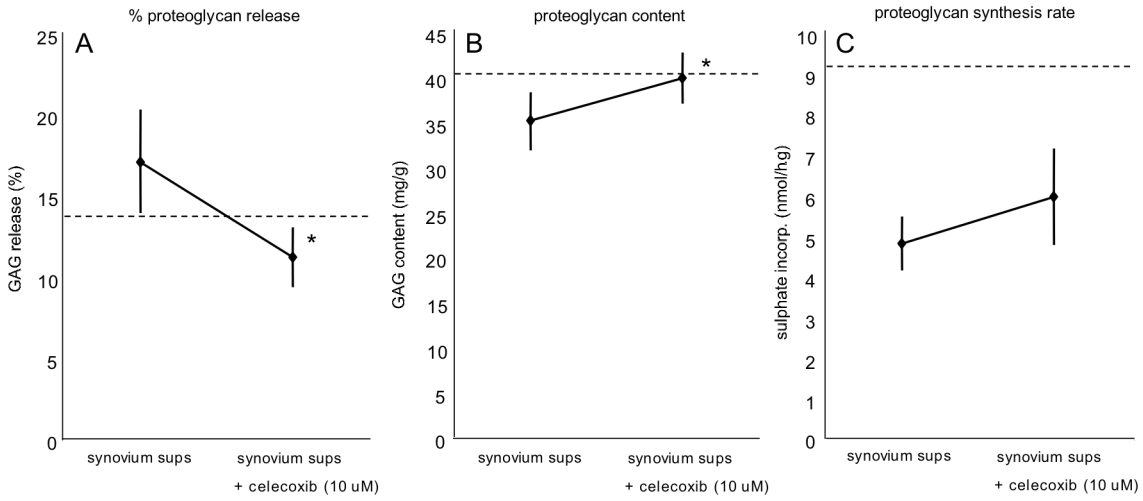

Figure 5. *The in vitro effect of celecoxib on canine cartilage; changes in proteoglycan turnover.* Percentage total release of proteoglycans (A), proteoglycan content (B), and proteoglycan synthesis rate (C) are depicted. Mean ± SEM values (n=6) are presented for cartilage cultured with 50% v/v experimental OA synovial tissue culture supernatants in the absence or presence of celecoxib (10 μM). Dashed lines indicate levels of healthy canine cartilage cultured alone. Asterisks indicate statistically significant ($p<0.05$) change induced by celecoxib compared to cartilage cultured in presence of 50% synovium culture supernatant alone.

Discussion

This study describes to our knowledge for the first time the *in vivo* effect of celecoxib on experimental OA cartilage, using the canine groove model of OA. Several recent *in vitro* studies showed that the selective COX-2 inhibitor celecoxib is capable to improve and even restore a disturbed cartilage turnover, both under inflammatory conditions (139) and under OA conditions (140, 173). Normal healthy cartilage was not influenced by celecoxib in these studies (139, 173). However, we could not confirm the positive *in vitro* results of celecoxib in our *in vivo* canine model of OA.

An increased synthesis of PGs, a decreased retention of these newly formed PGs, and an increased release of PGs combined with decreased PG content as found in the placebo treated OA group, characteristics of OA, are comparable to previous studies performed with this model (228, 229) and therefore are not expected to be the cause of the absence of an effect of celecoxib.

There is evidence for a polymorphism in the metabolism of celecoxib in Beagle dogs (245). There are at least two populations of dogs, distinguished by their capacity to eliminate celecoxib from plasma at either a fast or a slow rate after intravenous administration. About halve of the 242 tested Beagles were of the fast phenotype, the other halve of the slow phenotype. There was also an equal distribution of the two phenotypes within males and females. The difference between the two populations was due to a difference in the rate of metabolism of

celecoxib by liver cytochrome P-450. We were not able to check our Beagle dogs for this polymorphism. As we used animals with the same genetic background we assumed that they are either slow or fast metabolizers. Therefore the doses celecoxib were chosen in such a way that in one of the two dosages (100 or 200 mg) an effective dose, resulting in adequate plasma levels, was reached (245, 246). The other dose may as a consequence have been either too low or too high. Nevertheless, celecoxib treatment did not change proteoglycan turnover of the OA cartilage in either of the two concentrations.

Because of a lack of effect despite the concentrations chosen, we questioned whether celecoxib had reached the joint in an adequate dose. Therefore, PGE_2 levels in the synovial fluid at the end of the study were measured. PGE_2 is one of the main products formed by cyclooxygenase-2 (COX-2) and is an indirect measure of COX-2 activity. In the synovial fluid of the placebo treated group, we found increased levels of PGE_2. This corroborates the human OA condition, where also increased levels of PGE_2 in different joint tissues are found (137, 173). More interestingly, we found in the celecoxib treated groups, dose dependently, much lower levels of PGE_2. This indirectly proves that celecoxib had entered the joints in a dose that was effective in inhibiting COX-2 activity. Also the control joints of the celecoxib treated animals showed lower PGE_2 levels (although not statistically significant). This suggests that also in the contra-lateral control joints some COX inhibition had occurred by the treatment. Nevertheless, despite functional COX(-2) inhibition in the joint, no effects on cartilage were found.

This raised the question whether canine articular chondrocytes are sensitive to "human" celecoxib. It appeared that canine cartilage under the influence of catabolic mediators released by OA synovial tissue was beneficially influenced by celecoxib. These *in vitro* effects of celecoxib were comparable to results obtained with human cartilage *in vitro* exposed to celecoxib (139, 140, 173).

Thus, although celecoxib had reached the joint and COX-2 inhibition can be beneficial for canine cartilage under degenerative conditions, in our *in vivo* study no effects of celecoxib were observed. A possible explanation could be found in the different walking patterns as observed in the different treatment groups. The celecoxib treated dogs (both 100 mg and 200 mg) seemed to walk better and more when compared to the placebo group. This fits well with the analgesic effects of celecoxib. It could be that celecoxib had been beneficial to degenerated cartilage *in vivo* but that these effects were counteracted by the increased loading of the affected joint and with that progression of OA, because of the analgesic effects of celecoxib. The efficacy of celecoxib in reducing knee pain in patients with OA has been demonstrated in previous studies (117, 248, 249). Recently the effect of celecoxib *vs.* placebo treatment was compared on clinical and gait variables in knee OA patients; focussing on the efficiency of the locomotor mechanism (250). In this study celecoxib treatment improved the efficiency of the

locomotor mechanism significantly. Among the secondary outcome measures assessed, celecoxib treatment improved walking cadence and reduced the knee pain significantly.
Improved walking and loading of the animals treated with celecoxib might have resulted in undesired progression of OA in our experiments counteracting the direct beneficial effects on cartilage. Increased loading of a joint is associated with OA pathology (251-256). *In vivo* animal experiments indicate that impulsive loading and joint instability results in bone or cartilage changes commonly associated with OA (252, 257-259). Severe OA rapidly developed in dogs following anterior cruciate ligament transaction combined with a dorsal root ganglionectomy (257). Rapid development of OA was attributed to the increased ground reaction forces, which were 30% higher in these dogs compared with ACLT dogs with an additional sham procedure (260), a difference that may have resulted from the impaired ability of the dogs to experience knee instability and pain.
In this perspective it might have been better to treat the placebo group also with an analgesic. However, this is difficult because gastro-intestinal side effects of conventional NSAIDs are much more severe in dogs than in humans (261). Furthermore, conventional NSAIDs could have had an adverse direct effect on the cartilage (67, 134). Opioids are also no option because they will cause interference with their walking / joint loading activity. Buprenorphine treatment, which is used as a painkiller for 3 days after surgery, showed loss of appetite. Paracetamol (acetominophen) causes in dogs toxicity after repetitive administration of therapeutic doses (262, 263). In this respect it is remarkable that the prolonged usage of celecoxib showed no signs of side-effects. The veterinary market might in this respect offer a new application of this selective COX-2 inhibitor.
All together this study showed no beneficial effect of celecoxib on the proteoglycan turnover of experimentally induced OA. It could be that celecoxib had been beneficial to degenerated cartilage *in vivo* but that these effects were counteracted by the increased loading of the affected joint and with that progression of OA, because of the analgesic effects of celecoxib. To establish whether this concept is plausible further research using e.g. gait analyses is needed.

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Chapter 8

Selective COX-2 inhibition is beneficial for matrix turnover of osteoarthritic cartilage: a clinical study.

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Abstract

Objectives: Selective COX-2 inhibitors are prescribed for many disorders including osteoarthritis (OA), a degenerative joint disease with an incidence exceeding 10% of the adult population. Recent *in vitro* studies showed a positive direct effect of celecoxib, one of the selective COX-2 inhibitors, on human OA cartilage. Such effects are difficult to verify in a clinical trial because changes in OA cartilage, degenerative and reparative, are slow and evaluation of articular cartilage by imaging techniques is still hampered by their limited sensitivity. Therefore, we used a novel approach in which the benefits of *in vivo* treatment are combined with the benefits of *ex vivo* biochemical analyses of the cartilage.

Methods: Patients with knee OA were treated 4 weeks prior to planned knee replacement surgery with celecoxib 2dd200mg, naproxen 3dd250mg, indomethacin 2dd50mg, or received no treatment. During surgery cartilage was collected and analyzed *ex vivo*.

Results: When compared to non-treated patients, celecoxib treated patients showed significant beneficial effects on proteoglycan synthesis, -release, and -content, confirming our *in vitro* data. Naproxen showed similar although less outspoken, positive results. In the indomethacin group, no statistical differences were found compared to the control group, but a tendency towards lower proteoglycan content was observed. In all treated groups prostaglandin-E_2 levels were lower than in the control group, indicating COX-2 inhibition; in the celecoxib group this effect was most outspoken.

Conclusions: Using this novel approach we were able to demonstrate an *in vivo* generated beneficial effect of celecoxib and naproxen, in contrast to indomethacin, on OA cartilage proteoglycan turnover.

Introduction

Osteoarthritis (OA) is a slowly progressive degenerative joint disease, with a high incidence, characterized by gradual loss of articular cartilage. Pain is the most important symptom in patients with OA (2, 9). The first choice for pharmacological pain management in patients with OA, considering the guidelines of the European League Against Rheumatism (Eular) and the American College of Rheumatology (ACR) (264, 265), is still acetaminophen. But recognized studies indicate that, especially in patients with severe pain, nonsteroidal anti-inflammatory drugs (NSAIDs) are superior (266) and patients with OA showed a greater preference for NSAIDs than for acetaminophen (267, 268). Data from the period 1995-1998 showed that 33% of the patients with OA used an NSAID, at least three times more than acetaminophen was used (269).

The anti-inflammatory and analgesic effects of NSAIDs are mainly due to their ability to suppress cyclooxygenase (COX), an enzyme involved in the production of prostaglandins. The clinical efficacy and side-effects with respect to

gastrointestinal problems are well known (191). Cardiovascular side-effects of the second generation NSAIDs, the COXIBs or selective COX-2 inhibitors, became recently evident (84, 192, 270, 271). However, this has always been a concern for the conventional NSAIDs as well (193, 272).
Another problem of NSAIDs might be their direct (adverse) effects on cartilage. Direct effects of NSAIDs on cartilage may be of importance, specifically in prolonged treatment of joint disease in which inflammation is only mild and secondary as in OA.
Data on direct effects of conventional NSAIDs on cartilage are numerous, but results are far from conclusive (60, 134). Two frequently used NSAIDs that have been studied regarding their direct effects on cartilage are naproxen and indomethacin. Studies on the influence of naproxen show conflicting results; there are signs that naproxen suppresses cartilage proteoglycan synthesis in vitro (60, 273), other studies however show suppression of proteoglycan degradation (199, 274). Indomethacin shows mainly negative effects on the biochemical parameters of cartilage, in in vitro (60, 273) and animal studies (275, 276), but there are also studies that could not demonstrate any effect of indomethacin on cartilage (277, 278).
Data on selective COX-2 inhibitors are scarce. Recent *in vitro* data on celecoxib show positive effects on cartilage from OA patients (139, 173). Celecoxib had favorable effects on the turnover of proteoglycans in OA cartilage, especially by diminishing the enhanced release of proteoglycans, and to a lesser extend by an increase of synthesis of proteoglycans, while retention of newly synthesized proteoglycans was beneficially influenced as well. However, these data could not be confirmed in an animal study using the canine Groove model of OA (228) in which no effect of celecoxib on the characteristics of experimentally induced OA *in vivo* could be demonstrated, as described in chapter 7. It was suggested that celecoxib could have been beneficial to degenerated cartilage *in vivo* but that these effects were counteracted by the increased loading of the affected joint because of the analgesic effects of celecoxib.
Direct effects of NSAIDs on cartilage cannot be studied easily in clinical trials and therefore they are generally ignored in clinical practice. Effects of NSAIDs on inflammation shade their direct effects on cartilage. In addition (intrinsic) cartilage changes, catabolic and anabolic are generally very slow processes in OA and evaluation of cartilage degeneration by imaging techniques is hampered by the limited sensitivity of these techniques. Therefore we used a novel approach in which the benefits of *in vivo* treatment are combined with the benefits of *ex vivo* analyses of the cartilage. By treating patients with NSAIDs/COXIBs shortly before joint replacement surgery, significant amounts of cartilage tissue that have been exposed *in vivo* to the medications become available at the moment of replacement for *ex vivo* detailed biochemical and histochemical analyses. The

benefit over a clinical trial is that it gives us the opportunity to study the effects of NSAIDs/COXIBs on OA cartilage treated *in vivo* in detail and in a relatively short period of time.
The aim of this study was to investigate the effects of treatment of patients with severe knee OA for four weeks with celecoxib, in comparison with the conventional NSAIDs, naproxen and indomethacin, and compared to no treatment.

Material and methods

Patients

Thirty-nine patients with knee osteoarthritis, who were eligible for total knee replacement surgery, have been included between December 2004 and June 2005. Patients were treated at the University Medical Center of Utrecht (UMCU n=17), the Sint Franciscus Gasthuis Hospital in Rotterdam (n=11), and the Diakonessenhuis Hospital in Utrecht (n=4), all in the Netherlands.
Exclusion criteria were a total knee replacement for other reasons than OA, patients with a history of gastrointestinal bleedings or perforation, and patients with an increased risk for cardiovascular diseases (cardiovascular diseases in history, patients with untreated hypertension, patients with angina pectoris, and patients on oral anticoagulantia). Patients already on NSAIDs had to stop their medication and switch to the study-medication. The study was conducted according to the declaration of Helsinki and received ethical approval in all centers. Each patient put on medication gave written informed consent before participating in the study.

Study design

Patients with knee OA were treated 4 weeks prior to knee replacement surgery with celecoxib 2dd200mg, naproxen 3dd250mg, indomethacin 2dd50mg, or received no treatment. Because of their platelet-inhibiting effect, naproxen- and indomethacin-usage had to be stopped three days prior to surgery and because of the increased risk for gastrointestinal adverse effects with the use of naproxen and indomethacin, all patients on these medications also received omeprazol (1dd20 mg). Controls did not use NSAIDs according to their medical records and were informed explicitly not to take NSAIDs for at least 7 days prior to surgery.
At joint replacement surgery, cartilage with underling bone was obtained from femoral condyles and tibial plateaus. This cartilage was kept in phosphate buffered saline (PBS) for less than 4 hours and subsequently processed under laminar flow conditions at the UMC Utrecht. The investigators who performed the experiments and analyses were blinded to the patients' clinical data and medication use. The cartilage surface (collected) was graded by a rough scale to verify that replacement was performed on joints with a comparable degree of

degeneration. 0 = fibrillation or focal degeneration, 1= degeneration at multiple locations, 2 = degeneration at multiple locations with focal lesions, and 3 = degeneration throughout the tissue with severe focal lesions and focally full cartilage abrasion. After scoring, cartilage with sufficient thickness despite significant degradation was cut aseptically from the underlying bone. The slices were cut into squares and weighted (range 5-15 mg, accuracy 0.1 mg). Four randomly taken samples of each donor were fixed in 4% phosphate buffered formalin in 2% sucrose and stained with safranin-O fast green-iron haematoxylin for histochemistry. In addition twenty randomly taken cartilage samples of each donor were used for biochemical analysis of proteoglycan synthesis, -retention, -release, -content, prostaglandin-E_2 (PGE_2) production. Because of focal differences in composition and bioactivity of the knee cartilage, specifically in case of severe OA, the results of 20 cartilage samples per parameter per patient, obtained at random and handled individually, were averaged and taken as a representative value for the cartilage of that patient.

Histochemistry

The four randomly taken cartilage samples were graded for features of OA, using the modified Mankin criteria (141, 142). Two observers blinded to the source of the cartilage graded the cartilage and the averages of the two observers and the four samples were taken as representative score of each donor.

Proteoglycan analysis

As measure of the proteoglycan synthesis rate, sulfate incorporation rate was determined over a 4-hour period. After 1 hour of pre-culture (equilibration) culture medium was refreshed and $Na_2SO_4^{2-}$ (DuPont NEX-041-H, carrier free) 370 kBq added (96-well round bottomed microtiter plate; 200 μl culture medium/well 37°C, 5% CO_2 in air). Culture medium consisted of Dulbecco's modified Eagle's medium (DMEM), supplemented with glutamine (2 mM), penicillin (100 IU/ml), streptomycin sulfate (100 μg/ml) ascorbic acid (0,085 mM) and 10% heat inactivated pooled human male AB^+ serum. After the 4 hour labeling period, the cartilage explants were rinsed once, washed three times for 45 min in culture medium under culture conditions, and subsequently cultured, individually for an additional period of 3 days. After this period cartilage and culture medium were separated, cartilage was rinsed in cold PBS and both, cartilage samples and culture medium were snap frozen and stored at -20°C for no longer than 7 days.

For proteoglycan synthesis and -content, cartilage samples were digested (2h, 65 °C) in papain buffer as described before (143). Papain digests were diluted to the appropiate concentrations and glycosaminoglycans (GAGs) were stained and precipitated with Alcian Blue dye solution (144). The pellet obtained after centrifugation (9000 g, 10 min) was washed once (sodium acetate (NaAc) buffer

containing 0,1 M $MgCl_2$) and subsequently dissolved (sodium dodecyl sulphate (SDS). The $^{35}SO_4^{2-}$ radioactivity of the sample was measured by liquid scintillation analysis after addition of Picofluor-40 (Packard). $^{35}SO_4^{2-}$ incorporation was normalized to the specific activity of the medium, labeling time and wet weight of the cartilage samples. The proteoglycan synthesis rate is expressed as nanomoles of sulphate incorporated per hour per gram wet weight of the cartilage (nmol/h.g). 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 per gram wet weight of cartilage tissue (mg/g).
Part of the culture medium was used to analyze release of newly formed proteoglycans and total proteoglycan release. For release of proteoglycans, GAGs were precipitated from the 3-day culture medium and stained with Alcian blue (144). The radio-labeled GAGs were measured by liquid scintillation analysis and normalized to the proteoglycan synthesis rate and expressed as percentage release of newly formed proteoglycans, being a measure of retention of the newly formed proteoglycans. For the total release of proteoglycans blue staining was quantified photometrically by the change in absorbance at 620 nm (chondroitin sulphate used as a reference). Values were normalized to the GAG content of the explants and expressed as a percentage release.

Prostaglandin-E_2 analyses
Prostaglandin-E_2 (PGE_2) was determined in the remaining part of the 3 day culture medium. Prostaglandin-E_2 was determined by Enzyme Immuno Assay (EIA, Caymann Chemical) and expressed as pg per mg wet weight of cartilage tissue.

Calculations and statistical analysis
The averages of the results of the 20 samples for biochemical parameters and 4 samples for histochemistry were taken as representative values of each cartilage donor. Means of at least 8 donors per group ± SEM are given. Statistical evaluation of the effects of treatment was performed with an independent-sample T-test. P values less than or equal to 0,05 were considered statistically significant.

Results

Baseline characteristics
Average age and gender were comparable between the different treatment groups (table 1). All donors had a score of 3 for macroscopic damage. The average histological grade of cartilage damage ranged from 4.5 to 5.5 in the different groups, not statistically different between groups (table 1). It should be kept in mind that only the cartilage with sufficient thickness that could be cut from the joint surfaces after replacement surgery was used. Thus in fact the entire joint

had a worse appearance as represented by the macroscopic score, than represented by the modified Mankin score of the cartilage used. A representative light micrograph of the cartilage that was biochemically analysed in depicted in figure 1. It clearly shows the typical characteristics of OA cartilage including loss of safranin-O staining, fissures and irregularities of the articular surface and disturbance of the chondrocyte distribution (clusters of chondrocytes).

Table 1. *Baseline characteristics for the different treatment groups*

	controls (n=8)	indomethacin (n=8)	naproxen (n=11)	celecoxib (n=12)
Age (years)	65 ± 3	69 ± 3	65 ± 3	70 ± 3
Sex (female/male)	8/0	5/3	9/2	10/2
Macroscopic cartilage damage	3	3	3	3
Histological cartilage damage	5 ± 1	5 ± 1	4 ± 1	5± 1

Age, macroscopic appearance of the cartilage surface, and histological cartilage damage are presented as means ± SEM, sex is presented as a ratio. There were no statistical significant differences between different groups for either of the parameters.

Effects of NSAIDs on prostaglandin-E_2 production

To evaluate whether the different NSAIDs had been able to modulate the enhanced cyclooxygenase (COX)-2 production of the cartilage, the prostaglandin-E_2 production during the first 3 days of *ex vivo* culture was measured. As shown in figure 2, prostaglandin-E_2 production was diminished for cartilage obtained from all three treated groups when compared to the controls ($p \leq 0.004$, $p \leq 0.009$ and $p \leq 0.001$, respectively). Although not statistically different from indomethacin and naproxen, the effect of celecoxib treated patients was the most pronounced.

Effects of NSAIDs on proteoglycan turnover

The non-treated patients (controls) showed a proteoglycan synthesis rate, -retention, -release, and -content typical for osteoarthritic cartilage, as compared to previous studies (143) (figure 3A-D). Patients who had used celecoxib for a period of 4 weeks showed a statistically significant higher proteoglycan synthesis rate compared to the controls ($p \leq 0.03$) and a tendency towards a higher synthesis compared to the indomethacin group ($p \leq 0.09$). Also the naproxen group showed a trend towards a higher proteoglycan synthesis ($p < 0.10$) compared to the controls. The indomethacin treated group did not differ from the controls.

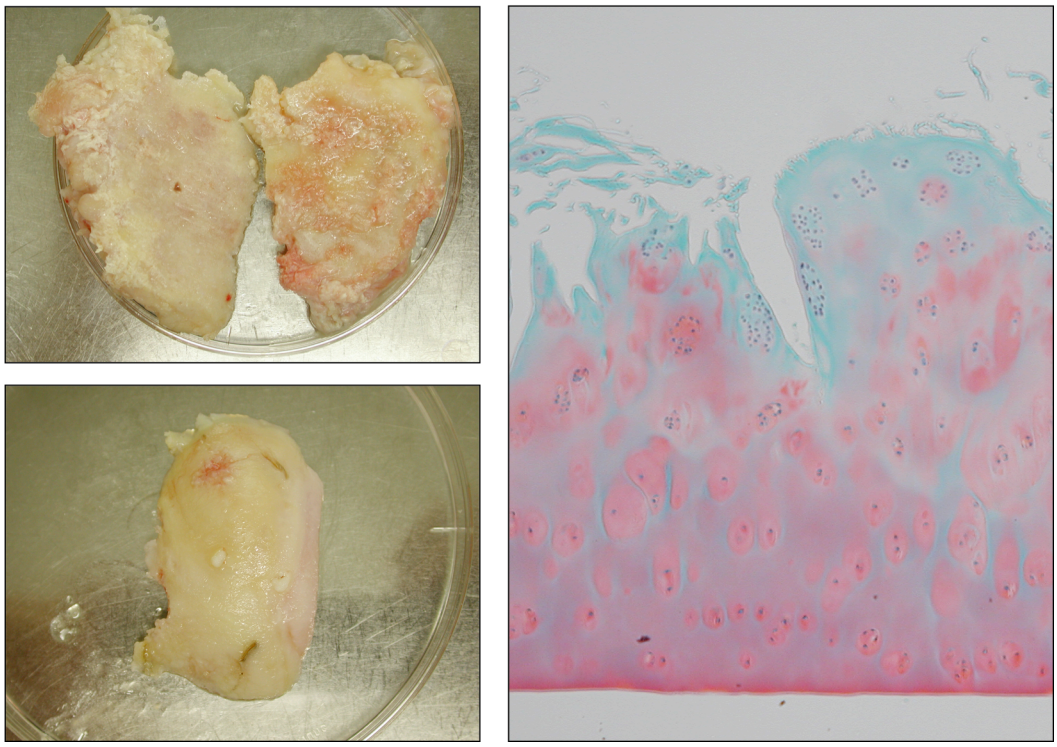

Figure 1. *Macroscopic and histological image of the osteoarthritic cartilage.*
Representative picture of plateau and condylar cartilage obtained at joint replacement surgery and light micrograph of this cartilage. Macroscopic score is 3. Sections are stained with safranin-O fast green-iron haematoxylin and graded for features of osteoarthritis according to the slightly modified criteria described by Mankin. Score for this sample is 6 (see appendix).

This increased proteoglycan synthesis was accompanied by a better retention of the newly formed proteoglycans; both celecoxib and naproxen had a diminished release of newly formed PGs compared to the untreated controls ($p \leq 0.008$ and $p \leq 0.018$, respectively), whereas indomethacin showed no difference compared to controls (figure 3B).
Also for the total release of proteoglycans, the newly formed and the resident ones, significant lower values were found for both the celecoxib ($p<0.01$) and naproxen ($p \leq 0.03$) treated patients compared to the non-treated controls. Indomethacin treatment showed a tendency towards a lower total proteoglycan release (figure 3C; $p<0.10$)
Most interestingly, a treatment period of 4 weeks resulted even in a significant higher proteoglycan content in the celecoxib and naproxen group compared to the non-treated patients ($p<0.05$ and $p<0.01$, respectively) and compared to the indomethacin group ($p \leq 0.026$ and $p \leq 0.022$, respectively) (figure 2D). The indomethacin group showed even a tendency towards a lower content compared to the non-treated controls ($p \leq 0.14$).

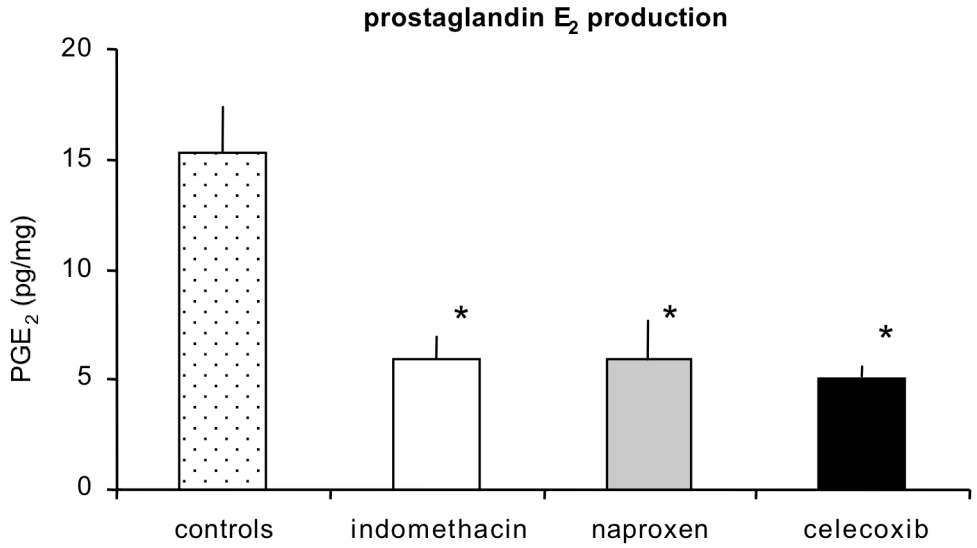

Figure 2. *Effects of three NSAIDs on PGE_2 production*
Ex vivo production of prostaglandin-E_2 by cartilage of the four different treatment groups. The results are presented as means ± SEM. Statistically differences for the effect of an NSAID compared to non-treated patients (controls) is depicted by an asterisk.

Discussion

This study evaluated the *in vivo* effect of celecoxib, a selective COX-2 inhibitor, compared to frequently used conventional NSAIDs on human osteoarthritic cartilage using detailed biochemical *ex vivo* evaluation.
To our knowledge this is the first study showing *in vivo* that celecoxib has a favorable effect on multiple cartilage matrix proteoglycan parameters of human osteoarthritic cartilage using an *ex vivo* evaluation. Moreover, it is the first time that the *in vitro* obtained data on human cartilage tissue in this respect (139, 173) have been verified *in vivo/ex vivo*.
A great advantage of using our approach is the opportunity to perform a full detailed biochemical analysis of the articular cartilage while the treatment was given *in vivo*. A drawback is that we cannot rule out the indirect effects of the NSAID /COXIB treatment on inflammation in the joint and with that indirect effect on cartilage. Another drawback of the approach might be that the condition of the cartilage is not known before start of treatment. However, it is expected that histochemical grading of the cartilage will not change significantly in a 4-week period. Moreover, the different treatments were assigned at random.
Interestingly, the changes in prostaglandin-E_2 production *ex vivo* determined after *in vivo* exposure demonstrated an almost identical pattern as obtained after *in vitro* exposure of human cartilage to the NSAIDs used as described in chapter 5. Celecoxib treatment resulted in the clearest reduction in prostaglandin-E_2 production (with the lowest p value).

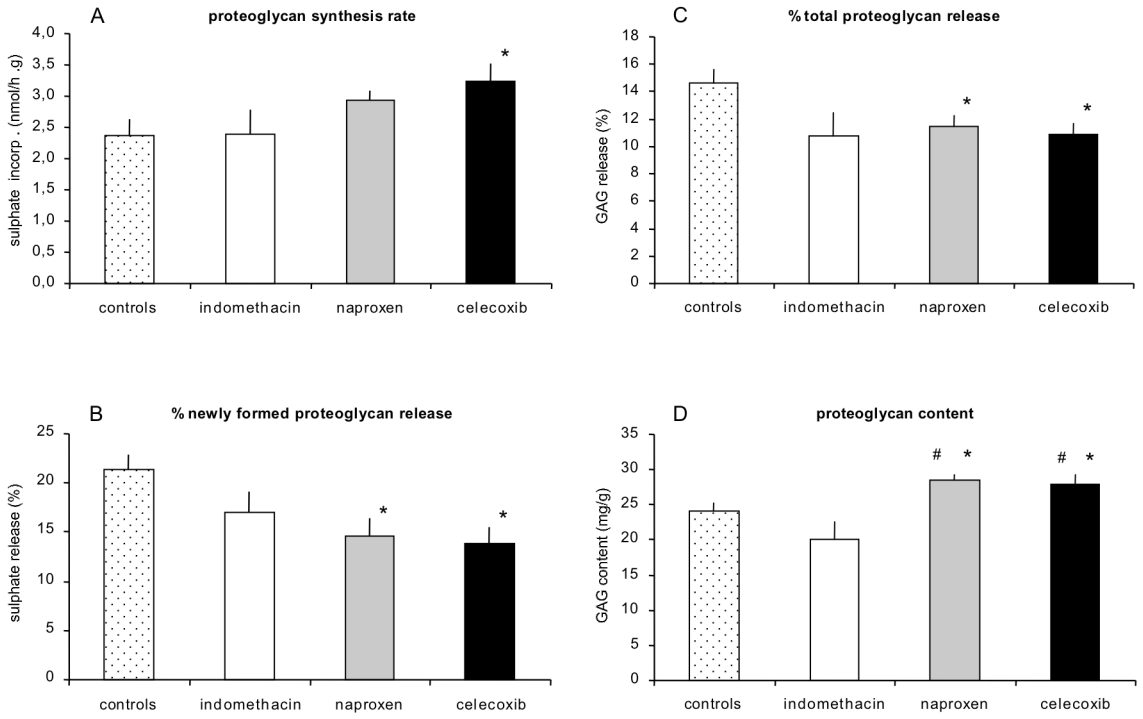

Figure 3. *Effects of three NSAIDs on proteoglycan turnover and content of OA cartilage.*
PG synthesis rate (A), percentage of newly formed proteoglycan release (B), percentage total proteoglycan release (C), and proteoglycan content (D) are shown. The results are presented as means ± SEM. Statistically differences for the effect of an NSAID compared to non-treated patients is indicated by an asterisk (*), and compared to the indometacin group by a wicket (#).

Based on the classification of Warner *et al.* (201) naproxen and indomethacin, as non-selective NSAIDs, have a relative selectivity for COX-1 while celecoxib is COX-2 selective. This might explain while celecoxib inhibits more pronounced the prostaglandin-E_2 production, since celecoxib is more effective in inhibiting COX-2, which produces these huge amounts of prostaglandin-E_2; COX-1 produces only small amounts of prostaglandins.
The effects of celecoxib on cartilage are very similar as those observed after *in vitro* exposure of human cartilage to celecoxib (139, 173). A beneficial effect of celecoxib on normal human cartilage under the influence of IL-1β and TNFα (139), as well as on human osteoarthritic cartilage, both early and late osteoarthritic (140, 173) was shown. For the first time the supposed chondro-reparative effects of celecoxib are now confirmed *in vivo*.

The positive effects of naproxen on proteoglycan synthesis, -release, and -content, however, are not as clear as in our *in vitro* study as described in chapter 5. In that study naproxen was found to be neutral with a tendency towards a negative influence on cartilage. However, available literature on the effects of naproxen on osteoarthritic cartilage is contradictory; other *in vitro* research shows inhibitory effects on proteoglycan synthesis (60, 273), and on proteoglycan release (199, 274). In the present study the effect of treatment on synovial inflammation, and thus indirectly on cartilage, certainly plays a role. In that respect, naproxen might have contributed to cartilage repair via inhibition of inflammation as well, explaining the difference with the less favorable effects obtained *in vitro*.
For indomethacin a tendency towards a negative effect on proteoglycan content was found when compared to controls. When compared to the celecoxib and naproxen treated group this effect was even statistically significant. Indomethacin showed a trend towards a better retention of newly formed proteoglycans and a diminished release, although both not statistically significant. Effects on the prostaglandin-E_2 pathway cannot solely explain this, as production of prostaglandin-E_2 did not differ from the naproxen treated group. This suggests involvement of additional, COX independent, pathways.
The lack of an effect of indomethacin could be due to the fact that indomethacin was (unavoidably) stopped three days prior to operation. With a relatively short half-life time, detectable effects of indomethacin on cartilage might have been missed. However the prostaglandin-E_2 levels of the indomethacin treated patients were significantly decreased demonstrating detectable efficacy of indomethacin even when stopped for 3 days prior to cartilage evaluation. Moreover, also naproxen, showing effects on cartilage, was stopped 3 days before surgery. However, naproxen has a longer half-life time and the effect of naproxen on the cartilage might thus have been extended during the last 3 days. Naproxen treated patients and the celecoxib treated patients, the latter treated until the last day before surgery, showed similar beneficial results. This suggests that cessation of treatment 3 days prior to surgery did not obstruct detection of *ex vivo* effects on the proteoglycan turnover.
Although it is clear that chondro-reparative effects can be obtained by NSAID treatment, most clearly by treatment with celecoxib, further research is indicated to evaluate the effects on synovial inflammation in relation to changes in cartilage and to evaluate the possible difference in effects when treatment is stopped 3 days prior to surgery.
The recent developments about the increased risk for cardiovascular side-effects with the use of selective COX-2 inhibitors and possible all conventional NSAIDs have put a new light on the use of these medications. Further research is now on the way to clarify the risk of the individual NSAIDs at cardiovascular events. In the

meantime the risk and benefits of different NSAIDs should be weighted very carefully in the treatment of OA patients.
Based on the present results, we can conclude that celecoxib most outspoken, but also naproxen, in contrast to indomethacin, have a chondro-reparative effect of human osteoarthritic cartilage *in vivo*. To what extend these effects are direct effects on cartilage or are obtained indirectly via control of synovial inflammation needs further study. Notwithstanding, these effects should be kept in mind when prescribing NSAIDs/COXIBs in treatment of osteoarthritis.

Chapter 9

Summary and General Discussion

Summary

The purpose of this PhD study was to reveal the direct effects of the selective COX-2 inhibitor, celecoxib (a second generation NSAID, viz. COXIB), on degenerated articular cartilage. The approach by using **human *in vitro*** studies, an **animal *in vivo*** studies and a **human clinical study** enabled me to answer this question. In addition this approach added to the insight in the role of inflammatory mediators including COX-2 in degenerated cartilage, added to the understanding of the balance between pain relieve and a possible risk of further joint damage due to increased joint use, and added to the translation of *in vitro* results to clinical practice. The overall results of the different approaches are complementary and should be taken into account when prescribing NSAIDs in clinical practice.

NSAIDs diminish inflammation and inflammatory mediators, which make NSAIDs indirectly beneficial to cartilage under inflammatory conditions, as in rheumatoid arthritis. However, direct effects of NSAIDs on cartilage have frequently been reported to be adverse, though beneficial and neutral effects have been reported as well. Direct effects of NSAIDs on cartilage are of specific importance in case treatment is prolonged and when inflammation is only mild and secondary as in osteoarthritis. Because these direct effects are not visible during clinical evaluation and are shadowed by the effects on inflammation, they are generally ignored. Moreover, in a clinical study set-up evaluation of such effects remain difficult to assess. Changes in cartilage, catabolic and anabolic, are in general very slow processes and remain undetectable for months up to years. There is still a lack of tools that can detect the subtle changes *in vivo*; in general it takes 2 years before radiographic analysis can reliably detect changes in cartilage integrity. Therefore, development of novel techniques, such as MRI and biomarker evaluation, remains an important issue in the evaluation of cartilage changes *in vivo*.

While studies on the direct effects of NSAIDs on cartilage *in vitro* are numerous, but far from conclusive, studies on the second generation NSAIDs, the selective COX-2 inhibitors (COXIBs), are scarce. The topic of this thesis was to provide more insight in the direct effects of one of these selective COX-2 inhibitors, celecoxib on articular cartilage. Issues of major relevance to clinical practice since it is essential that compounds used to treat osteoarthritis do not impair the ability of chondrocytes to repair the already damaged extracellular matrix.

The first step was to study the ***in vitro* effect of celecoxib on cartilage matrix turnover under normal and inflammatory conditions (Chapter 2).** It was found that celecoxib had no direct effects on healthy human cartilage, which contrasts with the reported effects of some of the non-selective NSAIDs. In co-

cultures of healthy cartilage with inflammatory cells celecoxib reversed the disturbed proteoglycan turnover of the cartilage. When the proinflammatory cytokines IL-1β and TNFα were added to healthy cartilage this resulted in adverse changes of cartilage proteoglycan turnover. Celecoxib reversed the adverse effects on proteoglycan release (both newly formed and resident proteoglycans), -content, and (although less outspoken) -synthesis. These data demonstrated that under the influence of inflammatory mediators, COX-2 is upregulated in human cartilage, which results in a disturbed cartilage matrix turnover that can be prevented by celecoxib.

NSAIDs are widely used in treatment of osteoarthritis. The results obtained in chapter 2 raised the question whether these results were also applicable for osteoarthritic cartilage. For clinical practice it is relevant to study effects of NSAIDs on early stages of osteoarthritis, when treatment might be most effective. In **chapter 3** we therefore studied the ***in vitro* effects of celecoxib on human degenerated cartilage,** an early pre-clinical form of osteoarthritis (101), compared to human healthy cartilage and human end stage (clinically defined) osteoarthritic cartilage. Both degenerated and osteoarthritic cartilage showed characteristic changes in proteoglycan turnover. Celecoxib was able to improve proteoglycan synthesis of degenerated cartilage and to normalize proteoglycan release. Importantly, celecoxib was able to improve cartilage matrix integrity by enhancing proteoglycan content. Similar results were found for end-stage osteoarthritic cartilage; normal cartilage remained unaffected. These results demonstrate *in vitro* that celecoxib has a significant beneficial effect on proteoglycan turnover and content, not only on end-stage osteoarthritic cartilage, but also on more early stages of osteoarthritis, whereas healthy cartilage remains unaffected. This suggests chondroprotective properties of celecoxib in treatment of degenerative joint disorders.

Although proteoglycan release and content normalized under the influence of COX-2 inhibition, proteoglycan synthesis was only partially influenced. This led to the hypothesis that **prostaglandin-E_2 (PGE_2) plays an important role in disturbance of cartilage proteoglycan release** and to a lesser extent in cartilage proteoglycan synthesis, which was subject of study in **chapter 4**. Only when healthy cartilage was sensitized by mediators of cartilage degeneration, such as IL-1β plus TNFα, prostaglandin-E_2 was able to further enhance proteoglycan release. However, the enhanced proteoglycan synthesis was not further influenced by PGE_2. Addition of celecoxib as a selective COX-2 inhibitor, inhibited the by IL-1β/TNFα enhanced prostaglandin-E_2 production and almost complete normalized proteoglycan release, but synthesis remained unaffected again. Also the enhanced nitric oxide levels remained elevated. It appeared that

prostaglandin-E_2 levels correlated significantly with proteoglycan release whereas nitric oxide levels correlated significantly with proteoglycan synthesis. These data imply involvement of prostaglandin-E_2 in enhanced cartilage proteoglycan release but not synthesis; IL-1β/TNFα induced nitric oxide elevation seems to be involved in inhibition of proteoglycan synthesis, independent of prostaglandin-E_2 and with that is probably less sensitive to regulation by (selective) COX-2 inhibitors

NSAIDs inhibit both COX-1 and COX-2. This appears to be associated with the well-known gastrointestinal adverse events: the more COX-1-selective, drugs appear to have the tendency to cause more gastrointestinal damage. A more recent debate has started whether all NSAIDs, including more COX-2 selective drugs, may cause cardio-vascular side effects. Maybe also the direct effects on cartilage by NSAIDs are dependent on their COX-2 (un)selectivity. It could well be that the adverse effects on cartilage of some of the conventional NSAIDs results from inhibition of COX-1. For this reason, **chapter 5** evaluates the ***in vitro* effect of some frequently used NSAIDs on human osteoarthritic articular cartilage**. Effects of indomethacin and naproxen as non-selective NSAIDs with moderately COX-1 selectivity were compared with effects of aceclofenac (as moderately selective for COX-2) and the selective COX-2 inhibitor celecoxib, covering a range from COX-1 to COX-2 selectivity. Osteoarthritic cartilage showed the characteristic proteoglycan turnover; addition of indomethacin further inhibited proteoglycan synthesis. No significant effect on the proteoglycan release was found; proteoglycan content tended to decrease. Naproxen did not show changes for all parameters. In contrast, aceclofenac and, most outspoken, celecoxib were beneficial to OA cartilage. Both showed an increase in proteoglycan synthesis, and normalization of release. Importantly, both NSAIDs improved proteoglycan content. Inhibition of prostaglandin-E_2 production indirectly showed that all NSAIDs inhibited cyclooxygenase, the more COX-2 specific, the more pronounced. It was concluded that NSAIDs with low COX-2/COX-1 selectivity show adverse direct effects on osteoarthritic cartilage (which was confirmed by use of an experimental selective COX-1 inhibitor), whereas high COX-2/COX-1 selective NSAIDs did not show such effects and even might induce cartilage reparative effects.

The described data are promising, but they are obtained using an *in vitro* model. Although such *in vitro* systems have the advantage that the direct effects on osteoarthritic cartilage can be studied, without interference of synovial inflammation, extrapolation to the *in vivo* situation has its limitations. Therefore we used an animal model of osteoarthritis, the canine "groove" model. This model is based on surgically applied damage of the articular cartilage followed by transient forced loading of the affected joint and shows characteristics mimicking

human osteoarthritis. To establish whether the observed characteristics of degeneration in this model represents the surgically applied damage or whether they are the results of progressive features of osteoarthritis we evaluated this **"groove" model shortly after surgery**. This is described in **chapter 6**. Ten weeks after surgery osteoarthritic features were found, as originally described for the model. Proteoglycan synthesis, percentage release of newly formed proteoglycans, and that of total amount of proteoglycans were enhanced, whereas proteoglycan content was significantly diminished. Importantly, three weeks after surgery these characteristics of osteoarthritis were not yet evident. This clearly show that the characteristics observed 10 and 20 weeks after induction of joint degeneration in the groove model are not just the expression of the surgically applied damage but are the result of progressive features of (experimental) osteoarthritis.

In **chapter 7** we actually evaluated whether the beneficial *in vitro* **effects of celecoxib** could also be **demonstrated *in vivo***. Dogs received daily placebo, 100 mg celecoxib or 200 mg celecoxib orally. Induction of osteoarthritis using the groove model resulted in macroscopic and histological damage of cartilage, changes in cartilage proteoglycan turnover, loss of cartilage matrix proteoglycans, and slight synovial inflammation, all characteristic of early osteoarthritis. Surprisingly, none of the parameters was significantly changed upon celecoxib treatment. Nevertheless, synovial fluid prostaglandin-E_2 levels were dose-dependently diminished by celecoxib demonstrating that celecoxib had reached the joint in sufficient amounts. Using an *in vitro* set-up, canine cartilage under degenerative conditions was favourably influenced by celecoxib demonstrating that canine cartilage is sensitive to celecoxib. It could be that celecoxib had been beneficial to degenerated cartilage *in vivo* but that these effects had been counteracted by increased loading of the affected joint because of the well-known analgesic effects of celecoxib and the important role of joint loading in progression of joint damage in this model. However, if this is indeed the case has to be established.

The groove model allows *in vivo* evaluation in an early stage of osteoarthritis but also here the extrapolation to the human situation is limited. Evaluation of human cartilage in a clinical trial is difficult because, as mentioned above, changes in osteoarthritic cartilage, degenerative and reparative, are slow and evaluation of the cartilage by imaging techniques is still hampered by the limited sensitivity of these techniques. Therefore, we used an approach in which the advantages of in vivo (clinical) treatment were combined with detailed biochemical evaluation of the cartilage as performed in vitro. This approach is described in **chapter 8** where we studied the **effects of selective COX-2 inhibition after short-term**

treatment of patients with severe knee osteoarthritis. Patients were treated 4 weeks prior to a planned knee replacement surgery with celecoxib 2dd200mg, naproxen 3dd250mg, indomethacin 2dd50mg, or received no treatment. When compared to non-treated patients, celecoxib treated patients showed significant beneficial effects on proteoglycan synthesis, release and content, confirming our *in vitro* data. Interestingly, naproxen also showed similar though less outspoken, positive effects. In the indomethacin group, no significant differences were found compared to the control group, and a tendency towards lower proteoglycan content was observed. In all treated groups prostaglandin-E_2 levels were lower than in the control group, indicating COX-2 inhibition. Using this approach we demonstrated an *in vivo* generated chondro-reparative effect of celecoxib (the most outspoken) and naproxen, in contrast to indomethacin, in OA cartilage.

Based on the presented *in vitro* and *in vivo* work as described in chapters 2-8 the following theory of the COX-2 involvement in cartilage degeneration is made (figure 1).

Discussion

The involvement of inflammatory mediators in osteoarthritis

Osteoarthritis is a chronic disease with slowly progressing destruction of articular cartilage as a result from the failure of chondrocytes to maintain the balance between synthesis and degradation of the extra cellular matrix. Although osteoarthritis differs from rheumatoid arthritis, synovitis in osteoarthritis does occur, although significantly less outspoken. Osteoarthritic cartilage itself is a rich source of inflammatory mediators, including cytokines, nitric oxide and prostaglandins (279). Recently, a role for proinflammatory cytokines in the pathogenesis of osteoarthritis was proposed based on data from studies using human osteoarthritic cartilage and various animal models (280, 281). Furthermore, direct evidence of cytokine production associated with degenerative changes in osteoarthritic cartilage has been reported in a recent study (282), in which IL-1β and TNFα were shown to co-localize with matrix metalloproteinases in the superficial zones of articular cartilage. COX-2 is also found to be expressed in osteoarthritic tissues (78, 137), such as chondrocytes and synovial cells. Also elevated levels of prostaglandin-E_2 have been found in osteoarthritic cartilage. The fact that selective COX-2 inhibition by celecoxib results in improvement of cartilage matrix turnover and structure as shown in chapter 2 and 3 corroborates this thought of a more central role of proinflammatory stimuli in the pathogenesis of osteoarthritis.

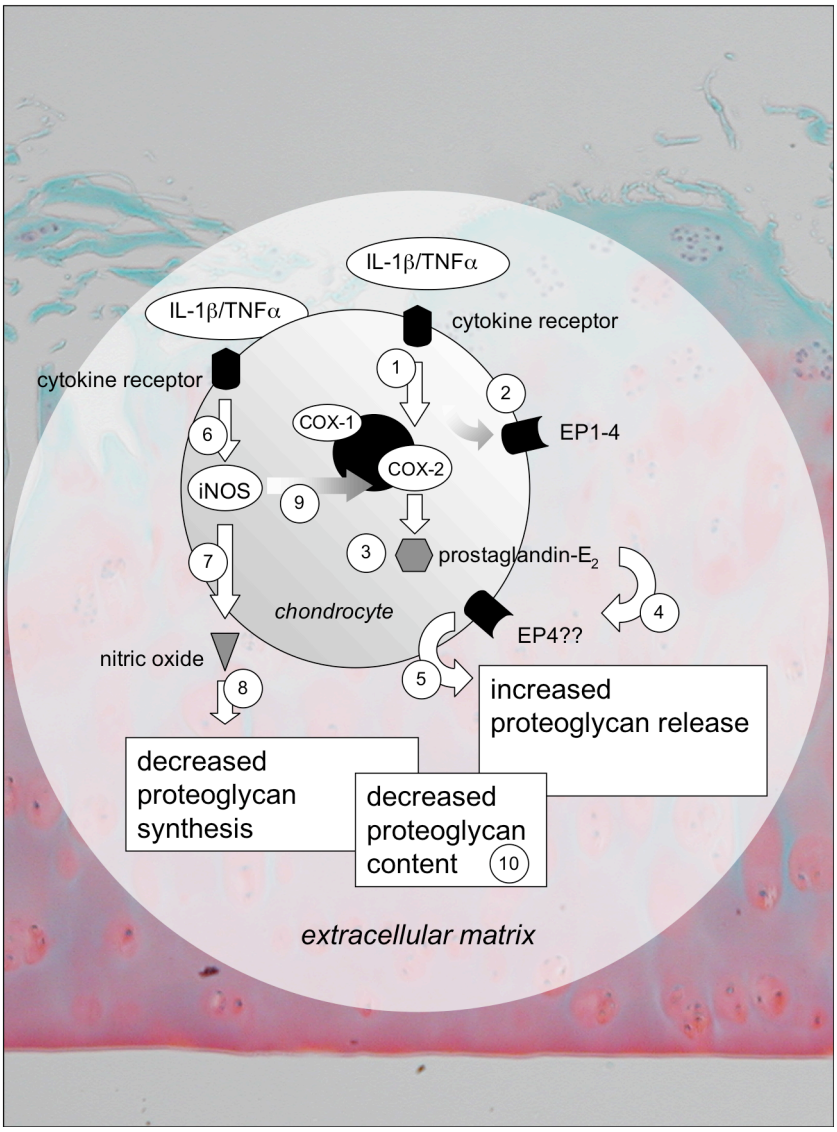

Figure 1: *Schematic representation of the mechanism of COX-2 mediated cartilage degeneration.* Upregulation of COX-2 by autocrine and paracrine produced mediators such as IL-1β/TNFα (1). Concomitant upregulation of the prostanoid receptors (EP 1-4) via COX-2 or other mechanisms (2). COX-2 upregulation results in large concentrations of prostaglandin-E_2 (3). Prostaglandin-E_2 stimulates, autocrine and paracrine, the EP4 receptor (4). This results in increased proteoglycan release (5). IL-1β/TNFα induces simultaneous activation of iNOS (6), which leads to formation of nitric oxide (7). This results among other effects, in a decreased proteoglycan synthesis (8) and may stimulate COX-2 production (9) concomitantly leading to enhanced release. The disturbance of proteoglycan synthesis and proteoglycan release will result in further loss of proteoglycan content (10) and with that to loss of cartilage matrix integrity. Selective COX-2 inhibition by celecoxib, amongst others, leads to inhibition of the COX-2 pathway and with that prostaglandin-E_2 production. This results in normalization of proteoglycan release, but not synthesis (see appendix).

However, the involvement of inflammatory mediators is not similar to the involvement of inflammation, as most of the mediators are produced by the chondrocytes themselves independent of inflammatory cells. Interestingly, as shown in chapter 3, selective COX-2 inhibition is already beneficial in an early phase of the osteoarthritis. This suggests an early involvement of COX-2 and related mediators in degenerative processes of cartilage. However, in chapter 4 we demonstrated that prostaglandin-E_2 alone has no effect on the proteoglycan turnover of healthy human articular cartilage. When cartilage is cultured in presence of IL-1β plus TNFα adverse changes in proteoglycan turnover of the cartilage are induced. When prostaglandin-E_2 is added to this sensitized cartilage prostaglandin-E_2 amplifies the adverse changes in proteoglycan turnover. A possible explanation for this lack of effect of protaglandin-E_2 on healthy cartilage could be the suppressed prostaglandin-E_2 receptor profile on healthy chondrocytes by which the biological actions of prostaglandin-E_2 are mediated.

COX-2 does not solely explain all adverse effects in cartilage degeneration

Interestingly, as demonstrated in chapter 4, when sensitized cartilage is treated with prostaglandin-E_2, only proteoglycan release is amplified, not proteoglycan synthesis. The other way around, when prostaglandin-E_2 production is inhibited (by selective COX-2 inhibition) only the enhanced release is diminished, not synthesis. We found a clear correlation between prostaglandin-E_2 production and proteoglycan release whereas the proteoglycan synthesis did not display such a correlation with prostaglandin-E_2 production. This regulation of proteoglycans release by prostaglandin-E_2 is in concordance with the studies of chapter 2 and 3 where inhibition of COX-2 resulted in normalization of proteoglycan release and only marginally influences proteoglycan synthesis. However, it is known that prostaglandin-E_2 is capable to inhibit the synthesis of collagens (182). There is also evidence that prostaglandin-E_2 has the capacity to activate matrix metalloproteinases (MMPs) (183), to stimulate collagenase gene expression in human synoviocytes (184), and to increase MMP-3 production of osteoarthritic cartilage explants (146). These effects might play a role in the increased proteoglycan release and decreased proteoglycan content as found in this thesis. Apparently, prostaglandin-E_2 (regulation) has not a direct effect on proteoglycan synthesis, suggesting other prostaglandin-E_2 independent pathways that regulate chondrocyte proteoglycan synthesis.

Previous studies showed that nitric oxide, being a product of affected chondrocytes as well, exerts a number of effects on synovial cell and chondrocyte functions which would be expected to promote degradation of cartilage including inhibition of collagen and proteoglycan synthesis (161), activation of MMPs (185), and apoptosis of cells (186, 187). A clear correlation between the nitric oxide production and proteoglycan synthesis and also proteoglycan release was

evident. Inhibition of prostaglandin-E_2 by using selective COX-2 inhibition has no effect on the nitric oxide production. There are a number of reports, corroborating this observation, indicating that inhibition of prostaglandin-E_2 production has no influence on nitric oxide production. However, inhibition of nitric oxide (not studied in our set-up) can lead to a change in prostaglandin-E_2 production (145, 146, 188). This may explain the correlation of nitric oxide production with proteoglycan release in addition to its correlation with synthesis.
Interestingly, in experiments using clinically defined osteoarthritic cartilage, correlations between prostaglandin-E_2 and proteoglycan release and nitric oxide production and proteoglycan synthesis as found in IL-1β/TNFα modulated cartilage, were not found (data not shown). This indicates that the more complex cartilage degeneration (and repair activity in the mean time) becomes, the more intermingled the different pathways of regulation of synthesis and release become.

The benefits of celecoxib treatment
In all cases selective COX-2 inhibition by celecoxib had no effect on healthy cartilage with respect to proteoglycan turnover. This contrasts several other conventional NSAIDs tested under comparable conditions, which showed adverse effects (60, 67, 99). This absence of an adverse effect of selective COX-2 inhibition was to be expected, as COX-2 is not produced in significant amounts in normal healthy cartilage. A possible explanation for this contrast is the expression of COX-1 in cartilage (both normal and osteoarthritic) and its inhibition by conventional NSAIDs. In chapter 5 it appeared that COX-2 selectivity, as recently classified by Warner *et al* (201), results in cartilage reparative properties, whereas the absence of COX-2 selectivity even results in negative effects. The adverse effects of an experimental COX-1 selective compound underscored the latter. These results underscore the important role for COX-2 in the disturbed proteoglycan turnover in osteoarthritis whereas COX-1 is involved in a more physiological role in the chondrocytes. This in concordance with the general accepted thought that COX-1 is regarded as the "housekeeping" isoform of cyclooxygenase and has clear physiological functions. Its activation leads, for instance, to the production of prostacyclin, which when released by the endothelium, is antithrombogenic (209), and when released by the gastric mucosa is cytoprotective (209). In contrast, COX-2 is excessively induced under inflammatory and detrimental conditions, such as osteoarthritis. This established concept has been modified by recent investigations demonstrating a significant participation of prostaglandins derived via the COX-1 pathway in some inflammatory processes (210-212), especially pain. Also the recent development with respect to cardiovascular side effect in relation to selective COX-2 inhibitors, and NSAIDs in general, forces us to reconsider the current concept. Nevertheless,

in the case of proteoglycan turnover in osteoarthritic cartilage the concept apparently still holds true.
Although the present results do not argue a conclusion that COX-1/COX-2 selectivity determines the harm/benefit ratio to cartilage, other COX selectivity unrelated characteristics of the tested NSAIDs might have been involved as well. Additional studies with other, even more selective COX-2 drugs may provide the answers.

Analgesic versus chondroprotective effects of celecoxib
The promising *in vitro* data and the lack of *in vivo* data from animal models of osteoarthritis urged to study selective COX-2 inhibition in such a model. The groove model of osteoarthritis (283) has features representative of human osteoarthritis (228). The model is distinctive in that the degenerative changes are progressive while synovial inflammation diminishes over time (229). Because of this, evaluation of direct effects of medication on cartilage is less hampered by a possible anti-inflammatory effect of treatment. Additionally the model is distinctive because there is no permanent trigger causing joint damage, making the model more sensitive to treatment. A permanent trigger for joint damage, such as joint instability used in the ACLT model, will counteract the possible beneficial effects of treatment. Assuming that cartilage repair is possible (64, 243, 244), the trigger, intrinsic to the cartilage damage itself, can be eliminated by treatment. In addition as described in chapter 6, the characteristics observed after induction of joint degeneration in the groove model are not just the expression of the surgically applied damage but are the result of progressive features of (experimental) osteoarthritis. However, this progression will largely depend on the forced (intensified) loading of the affected joint.
This canine groove model of osteoarthritis was chosen to study the effect of celecoxib on osteoarthritic cartilage *in vivo*. Chapter 7 describes the first study in which the *in vivo* effect of celecoxib on experimental osteoarthritic cartilage is evaluated. We were not able to confirm our positive *in vitro* results of celecoxib in this model. The osteoarthritic characteristics in the celecoxib treated groups were the same as in the placebo treated group, no effect of celecoxib on the severity of osteoarthritis was observed. It was proven in these experiments that celecoxib had reached the joint. In additional *in vitro* experiments it was demonstrated that COX-2 inhibition can be beneficial for canine cartilage under degenerative conditions. A possible explanation for the absence of an effect could be found in the different walking patterns as observed in the different treatment groups. Celecoxib treated dogs seemed to walk better and more when compared to the placebo group. This fits well with the analgetic effects of celecoxib. Knowing that loading is a prerequisite for the progression of osteoarthritis in this model, it could be that this analgesics effect had worsened the severity of cartilage degeneration. In that

respect, it could have been that celecoxib had been beneficial to degenerated cartilage *in vivo* but that these effects were counteracted by the increased loading of the affected joint. Might this observation have clinical implications? The efficacy of celecoxib in reducing knee pain in patients with osteoarthritis has been demonstrated in several studies (117, 248, 249). Recently, the effect of celecoxib *vs.* placebo treatment was compared on clinical and gait variables in knee osteoarthritis patients; focussing on the efficiency of the locomotor mechanism (250). In this study celecoxib treatment improved the efficiency of the locomotor mechanism significantly. Among the secondary outcome measures assessed, celecoxib treatment improved walking cadence and reduced the knee pain significantly. Increased loading of a joint is associated with OA pathology (251-256).
So, improved walking and loading in case of celecoxib treatment might result in undesired progression of osteoarthritis counteracting the direct beneficial effects on cartilage. Luckily the balance does not change to adverse effects.
Studies using animal model of osteoarthritis less depending on mechanical properties for progression of degeneration and more dependent on an inflammatory component might demonstrate whether our concept is true in this respect.

Translation of the in vitro results to clinical practice
Although the chondroprotective effects of celecoxib *in vitro* could not be confirmed in an *in vivo* animal model of osteoarthritis, they could be confirmed in vivo as described in chapter 8. Here we treated patients with knee osteoarthritis 4 weeks prior to planned knee replacement surgery with celecoxib, naproxen, indomethacin, or no treatment. *Ex vivo* analyses showed beneficial effects on proteoglycan synthesis, release and content in celecoxib treated patients, confirming our in vitro data. The fact that in this *in vivo* study chondroprotective effects were found may have several reasons. First, in this study indirect effects on inflammation might have been involved. Future analyses of the severity of inflammation based on histochemistry of the synovial tissue of these patients in comparison with the animal data might support this. Secondly, there might have been direct effects on cartilage. It is not expected, in contrast to the *in vivo* treated animals, that these patients changed their loading pattern of the affected knee significantly in this short period of treatment. Dogs can easily walk on three legs enabling them to adapt loading of the affected joint, much more easily than humans.
The indomethacin treated group, showed no significant differences compared to the control group, but a tendency towards lower proteoglycan content. The naproxen treated group also showed beneficial changes, although less pronounced as in the celecoxib treated group. The relative mild negative effect of

indomethacin and beneficial effect of naproxen contrast the in vitro findings and might be explained by the fact that the medication also influences synovial inflammation during the 4 weeks of treatment. By diminishing the synovial inflammation, indirect effects on cartilage proteoglycan turnover occur. This is different compared to the *in vitro* approach, where exclusively the direct effects on cartilage were studied.
Interestingly, the changes in prostaglandin-E_2 production of the cartilage *ex vivo* determined after *in vivo* exposure and obtained after *in vitro* exposure of human cartilage to the NSAIDs in chapter 5 displayed identical patterns. Celecoxib treatment resulted in the most outspoken reduction in prostaglandin-E_2 production. Based on the classification of Warner *et al* (201) naproxen and indomethacin, as non-selective NSAIDs, have a relative selectivity for COX-1 while celecoxib is only selective for COX-2 (as expected). This might explain while celecoxib inhibits more pronounced the prostaglandin-E_2 production, as celecoxib is more effective in inhibiting COX-2.
Although *in vitro* models cannot address all the events that occur in a (osteoarthritic) joint, they allow quantification of specific events and mechanisms involving direct effects of NSAIDs on cartilage. The fact that the results obtained in chapter 8 are in general confirming the results obtained with the use of our *in vitro* model supports that the usage of cartilage tissue explants *in vitro* is, even with its restrictions, a useful model.

Celecoxib being the choice of treatment of osteoarthritis?
The present results favor the use of selective COX-2 inhibitors in osteoarthritis, preferably in an early stage of the disease. However, the recent worries about an increased risk for cardiovascular side-effects with the use of NSAIDs, including selective COX-2 inhibitors, have thrown a new light on the use of these drugs in elderly (osteoarthritic) patients. Among NSAIDs, only aspirin has been proven to significantly reduce cardiovascular risk (284, 285), primarily through inhibition of COX-1 mediated platelet aggregation (286). It has been suggested that other nonselective agents, especially naproxen, may provide some lesser degree of cardioprotection (75, 193) but conclusive evidence is lacking (75). Conversely, there are concerns that the COX-2 inhibitors may increase cardiovascular risk (193, 287). However mechanisms for this potentially adverse cardiovascular effect are unknown, and it is becoming increasingly clear that our understanding of the role of COX-2 in cardiovascular function is incomplete. Some studies have demonstrated a potentially beneficial effect of COX-2 on cardiovascular function that could be negated by COX-2 inhibition (76), while other studies have reported improved endothelial function with COX-2 inhibitors (76). Additionally, the impact of combined therapy with aspirin and other COX-inhibitors is not yet clear. It is clear that further research is needed to clarify the risk of the individual NSAIDs for

cardiovascular events. In the meantime the risk and benefits of different NSAIDs should be weighted very carefully in the treatment of OA patients.
In that respect also, the beneficial effect of a selective COX-2 inhibitor on cartilage, irrespective of further side effects, is something that should be kept in mind in making a decision in prescribing NSAIDs in osteoarthritis, especially when long-term use is needed.

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Nederlandse samenvatting

COX-2 inhibitie in artrose: effecten op kraakbeen

COX-2 inhibitie in artrose: effecten op kraakbeen

Artrose

Artrose is een veel voorkomende degeneratieve gewrichtsaandoening waarvan de oorzaak niet volledig bekend is. Meer dan de helft van 65-plussers heeft artrose in één of meer gewrichten. Waarschijnlijk zijn er verschillende factoren nodig om artrose te ontwikkelen. Artrose wordt gekenmerkt door beschadiging van het kraakbeen in de gewrichten, verharding van het onderliggende bot en botwoekeringen op de randen van het gewricht. Tevens treedt regelmatig een milde ontsteking op in de binnenbekleding van het gewricht, het synovium. Deze ontsteking kan bijdragen aan de gewrichtsschade. Tot nu toe kan artrose niet worden genezen en is behandeling ervan meestal gericht op het verminderen van pijn, het versterken van spieren rond het gewricht en indien nodig het onderdrukken van ontsteking. In eindstadia van artrose is het vastzetten of vervangen van het gewricht door een kunstgewricht vaak de enige oplossing.

Niet Steroide Anti-Inflammatoire Drugs (NSAIDs) staan bekend om hun pijnstillende en ontstekingsremmende werking. Deze middelen hebben naast bijwerkingen op het maagdarmkanaal en de nieren, mogelijk ook een negatief direct effect op kraakbeen.
De bijwerkingen van NSAIDs worden onder andere veroorzaakt door het werkingsmechanisme van deze groep medicamenten. Dit werkingsmechanisme is gebaseerd op remming van het enzym cyclooxygenase (COX). Dit enzym bestaat in twee vormen, namelijk COX-1 en COX-2. COX-1 heeft een belangrijke rol in allerlei "huishoudelijke" processen (waardoor bij remming ook de bijwerkingen ontstaan), terwijl COX-2 een grote rol speelt bij ontstekingen. Dit heeft tot de ontwikkeling van zogenaamde selectieve COX-2 remmers geleid. Deze nieuwe groep NSAIDs hebben dezelfde ontstekingsremmende en pijnstillende werking maar hebben veel minder bijwerkingen op het maagdarmkanaal en de nieren. Dit is al in verschillende grote studies aangetoond. Het effect van deze middelen op kraakbeen is echter minder goed bekend.
Bij reumatoïde artritis (RA) ontstaat kraakbeenschade als gevolg van ontsteking. Het onderdrukken van ontsteking ter voorkoming van kraakbeenschade bij RA staat centraal in de behandeling van RA. Dit in tegenstelling tot de behandeling van gewrichtschade bij artrose. De kraakbeenschade bij artrose is een intrinsiek proces van het kraakbeen waaraan ontsteking slechts bijdraagt. Een mogelijk negatief direct effect van NSAIDs op kraakbeen kan daardoor bij artrose bijzonder nadelig zijn.

Het doel van dit proefschrift was het onderzoeken van de directe effecten van de selectieve COX-2 remmer celecoxib op artrotisch kraakbeen. Door gebruik te maken van *in vitro* (laboratorium) studies, een *in vivo* dierstudie en een klinische studie was het mogelijk om hier een antwoord op te krijgen. Daarnaast leverde deze benadering meer inzicht in de rol van verschillende ontstekingsmediatoren,

waaronder COX-2, in kraakbeenschade. Ook gaf deze benadering meer inzicht in de rol van pijnstilling, en daardoor toegenomen gebruik van het gewricht, in relatie tot een mogelijk risico van verdere beschadiging van het gewricht. Eveneens resulteerde dit onderzoek in translatie van *in vitro* resultaten naar de klinische praktijk. De resultaten van al deze verschillende benaderingen zijn complementair en zouden in overweging moeten worden genomen wanneer deze middelen in de kliniek worden voorgeschreven.

De rol van ontstekingsmediatoren in artrose

Artrose is een chronische ziekte waarbij langzaam het gewrichtskraakbeen degenereert als gevolg van het falen van kraakbeencellen in het handhaven van een balans tussen aan- en afbraak van de kraakbeenmatrix. Hoewel artrose anders is dan reumatoïde artritis, is er ook ontsteking van de binnenbekleding van het gewricht (synovitis), maar in mindere mate. Artrotisch kraakbeen zelf is een rijke bron van mediatoren die ontsteking induceren en/of handhaven, bijvoorbeeld cytokinen, stikstof oxide en prostaglandinen. Deze mediatoren spelen dan ook een belangrijke rol in de afbraak van kraakbeen. Een andere mediator die gevonden wordt in artrotisch weefsel zoals kraakbeen en synovium, is cyclooxygenase-2, ook wel COX-2 genoemd. Ook verhoogde hoeveelheden van prostaglandine-E_2, het belangrijkste product van COX-2, worden hier gevonden.
In hoofdstuk 2 staan beschreven de *in vitro* effecten van celecoxib (een van de selectieve COX-2 remmers) op de aan- en afbraak van humaan kraakbeen en zijn matrix onder normale en ontstekingscondities. Proteoglycanen zijn een belangrijk bestandsdeel van deze kraakbeenmatrix. Het bleek dat celecoxib geen directe effecten had op gezond kraakbeen, wat in tegenstelling is met sommigen van de veel gebruikte oudere niet-selectieve NSAIDs.
Wanneer gezond kraakbeen in combinatie met ontstekingscellen werd gekweekt ontstond er een verstoorde proteoglycaan aan- en afbraak en dus een verstoorde kraakbeenmatrix. Wanneer celecoxib werd toegevoegd aan deze kweken werd de verstoorde proteoglycaan aan- en afbraak voorkomen en bleef normaal.
Ook wanneer de ontstekingsmediatoren IL-1β en TNFα aan gezond humaan kraakbeen werd toegevoegd ontstond er wederom een verstoorde proteoglycaan aan- en afbraak. Ook deze verstoring kon door de toevoeging van celecoxib worden voorkomen.
Het feit dat remming van COX-2 met de selectieve COX-2 remmer celecoxib resulteert in een verbetering van de aan- en afbraak van de kraakbeenmatrix, ondersteunt de gedachte van een meer centrale rol van deze ontstekingsmediator in het ontstaan van artrose.

Naar aanleiding van de resultaten beschreven in hoofdstuk 2 kwam dan ook de vraag naar voren of celecoxib ook gunstig zou zijn voor artrotisch kraakbeen, gekenmerkt door een verstoorde proteoglycaan aan- en afbraak. In de klinische praktijk is het van belang om zo vroeg mogelijk met behandeling te kunnen beginnen omdat dan de behandeling waarschijnlijk het meest effectief zal zijn. In

hoofdstuk 3 hebben we de studie beschreven waarin we de *in vitro* effecten van celecoxib op humaan pre-klinische (vroege vorm van) artrotisch kraakbeen hebben bestudeerd. De effecten hebben we vergeleken met die gevonden bij humaan gezond kraakbeen en humaan artrotisch kraakbeen (klinisch gediagnosticeerd). Zowel het pre-klinische als het artrotisch kraakbeen lieten hun karakteristieke verandering in proteoglycaan aan- en afbraak zien ten opzichte van het gezonde kraakbeen. Dit kenmerkte zich door een verlaging van de aanmaak van proteoglycanen, een verhoogd verlies van deze proteoglycanen, en een verlaagd proteoglycaan gehalte, dus een duidelijk verstoorde kraakbeenmatrix. Toevoeging van celecoxib aan pre-klinisch artrotisch kraakbeen resulteerde in een verbetering van proteoglycaan aanmaak en een vermindering van verlies van proteoglycanen in dit type kraakbeen. Belangrijker, celecoxib was in staat om de structuur van het kraakbeen te verbeteren, zoals bleek uit een verhoging van het proteoglycaan gehalte. Overeenkomstige resultaten werden gevonden wanneer celecoxib aan artrotisch kraakbeen werd toegevoegd. Deze resultaten laten zien dat celecoxib *in vitro* een gunstig effect heeft op de proteoglycaan aan- en afbraak van artrotisch kraakbeen niet alleen in een eindfase maar ook in de meer vroege fase van artrose. Gezond kraakbeen werd wederom niet beïnvloed.

Hoofdstuk 2 en 3 ondersteunen de gedachte voor een centrale rol van ontstekingsmediatoren in het ontstaan van artrose. Echter deze rol van mediatoren is niet gelijk aan de rol van ontsteking, aangezien de meeste van deze mediatoren door het kraakbeen zelf gevormd worden en niet door specifieke ontstekingscellen. Zoals we hebben laten zien in hoofdstuk 3 is het remmen van COX-2 al in een vroeg stadium van artrose gunstig. Dit suggereert een vroege betrokkenheid van COX-2, en zijn product prostaglandine-E_2, in de afbraak van kraakbeen. Echter, in hoofdstuk 4 hebben we laten zien dat prostaglandine-E_2 alleen geen effect heeft op kraakbeen. Wanneer gezond kraakbeen gekweekt werd in aanwezigheid van de ontstekingsmediatoren IL-1β en TNFα resulteerde dit in een verstoorde kraakbeenmatrix; een verlaagde aanmaak en een verhoogd verlies van proteoglycanen. Wanneer dan prostaglandine-E_2 werd toegevoegd aan dit "beschadigde"kraakbeen verergerde de verstoorde kraakbeenmatrix.

COX-2 alleen verklaart niet alle ongunstige effecten in kraakbeenmatrix afbraak

Een opvallende bevinding in hoofdstuk 4 was dat vooral het verlies van proteoglycanen werd versterkt terwijl de verlaagde aanmaak van proteoglycanen niet werd beïnvloed. Andersom, wanneer prostaglandine-E_2 productie wordt verlaagd door remming van COX-2 zagen we het grootste effect ook op het verlies van proteoglycanen en niet of slechts gedeeltelijk op de aanmaak van proteoglycanen. Er bleek ook een duidelijke correlatie te zijn tussen het verlies van proteoglycanen en de hoeveelheid prostaglandine-E_2 gevormd door het kraakbeen. Deze regulatie van verlies van kraakbeenmatrix door prostaglandine-E_2 wordt eveneens ondersteund door de resultaten in hoofdstuk 2 en 3 waar

remming van COX-2 vooral tot normalisatie van het verlies van kraakbeenmatrix leidde en maar in beperkte mate een verbetering in de aanmaak van kraakbeenmatrix gevonden werd.
Aangezien prostaglandine-E_2 niet een direct effect heeft op de aanmaak van kraakbeenmatrix suggereert dit dat deze aanmaak door een andere route wordt gereguleerd.
Andere studies hebben aangetoond dat stikstof oxide, ook een mediator van aangedane kraakbeencellen, verschillende effecten heeft op synoviale- en kraakbeencel functies waarvan gedacht wordt dat deze bijdragen aan de afbraak van kraakbeen. Er werd door ons dan ook een duidelijke correlatie gevonden tussen het gevormde stikstof oxide en de verlaagde aanmaak van kraakbeenmatrix, zoals beschreven in hoofdstuk 4.
Opvallend is dat in experimenten waarbij artrotisch kraakbeen werd gebruikt de correlaties tussen prostaglandine-E_2 en verlies van kraakbeenmatrix en tussen stikstof oxide en aanmaak van kraakbeenmatrix niet werden gevonden (deze resultaten zijn niet in het proefschrift beschreven). Dit geeft aan dat hoe ernstiger de kraakbeenschade is, des te complexer de verschillende mechanismen van aanmaak en afbraak van kraakbeenmatrix worden.

De voordelen van celecoxib behandeling
In alle experimenten had COX-2 remming door celecoxib geen effect op de kraakbeenmatrix van humaan gezond kraakbeen. Dit is in tegenstelling met andere conventionele NSAIDs getest onder vergelijkbare omstandigheden, die wel een negatief effect op de kraakbeenmatrix kunnen hebben. De afwezigheid van een effect is ook wel te verwachten aangezien COX-2 niet tot expressie komt in normaal gezond kraakbeen. Een mogelijke verklaring voor het contrast tussen een selectieve COX-2 remmer en conventionele NSAIDs is de expressie van COX-1 in gezond en artrotisch kraakbeen en de remming hiervan door conventionele NSAIDs. Om dit verder te onderzoeken hebben we in hoofdstuk 5 het effect van NSAIDs met verschillende COX-2/COX-1 selectiviteit op humaan artrotisch kraakbeen onderzocht. Effecten van indometacine en naproxen, die enige mate van COX-1 selectiviteit hebben, werden vergeleken met effecten van aceclofenac (matig COX-2 selectief) en de selectieve COX-2 remmer celecoxib. Het artrotisch kraakbeen had zijn bekende verlaagde aanmaak en vergrote verlies van proteoglycanen. Toevoeging van indometacine verlaagde de aanmaak verder. Er was geen invloed op het verlies van de proteoglycanen. Naproxen liet voor geen van de parameters een significante verandering zien. Echter, aceclofenac en nog duidelijker celecoxib hadden een positieve invloed op het artrotisch kraakbeen. Beiden lieten ze een verbetering van de aanmaak van proteoglycanen zien en een normalisatie van het verlies van proteoglycanen. Uit dit hoofdstuk concluderen we dat COX-2 selectiviteit resulteert in kraakbeenmatrix herstellende eigenschappen. Afwezigheid van COX-2 selectiviteit resulteert zelfs in negatieve effecten. De negatieve effecten van een experimentele selectieve COX-1 remmer (eveneens bestudeerd in dit hoofdstuk) bevestigde dit. Daarnaast laten deze resultaten zien

dat COX-2 een belangrijke rol speelt in kraakbeenafbraak terwijl COX-1 meer een fysiologische functie heeft in de normale handhaving van gezond kraakbeen. Dit past ook goed in het algemene beeld van COX-1, waarbij COX-1 gezien wordt als een "huishoud" enzym en belangrijk is in allerlei fysiologische functies in verschillende celtypen. Activatie van COX-1 leidt tot, bijvoorbeeld, vorming van prostacycline, wat antitrombotisch werkt wanneer dit in een bloedvat gebeurt. Wanneer prostacycline in de maag wordt gevormd werkt dit beschermend voor de maagcellen. Dit in tegenstelling tot COX-2. COX-2 komt uitbundig tot expressie onder invloed van ontstekings- en stress-mediatoren, zoals artrose.
Dit beeld is echter recentelijk bijgesteld door onderzoek waarbij werd aangetoond dat prostaglandinen gevormd via COX-1 ook betrokken zijn in sommige processen van ontsteking, voornamelijk pijn. Ook de recentelijke waarneming dat gebruik van COX-2 remmers en NSAIDs zou bijdragen aan een verhoogd risico voor hart- en vaatschade nuanceert het oorspronkelijke concept dat COX-1 "goed" en COX-2 "slecht" zou zijn. Desalniettemin, in het geval van kraakbeenafbraak van artrotisch kraakbeen blijft dit concept gelden. Hoewel de huidige resultaten de conclusie ondersteunen dat de COX-1/COX-2 ratio de ongunstig/gunstig ratio voor kraakbeenmatrix bepaaldt, kunnen andere COX onafhankelijke effecten van celecoxib niet worden uitgesloten. Nieuwe studies met andere, wellicht zelfs selectievere COX-2 remmers zouden hier antwoord op kunnen geven.

Pijnstillende versus kraakbeenbeschermende effecten van celecoxib

De veelbelovende resultaten van de bovengenoemde *in vitro* studies en het ontbreken van gegevens verkregen uit diermodellen voor artrose maakte het zinvol om het effect van celecoxib te testen in een diermodel; het 'groove' model, een hondenmodel voor artrose. In dit model is de aard van de prikkel waarmee artrose geïnduceerd wordt anders dan in de veel andere modellen: het kraakbeen wordt operatief beschadigd ('grooves'). Deze kraakbeenschade wordt vervolgens gecombineerd met belasting van het aangedane gewricht. Dit resulteert in een periode van minimaal 10 weken in artrotische verschijnselen in het gewricht, die goed overeenkomen met de gewrichtsschade in andere bestaande modellen en met artrose bij mensen. Dit model kenmerkt zich door progressieve degeneratie van het kraakbeen in combinatie met een afnemende ontsteking van het synovium. Door deze eigenschappen is dit model bij uitstek geschikt om directe effecten van behandeling op kraakbeen te bestuderen. Daarnaast onderscheidt dit model zich omdat het geen permanente prikkel voor gewrichtsschade heeft, wat het model gevoeliger voor behandeling maakt. Een permanente prikkel voor gewrichtsschade kan een mogelijk gunstig effect van behandeling namelijk tegengaan.
De gewrichtsschade in het 'groove'model kan echter het effect zijn van de operatief aangebrachte krassen. In hoofdstuk 6 hebben we daarom dit model verder gekarakteriseerd. Tien weken na de het aanbrengen van de schade werden artrotische verschijnselen gevonden, zoals origineel beschreven voor het model. De proteoglycaan aanmaak en verlies waren verhoogd terwijl het proteoglycaan gehalte sterk verminderd was. Belangrijker, 3 weken na het aanbrengen van de

krassen waren deze veranderingen niet waarneembaar. Dit geeft aan dat de proteoglycaan veranderingen gevonden op 10 en 20 weken niet de weerspiegeling van de aangebrachte krassen is maar een weerspiegeling van progressieve (experimenteel geïnduceerde) artrotise is.
Dit 'groove'model hebben we gebruikt om in hoofdstuk 7 het *in vivo* effect van celecoxib op artrotisch kraakbeen te bestuderen. Helaas waren we niet in staat om met behulp van dit diermodel de eerdere bevonden *in vitro* resultaten te bevestigen. De honden kregen dagelijks oraal placebo, 100 mg celecoxib of 200 mg celecoxib. De inductie van artrose volgens het 'groove' model resulteerde in macroscopische histologische beschadiging van het kraakbeen, verandering in proteoglycaan aanmaak en afbraak, daling in proteoglycaan gehalte, en een milde synoviale ontsteking, allemaal kenmerken van vroege artrose. Geen van deze kenmerken was echter veranderd in de celecoxib behandelde honden. Echter de prostaglandine-E_2 waarden in synoviale vloeistof van deze honden waren dosis-afhankelijk verlaagd, wat aangeeft dat celecoxib in voldoende mate in het gewricht is gekomen om COX-2 te remmen. Gebruik makende van het *in vitro* model, bleek beschadigd hondenkraakbeen ook positief beïnvloed te worden door celecoxib, wat aangeeft dat hondenkraakbeen wel gevoelig is voor celecoxib. Een mogelijke verklaring voor het uitblijven van een effect van celecoxib *in vivo* kan zitten in de verschillende belasting van de artrotische knie. Celecoxib behandelde honden leken beter en meer te lopen dan de placebo behandelde honden. Dit zou mogelijk kunnen komen door de pijnstillende werking van celecoxib. Aangezien belasting van de aangedane poot van wezenlijk belang is voor het ontstaan van gewrichtsschade in dit diermodel, kan dit pijnstillende effect van celecoxib mogelijk de schade hebben verergerd en daarmee de gunstige effecten van celecoxib op het kraakbeen hebben tegengewerkt.
Het is in verschillende studies aangetoond dat celecoxib kniepijn vermindert in artrose patiënten. Daarnaast is recent aangetoond dat celecoxib behandeling bij artrose patiënten in een beter looppatroon resulteert. Het is algemeen bekend dat het ontwikkelen en de vooruitgang van artrose ook bij mensen af kan hangen van de belasting van het artrotische gewricht.
Dus een beter looppatroon en een zwaardere belasting van het aangedane gewricht door behandeling met celecoxib kan resulteren in een niet gewenste voortgang van de gewrichtsschade en het directe gunstige effect van celecoxib op het kraakbeen tegengaan.
Studies met diermodellen die minder afhankelijk zijn van een mechanische component voor het ontwikkelen artrose kunnen mogelijk aantonen of onze verklaring voor het uitblijven van een *in vivo* effect van celecoxib juist is.

Translatie van de in vitro resultaten naar de klinische praktijk

Hoewel de *in vitro* kraakbeenbeschermende effecten van celecoxib niet in een *in vivo* diermodel bevestigd konden worden, konden ze wel bevestigd worden in een speciale klinische set-up.

Evaluatie van kraakbeen in een humane klinische trial is erg lastig. Veranderingen in het kraakbeen zijn relatief langzaam en de veranderingen van het kraakbeen kunnen nog niet voldoende zichtbaar worden gemaakt met de huidige beeldvormende technieken (röntgen en MRI). Daarom hebben we in hoofdstuk 8 voor een benadering gekozen waarin we de voordelen van een klinische behandeling konden combineren met de gedetailleerde biochemische analyse van het kraakbeen zoals we dat bij de *in vitro* experimenten doen. In dit hoofdstuk hebben we gekeken naar de effecten van selectieve COX-2 inhibitie na een kortdurende behandeling van ernstige knieartrose patiënten. Deze patiënten werden 4 weken voordat de geplande vervangende knie operatie plaats vond behandeld met celecoxib, naproxen, indometacine of geen behandeling. De celecoxib behandelde patiënten lieten in vergelijking met onbehandelde patiënten significante verbeteringen zien in aan- en afbraak en gehalte van proteoglycanen, zoals eerder gevonden in de *in vitro* experimenten. Merkwaardig genoeg lieten de naproxen behandelde patiënten vergelijkbare gunstige resultaten zien, hoewel deze minder uitgesproken waren. De indometacine behandelde groep liet echter een trend tot negatieve werking zien. Dit ongunstige effect van indometacine en de relatief gunstige effecten van naproxen zijn in tegenstelling tot de gevonden *in vitro* effecten van deze NSAIDs zoals beschreven in hoofdstuk 5 waar ze beide, vooral indometacine, duidelijk ongunstig waren. Een mogelijke verklaring hiervoor is dat beide NSAIDs de aanwezige synoviale ontsteking gedurende 4 weken behandeling remt. Deze remming van ontsteking (en mediatoren) heeft weer een indirect gunstig effect op de kraakbeenmatrix aan- en afbraak.
In alle behandelde groepen was een daling van prostaglandine-E_2 productie te zien, een indirecte aanwijzing dat de waargenomen resultaten, in ieder geval voor een groot deel, via COX-2 inhibitie verlopen. Gebruik makende van deze benadering konden we dan ook een *in vivo* gegenereerde kraakbeen herstellend effect van celecoxib (en in mindere mate van naproxen) op artrotisch kraakbeen aantonen, wat duidelijk een voordeel is ten opzichte van indometacine.
In vitro modellen kunnen niet alle aspecten van een artrotisch gewricht weergeven. Deze modellen maken het wel mogelijk om slechts enkele specifieke aspecten of mechanismen te onderzoeken, zoals direct effecten van NSAIDs op kraakbeen. Het feit dat de resultaten van hoofdstuk 8 in grote lijn overeenkomen met de resultaten gevonden met het gebruik van ons *in vitro* model ondersteundt het gebruik van kraakbeenweefsel in kweek als bruikbaar model.

Celecoxib als keus voor de behandeling van artrose

De resultaten uit dit proefschrift ondersteunen het gebruik van een selectieve COX-2 remmer bij artrose, bij voorkeur in een vroege fase van de ziekte. Echter, de recente ontwikkelingen met betrekking tot een verhoogd risico op hart- en vaatschade bij gebruik van NSAIDs en selectieve COX-2 remmers, hebben de nodige vraagtekens bij het gebruik van deze middelen gezet. Van de NSAIDs is het alleen voor aspirine bewezen dat gebruik hiervan een duidelijke verlaging van risico op hart- en vaatschade geeft. Er zijn indicaties dat andere NSAIDs ook deze

verlaging op hart- en vaatschade geven maar duidelijk bewijs ontbreekt. Omgekeerd, sommige studies geven aan dat gebruik van COX-2 remmers juist dit risico vergroten. Het mechanisme hierachter is niet bekend en de rol van COX-2 in het functioneren van hart en vaten is bij lange na nog niet duidelijk. Ook zijn er studies waarbij COX-2 remmers juist beschermende eigenschappen lieten zien in relatie tot hart- en vaatfunctioneren. Daarnaast is de invloed van de veel toegepaste gecombineerde therapie van aspirine en COX-2 remmers ook nog niet duidelijk. Het is dus van belang om meer onderzoek te doen om het risico op hart- en vaatschade bij gebruik van NSAIDs beter in beeld te brengen. In de tussentijd moeten risico's en voordelen van de verschillende NSAIDs gewogen worden bij de behandeling van artrose. In dat kader hoort ook de gunstige werking van celecoxib voor kraakbeen, ongeacht de andere bijwerkingen, in het voorschrijven van NSAID als behandeling voor artrose, zeker als dit voor langdurig gebruik is.

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En dan nog mijn ex-kamergenoten. Mies, het was een geweldige tijd en ik mis het nog steeds. Het word weer eens tijd voor een stukje surf met bier, of zoiets. Jocea, jij was dit jaar de eerste van "onze lichting", we kunnen er een voorbeeld aan nemen, bedankt. Siska, al een tijd niet meer op de kamer maar wel in hetzelfde promotie-schuitje. Succes straks.

Ook wil ik graag de mensen van het Gemeenschappelijke Dierenlaboratorium (GDL) van de Unversiteit Utrecht noemen: Hester, Maurits, Helma, Aline, Hans Vosmeer, Nico, Jannie en Herman. Bedankt voor jullie nimmer aflatende zorg voor de honden en uitstekende hulp bij de experimenten.

Jeroen en Benno van TNO-PG Leiden ben ik ook dankbaar. Weliswaar waren jullie niet direct betrokken bij mijn promotie-onderzoek maar des te meer bij al die andere studies. Het is me waar genoegen met jullie samen te werken.

En dan de mensen buiten het werk. Bob, Ronald, Tom en Jouri. Een ieder op zijn manier, jullie waren het die mij het makkelijk maakte om op gepaste tijden eens met totaal iets anders bezig te zijn. Dat er nog maar veel van dat soort dagen mogen komen. We beginnen de 18$^{de.}$. Bob, misschien moeten we de Amerika band maar weer eens kijken. Het is fijn zo'n vriend te hebben. Ronald over mijn onderzoek hoeven we het niet te hebben, muziek des te meer. Dank hiervoor. Tom naar welk concert gaan we? Ik kijk al uit naar het volgende. Jouri, er zijn maar weinig mensen met wie ik mijn passie voor de Mustang kan delen. Dat we nog veel momenten op het circuit mogen beleven en wie weet rijden wij daar een keer. Mijn onderzoek kan geen excuus meer zijn.

Mijn paranimfen, Tom en Anne Karien. Tom, medestudent, mede-AIO en nu dan mede-postdoc, en nu mede-paranimf, maar vooral vriend. Jij weet als geen ander hoe turbulent een promotie kan zijn. Daarom heb ik alle vertrouwen in jou als paranimf, bedankt alvast voor je steun. Anne Karien als "professioneel" paranimf is het alleen maar logisch dat je straks aan mijn zijde staat. Bedankt voor al je goede zorgen en steun, niet alleen gedurende mijn onderzoek maar ook straks.

Pa en Ma, ook al was het vaak niet te begrijpen wat ik nu precies onderzocht en waarom ik weer eens tot diep in de nacht op het lab zat, dankzij jullie onvoorwaardelijke steun is het dat ik hier nu sta. Bedankt dat ik heb kunnen doen wat ik graag wil.

En tot slot Marinde, mijn held. Dank voor alle goede zorg. Jij maakt promoveren makkelijk. Op naar een nieuwe mijlpaal in ons leven. En o ja,....ik ook van jou.

Curriculum Vitae

Simon Mastbergen was born in Soest on October 24, 1972. In 1993 he finished his secondary school education at the "Oosterlicht College" in Nieuwegein, the Netherlands. From September of the same year until 1998 he studied Medical Biology at the Free University Amsterdam. During this period he obtained research experience at the department of Cell Biology and Immunology, Free University Amsterdam, at the department of Endocrinology VU University Medical Center Amsterdam, and at the department of Urology/Nuclear Medicine, VU University Medical Center Amsterdam.

In February 1999 he started his PhD study at the department of Rheumatology & Clinical Immunology at the University Medical Center Utrecht, entitled: COX-2 inhibition in osteoarthritis: effects on cartilage. From 1999 till now, this work was supervised by Dr. FPJG Lafeber and Prof. Dr. JWJ Bijlsma.

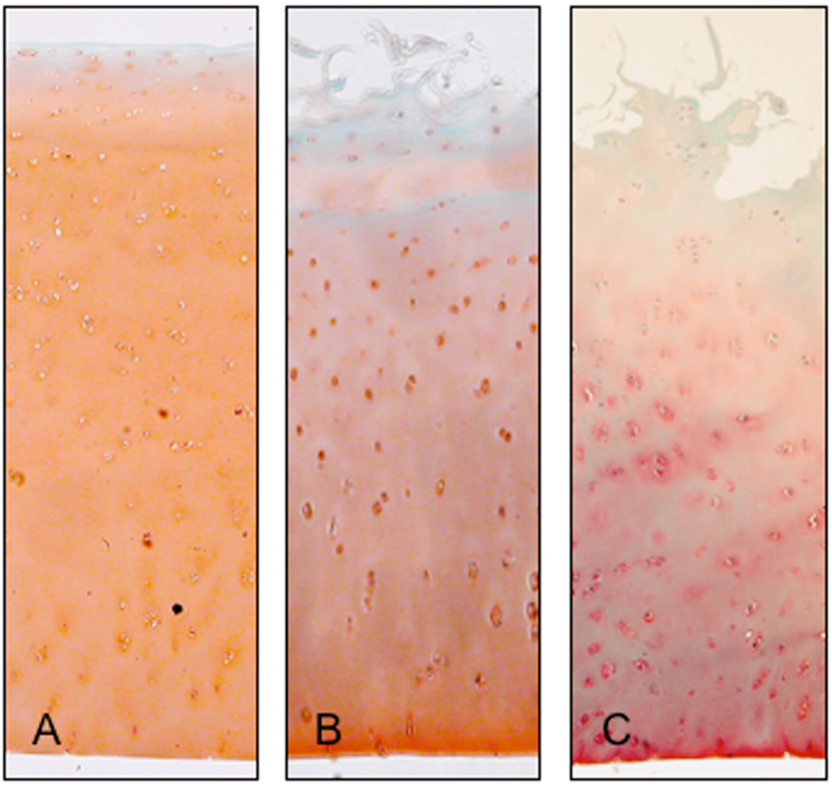
From June 2001 until July 2002 he coordinated a large animal in vivo study for an English company. During this period his PhD work was on a hold, although spin-off research resulted in Chapter 6 of this thesis.

From August 2002 till April 2005 he continued his PhD study on COX-2 regulation in cartilage for 4 days a week.

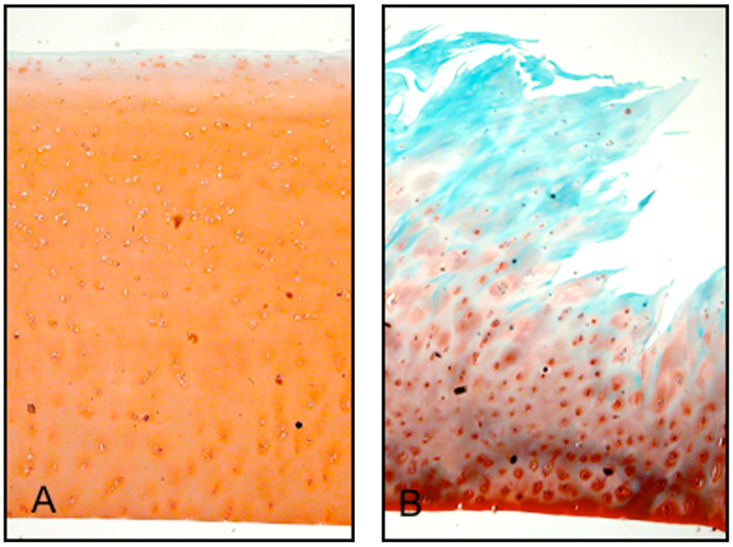
During this period, 1 day a week he coordinated contract animal and cartilage research performed at the department of Rheumatology & Clinical Immunology. Under his supervision several in vitro and in vivo cartilage studies have been performed during that period.

At present his is appointed at the department of Rheumatology & Clinical Immunology at the UMC Utrecht as coordinator "contract research for animal and cartilage research".

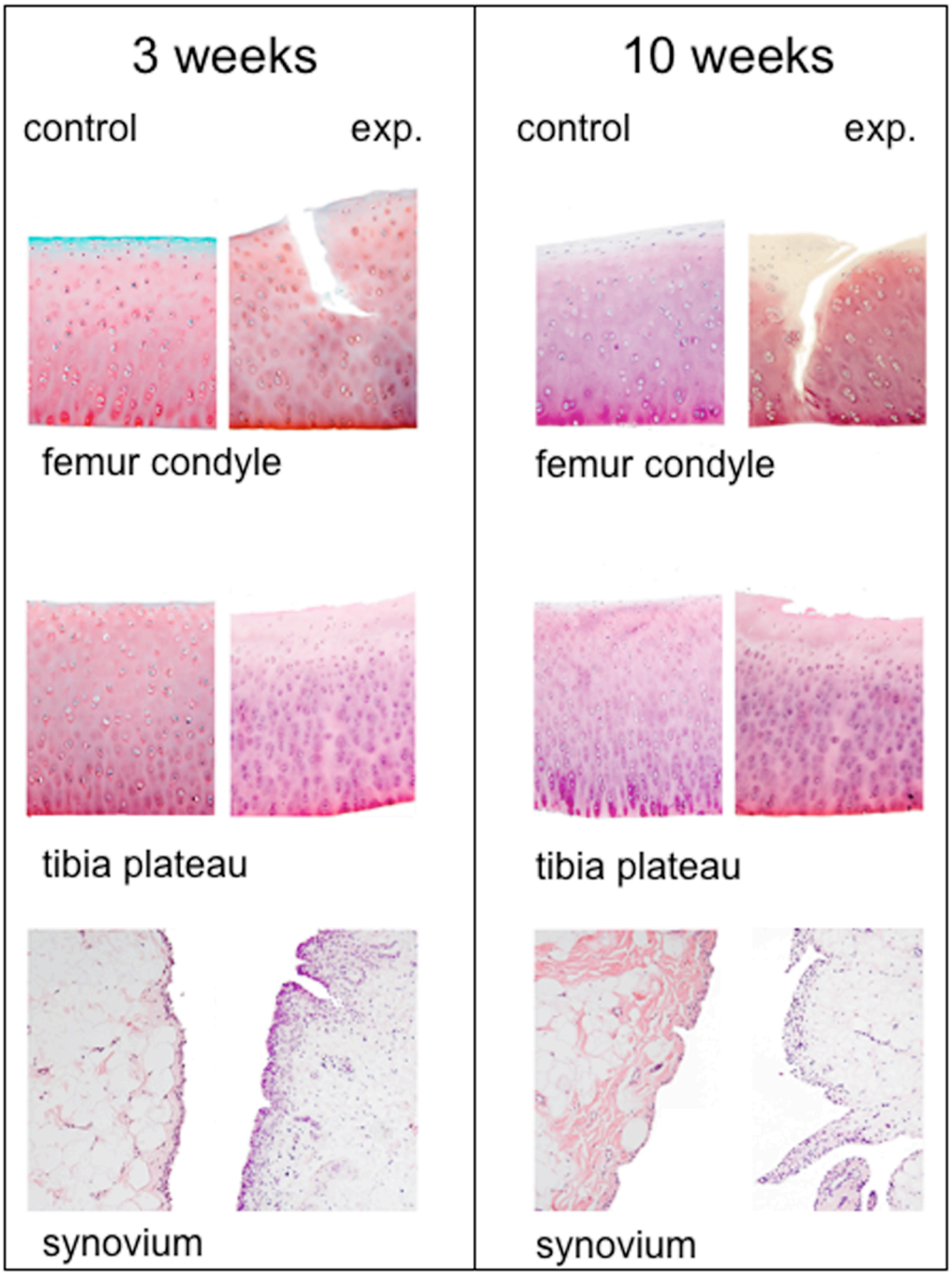
Appendix



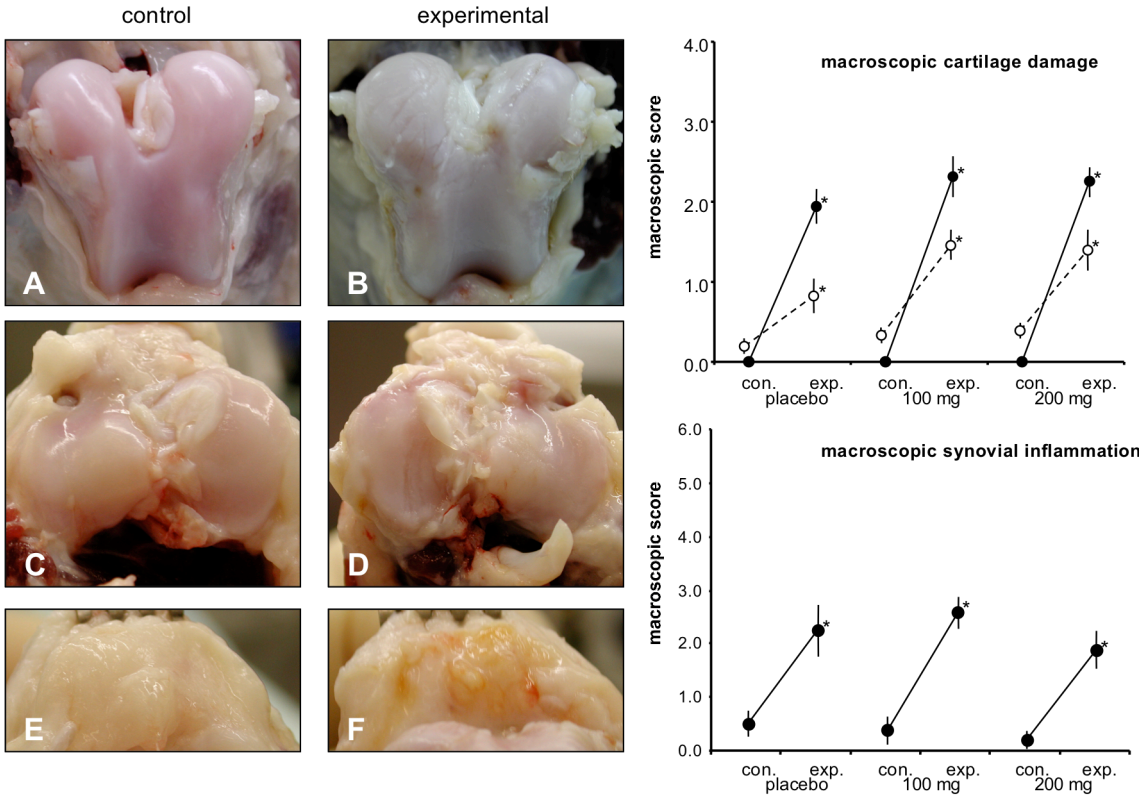
Chapter 3 Figure 1. *Normal healthy, degenerative and osteoarthritic cartilage histology.* Representive light micrographs of condylar cartilage obtained *post-mortem* from joints with normal healthy cartilage (A), or joints with degenerated cartilage (B) and cartilage obtained at joint replacement surgery (C). Sections are stained with safranin-O fast green-iron haematoxylin and graded for features of osteoarthritis according to the slightly modified criteria described by Mankin *et al*., scores for the depicted samples are 0, 4 and 6, respectively .



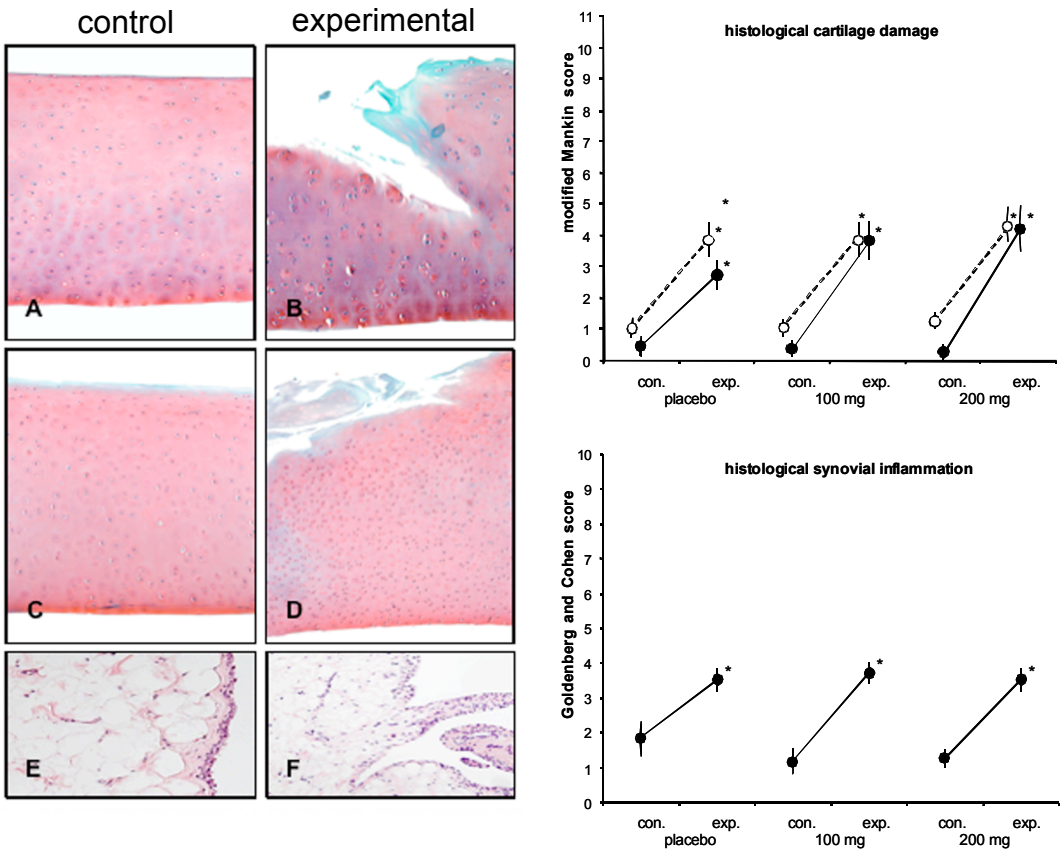
Chapter 5 Figure 1. *Normal healthy and osteoarthritic cartilage histology.*
Representive light micrographs of condylar cartilage obtained *post-mortem* from joints with normal healthy cartilage (A) and cartilage obtained at joint replacement surgery (B). Sections are stained with safranin-O fast green-iron haematoxylin and graded for features of OA according to the slightly modified criteria described by Mankin et al., scores for the depicted samples are 0 and 7, respectively.



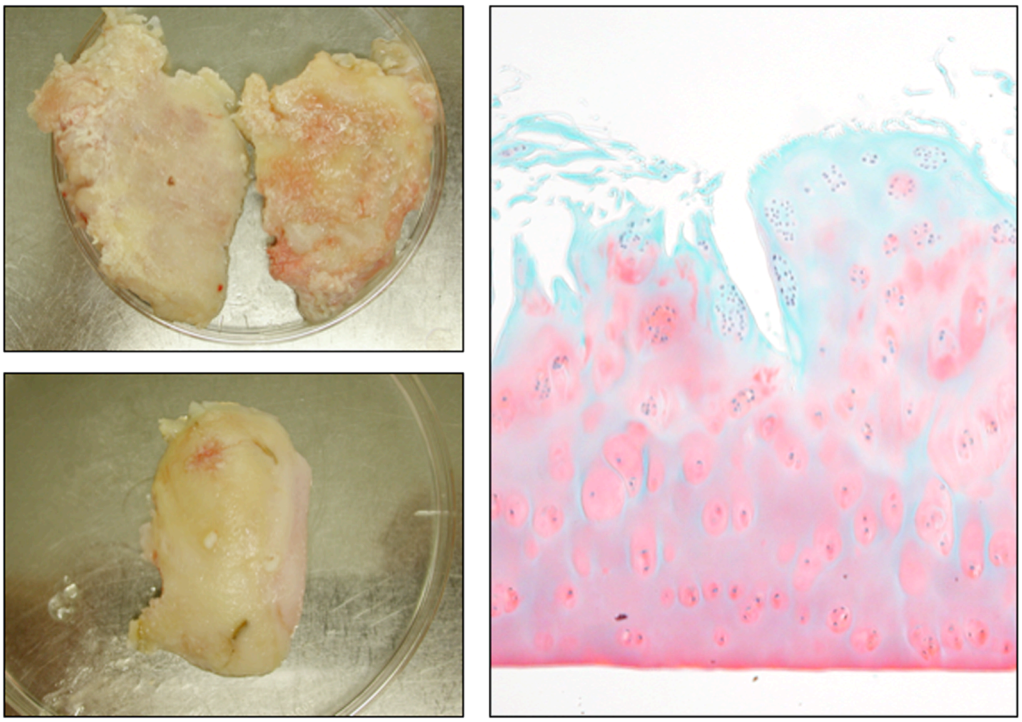
Chapter 6 Figure 1.
Representative light micrographs of condylar cartilage (top), tibial cartilage (middle;safranin-O stained) and synovial tissue (bottom; Hematoxylin-Eosine stained) 3weeks (left) and 10 weeks (right) post-surgery. Control joint (controls, left side) at 3 weeks and controls, right side at 10 weeks, had for cartilage a modified Mankin grade 0 and for synovial tissue a modified Goldenbeg and Cohen grade of 0. The experimental joint had at 3 weeks a modified Mankin grade 4 and 1 for condylar and plateau cartilage, respectively and for synovial inflammation a score of 3. The experimental joint at 10 weeks had a modified Mankin grade 5 and 4 for condylar and plateau cartilage, respectively and for synovial inflammation a score of 3.



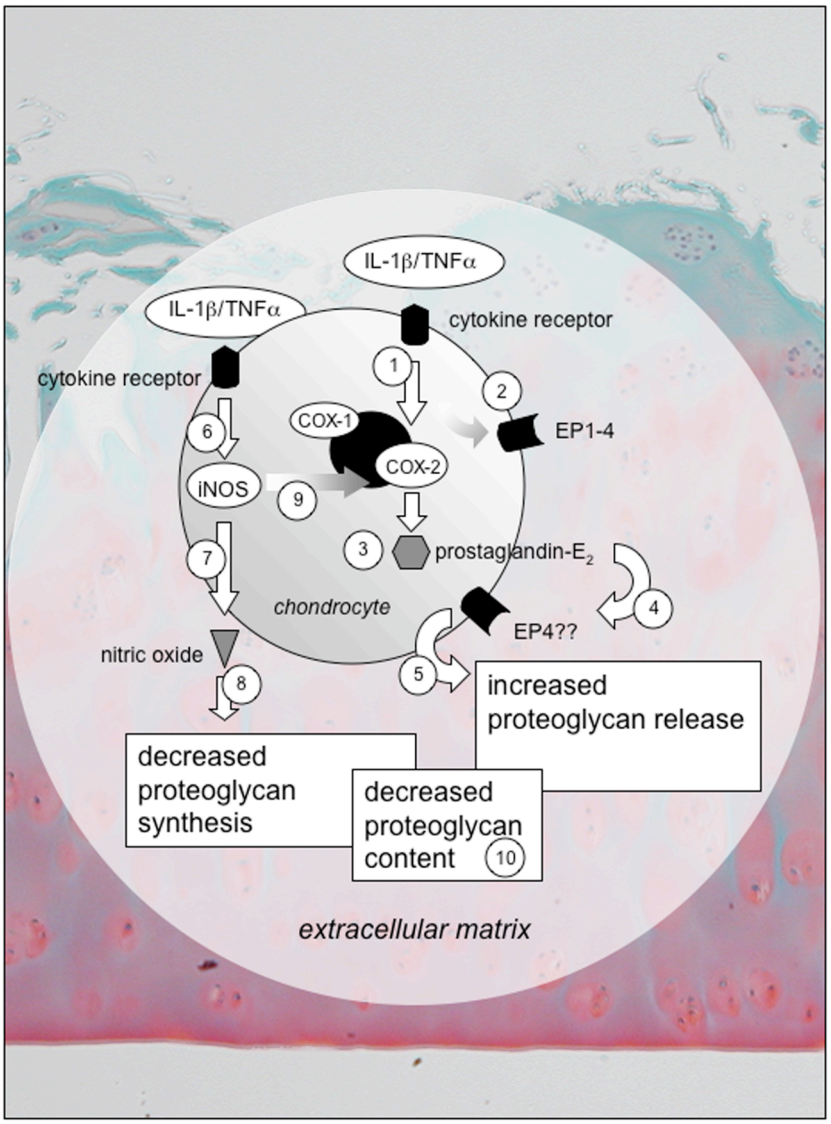
Chapter 7 Figure 1. *Macroscopic changes as a result of experimentally induced OA and celecoxib treatment.*
Representative macroscopic view of articular cartilage surfaces and synovium. Femoral condyles (A + B), tibial plateaus (C + D) and synovium (E + F) of the control (left) and experimental (right) joint of a placebo treated dog are shown. Average macroscopic score of cartilage damage and synovial inflammation are depicted in graphs (G + H). For figure G: Closed symbols with solid lines represent macroscopic damage of femoral condyles and open symbols with dashed lines represent macroscopic damage of tibial plateaus. Mean ± SEM values (n=8) are presented for placebo, celecoxib 100 mg and celecoxib 200 mg treated animals. Asterisks indicate statistically significant ($p<0.05$) changes compared to contralateral controls. No statistically significant differences between groups were found.



Chapter 7 Figure 2. *Histological changes as a result of experimentally induced OA and celecoxib treatment.*
Representative light micrographs of condylar cartilage (A + B), tibial cartilage (C + D) and synovial tissue (E + F) of the control (left) and experimental (right) joint of a placebo treated dog are shown. Average scores of histological determined cartilage damage (modified Mankin grade) for femoral condyles (closed circles, solid lines), tibial plateaus (open circles, dashed lines) and synovial inflammation (modified Goldenberg and Cohen score) are depicted in graphs (G + H). Mean ± SEM values (n=8) are presented for placebo, celecoxib 100 mg and celecoxib 200 mg treated animals. Asterisks indicate statistically significant ($p<0.05$) changes compared to contralateral controls. No statistically significant differences between groups were found.



Chapter 8 Figure 1. *Macroscopic and histological image of the osteoarthritic cartilage.* Representative picture of plateau and condylar cartilage obtained at joint replacement surgery and light micrograph of this cartilage. Macroscopic score is 3. Sections are stained with safranin-O fast green-iron haematoxylin and graded for features of osteoarthritis according to the slightly modified criteria described by Mankin. Score for this sample is 6.



Chapter 9 Figure 1: *Schematic representation of the mechanism of COX-2 mediated cartilage degeneration*. Upregulation of COX-2 by autocrine and paracrine produced mediators such as IL-1β/TNFα (1). Concomitant upregulation of the prostanoid receptors (EP 1-4) via COX-2 or other mechanisms (2). COX-2 upregulation results in large concentrations of prostaglandin-E_2 (3). Prostaglandin-E_2 stimulates, autocrine and paracrine, the EP4 receptor (4). This results in increased proteoglycan release (5). IL-1β/TNFα induces simultaneous activation of iNOS (6), which leads to formation of nitric oxide (7). This results among other effects, in a decreased proteoglycan synthesis (8) and may stimulate COX-2 production (9) concomitantly leading to enhanced release. The disturbance of proteoglycan synthesis and proteoglycan release will result in further loss of proteoglycan content (10) and with that to loss of cartilage matrix integrity. Selective COX-2 inhibition by celecoxib, amongst others, leads to inhibition of the COX-2 pathway and with that prostaglandin-E_2 production. This results in normalization of proteoglycan release, but not synthesis.

What does it mean when you
promise someone

that no matter how hard and
whatever may come

it means that I won't give in
won't give in, won't give in
everyone I love is here
play it once then we disappear

(won't give in, Neil Finn/Tim Finn)