Inflammatory mediators in osteoarthritis

-Identification and role in cartilage repair-

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Inflammatory mediators in osteoarthritis

-Identification and role in cartilage repair-

Ontstekingsmediatoren in artrose
-identificatie en rol in kraakbeen herstel-
(met een samenvatting in het Nederlands)

Proefschrift

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Co-promotor:	Dr. L.B. Creemers

Contents

List of abbreviations

ACR	American College of Rheumatology
ADAMTS	A disintegrin and metalloproteinase with trombospondin motifs
CCR	CC- Chemokine Receptor
CCL	Chemokine (C-C motif) ligand
COX-2	Cyclooxygenase-2
DMMB	Di-methlylmethylene Blue
DMEM	Dulbecco's Modified Eagle's medium
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
GAG	Glycosaminoglycans
IFNγ	Interferon-γ
ITS-X	Insulin Transferrine Selenium- X
IL	Interleukin
LDH	Lactate dehydrogenase
LIF	Leukemia inhibitory factor
MCP	Monocyte chemotactic factor
MMP	Matrix metalloproteinase
MCP	Monocyte chemotactic protein
MDC	Macrophage derived chemokine
MIP	Monocyte Inhibitory Protein
NSAID	Non-steroidal anti-inflammatory drug
NGF	Neural growth factor
OPG	Osteoprotegerin
OA	Osteoarthritis
OSM	Oncostatin M
PBS	Phosphate buffered saline
PGE2	Prostaglandin E2
PCA	Principal Component Analysis
RA	Rheumatoid Arthritis
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
SF	Synovial fluid
TNF-α	Tumor Necrosis Factor -α
VEGF	Vascular growth factor

Chapter 1

General introduction and aims

1.1 Background

Osteoarthritis (OA) is the most common degenerative joint disorder, affecting more than 1.2 million people in the Netherlands (Poos et al 2011; data RIVM incidentie en prevalentie aandoeningen 2011). With ageing and increasing obesity of the population, a growing incidence is anticipated in the near future. Currently, there is no cure for OA, only symptomatic pain relief and at end-stage OA, total joint arthroplasty. Key features of OA are degeneration of the articular cartilage, synovitis, thickening of the subchondral bone, osteophyte formation and degenerative changes of the meniscus and ligaments (figure 1). These features of OA will eventually lead to pain, loss of function of the joint, and disabilities. Diagnosis of OA is based on clinical symptoms, physical examination and/or radiological evaluation [1]. The most common risk factors include age, sex, joint injury, obesity (loading and systemic inflammation), genetic predisposition and mechanical factors [1]. The current American College of Rheumatology (ACR) criteria and definitions for OA do not discriminate between different sub- or phenotypes of OA. Recently, a new classification of osteoarthritis based on phenotype has been suggested [2] table 1.

Table 1. Phenotypes in osteoarthritis (adapted from Bijlsma et al. Lancet 2011)

	Post traumatic	Metabolic	Ageing	Genetic	Pain
Age	< 45 years	45-65	> 65	Variable	Variable
Main feature	Mechanical stress	Mechanical stress, inflammation e.g. adipokines	Chondrocyte senescence	Genetic factors	Inflammation, aberrant pain perception
Main site	Knee-ankle-wrist-shoulder	Knee- hand	Hip-knee-hand	Fingers-hand	Knee-hand

1.2 Tissues involved in the pathogenesis of osteoarthritis

Osteoarthritis is a disease of all the joint tissues, affecting not only the cartilage but also the bone, synovial tissue, meniscus and ligaments. As the entire joint is affected, it is important to consider OA as 'joint failure' and treat the entire joint and not only the affected tissues [3]. In the following chapters important joint tissues and the changes that occur in these tissues in OA are described below (figure 1).

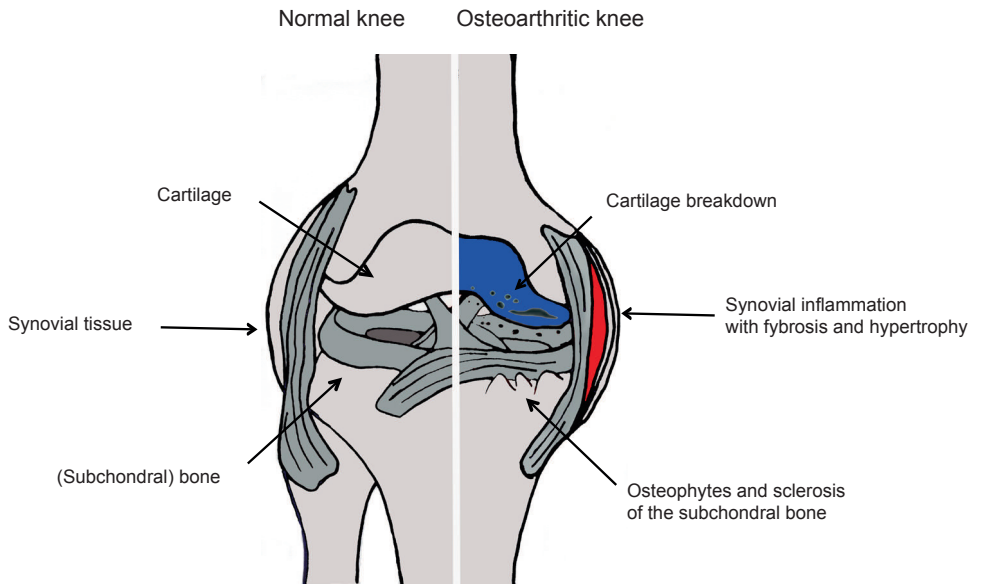

Figure 1. The healthy and osteoarthritic joint (Hunter et al. Osteoarthritis; BMJ 2011)
In osteoarthritis all joint tissues are affected. There is breakdown of the cartilage, fibrosis and hypertrophy of the synovial tissue and degeneration of the menisci. Bone changes include osteophyte formation, bone remodelling and sclerosis of the subchondral bone.

1.2.1 Cartilage

Cartilage is an avascular and aneural tissue. The articular cartilage provides smooth motion of the joint and shock absorption by its specific extracellular matrix (ECM). The ECM of cartilage consists predominantly of water (70 %), collagen type II and proteoglycans, with the chondrocyte as only cell type [4]. Collagen represents 50-60% of the cartilage dry weight. The main collagen is type II collagen, of which the main function is tensile strength en stiffness. During the OA process, type II collagen is replaced by type I collagen, thereby leading to inferior cartilage. The proteoglycans account for 5-10 % of cartilage dry weight. Proteoglycans are complex molecules consisting of a protein core to which multiple negatively charged glycosaminoglycans [5] are covalently attached, such as keratan sulfate and chondroitin sulfate, as side chains. Through binding to link protein, they bind to hyaluronic acid, thereby forming large aggregates. By attracting positive cations the proteoglycans entrap water in the cartilage. In the course of OA, proteolytic enzymes, such as ADAMTS', degrade the proteoglycans and matrix metalloproteinases (MMP) degrade the collagen network. The degradation rate and synthesis rate of ECM molecules can be used as readout parameter for cartilage repair and regeneration. The chondrocytes in healthy cartilage produce small amounts of matrix. In early OA, the chondrocyte becomes activated to initiate repair, characterized by proliferation, cluster formation of the chondrocytes and increased production of ECM. In end-stage OA, apoptosis occurs and the production of ECM is disturbed

[6]. Also the tidemark, denoting the border between calcified and uncalcified cartilage, changes during the OA process. The hypothesis is that these changes occur by vascular invasion into the cartilage under the influence of vascular endothelial growth factor [7].

1.2.2 Synovial tissue

The synovial tissue is the inner lining of the joint and is only a few cells thick. Its main functions are the production of hyaluronic acid (HA) and the clearance of waste products from the synovial fluid [8]. HA and lubricin (produced by the chondrocytes) are essential for frictionless movement of the joint. The synovial tissue consists of multiple cell types, e.g. macrophages and synoviocytes. The synovial lining cells, both the macrophages and the synoviocytes, are capable of producing multiple soluble mediators, e.g. cytokines, chemokines, growth factors, enzymes, which play a crucial role in joint homeostasis. Many previous studies have demonstrated the role of synovitis in rheumatoid arthritis (RA) and osteoarthritis. There is an increased production of catabolic enzymes and matrix degrading enzymes, such as matrix metalloproteinases (MMP) and aggrecanases, by the synovial tissue [8-11]. These studies have led to novel treatments (e.g. biologicals) for RA that improved the clinical course and outcome for patients. These therapeutics focus on blocking inflammatory mediators, e.g. Interleukin (IL)-1 and Tumor Necrosis Factor (TNF)-α, produced by the synovial tissue, by monoclonal antibodies. There is increasing evidence that synovitis is one of the key futures in OA and it is well recognized as one of the pathological changes in the osteoarthritic joint [12]. Synovitis in osteoarthritis is characterized by hyperplasia, influx of inflammatory cells and finally fibrosis of the synovial tissue [13]. Recently, the role of synovitis or synovial inflammation in OA has been studied to understand the pathogenesis of OA. Symptoms as pain are associated with synovitis as scored by magnetic resonance imaging or ultrasound. Moreover, Ayral et al demonstrated a relationship between synovial inflammation and cartilage degeneration [14]. Despite the clear association of synovitis with OA, it is still not clear whether it is a cause or an effect of the concurrent cartilage degeneration and if there is a causal relationship, whether indeed degradation is induced, or repair is inhibited. Better understanding of the role of the synovial tissue and the production of inflammatory mediators in OA may lead to new insights in the pathophysiology of OA and, ultimately aid in the treatment of OA.

1.2.3 Subchondral bone

The subchondral bone consists of cortical bone, which is supported by the underlying cancellous bone. It is a network of bone trabeculae that is responsible for its stiffness and strength to withstand mechanical loading. As bone is innervated and cartilage is not, bone may add to the perception of pain described in OA. Also the subchondral bone is important in bringing nutrients to the covering cartilage. Bone marrow lesions are among the first changes that occur in the joint [15]. During progression of OA, thickening of the subchondral bone, cyst formation, sclerosis and osteophyte formation are key processes in the pathogenesis of OA. These changes are easily observed on plain x-rays, and therefore belong to the principal features to diagnose OA. Because changes in the bone occur gradually over time, x-rays can also be used to monitor OA over time. However, there is almost no correlation between the severity of OA and the changes on x-ray [16]. Whether (subchondral) bone changes precede or follow the changes in the cartilage is still under debate [3]. Recent research has focussed on the complex interaction of cartilage and bone and their role in OA [17].

1.2.4 Synovial fluid

The synovial fluid is crucial for joint function. The synovial fluid is the lubricant of the joint. Normal synovial fluid is clear, viscous by the presence of hyaluronic acid, and contains very few cells. During inflammation the volume of synovial fluid increases, the viscosity decreases and there is an influx of inflammatory cells such as lymphocytes [18]. Since articular cartilage is avascular and aneural, the synovial fluid brings nutrients to the cartilage and helps clear their waste products. Importantly, since there is no direct contact between cartilage and synovial tissue, the synovial fluid aids in the crosstalk between both tissues. The synovial fluid carries a multitude of autocrine and paracrine factors to and from chondrocytes and other joint tissues. These mediators include cytokines, adipokines, enzymes and other mediators such as prostaglandins. In this way, the synovial fluid is the best representation of the joint environment and represents the catabolic or anabolic state of the joint. Moreover, treatments for OA could be monitored by analysis of the synovial fluid.

1.3 Inflammation and soluble mediators

The role of inflammatory mediators in OA is well recognized [9, 19, 20]. During the development and progression of OA, inflammation of the synovial tissue leads to the production of catabolic factors (figure 2; [8]. Also the cartilage itself is capable of producing inflammatory mediators, which may contribute to the progression of OA [21, 22]. Maintaining matrix homeostasis in the healthy adult cartilage requires turnover of matrix components, principally collagen and proteoglycan.

Soluble mediators are present in the synovial fluid and produced by the cartilage, synovial tissue and bone. The most well-known and studied cytokines are IL-1 and TNFα. Chondrocytes stimulated by these inflammatory mediators produce matrix proteases and suppress the synthesis of collagen and proteoglycan. Thus, cytokines not only favor tissue destruction, but also inhibit tissue repair.

Since the identification of IL-1 to induce degradation of cartilage *in vitro* [23], the focus has been on the role of IL-1 in degenerative joint disorders such as OA. Previous studies show that IL-1 suppresses aggrecan synthesis and collagen synthesis in chondrocytes and can enhance the production of matrix-degrading enzymes such as MMP-13. However, treatment with the IL-1 receptor antagonist does not improve the clinical outcome in OA patients [24, 25], in contrast to the clinical effectivity found in RA. TNFα has also been of great interest in RA research since the discovery of TNFα in the RA joint and its capability to induce cartilage degradation [26]. However, where biologicals that block TNFα (e.g. infliximab, etanercept) have been used to stop the progression of RA, in OA these have met with little clinical success [27]. One explanation for this lack of effects is the observation that IL-1 and TNFα are, if at all, present at very low levels in synovial fluid of OA patients (shown in this thesis). As inflammation seems crucial in OA, other inflammatory mediators, such as interferon-γ (IFNγ), IL-6 and oncostatin M [28], may rather play a role in OA. These mediators are known to interact with chondrocytes and other joint cells to release degrading enzymes. Chemokines, such as IL-8, RANTES, Monocyte chemotactic protein-1, have been implicated in OA. High levels of chemokines have been found in OA synovial fluid, which could explain the recruitment of inflammatory cells in synovitis [18]. However, it is not clear which of these factors play a causal role in the loss of cartilage matrix found in OA. Moreover, different mediators may be involved in different stages of OA or even at different joint locations. As the knee joint is most accessible for synovial fluid collection, most data on synovial fluid composition have been obtained at this anatomical location.

Prostaglandins are amongst the mediators from the lipid mediator cascade of which production is increased during inflammatory processes. The most studied prostaglandin is prostaglandin-E2 (PGE2). Cyclooxygenase (COX) enzymes are important in the conversion of arachidonic acid into PGE2. Medications that block COX activity, e.g. celecoxib, are used for the treatment of OA. Some in vitro studies showed a positive effect on cartilage metabolism, which could indicate that COX-inhibitors are potential disease modifying drugs [29, 30].

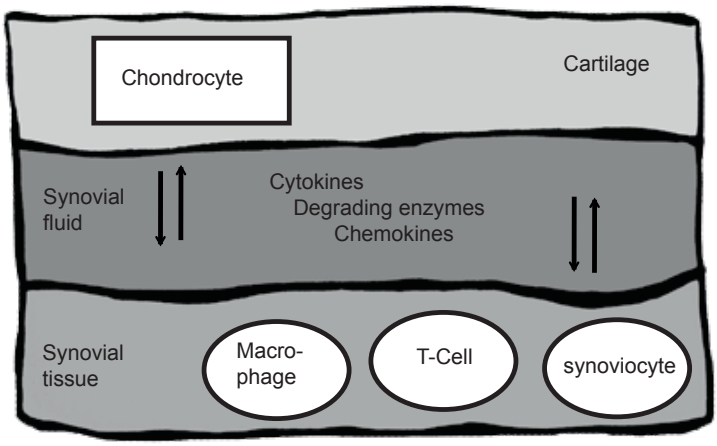

Figure 2. mediators in the osteoarthritic joint.
Inflammatory mediators secreted by osteoarthritic synovial tissue and osteoarthritic cartilage are present in the synovial fluid. Changes in joint environment affect both cartilages as synovial tissue.

As described above inflammatory mediators are among the key players in the pathogenesis of osteoarthritis. Many inflammatory mediators have been identified in osteoarthritic synovial fluid and associated with OA cartilage degeneration; however, this has not led to a cure for OA. Which inflammatory mediators are present in OA synovial fluids, and especially which role they play in osteoarthritis is still unclear.

1.4 Research aims

Further understanding of the effect of inflammatory mediators in the development and progression of OA, and especially in cartilage repair, may lead to the identification of a potential target to stop OA progression. We set out to identify inflammatory mediators present in synovial fluid of patients with osteoarthritis and examine the involvement of cartilage and synovial tissue in the production of these mediators. Moreover, with the goal to find targets to treat osteoarthritis, we used different culture models to study the role of inflammatory mediators in OA cartilage repair.

Specific aims:

1. Identify disease specific inflammatory soluble mediators present in the synovial fluid of osteoarthritic joints (knee and wrist);
2. Examine the involvement of OA synovial tissue and cartilage in the production of inflammatory mediators and the effect of OA synovial tissue on OA cartilage repair;
3. Study the effect of anti-inflammatory interventions on the production of inflammatory mediators and OA cartilage repair;

1.5 Outline of this thesis

As a first step to identify inflammatory mediators we compare the joint environment of the healthy and osteoarthritic knee joint, by measuring a panel of inflammatory cytokines in the synovial fluid and applying cluster analyses to identify different clusters associated with OA processes in **chapter 2.** Where the knee is a weight-bearing joint with different aetiologies of OA, e.g. trauma, ageing, obesity, the wrist is a non-weight bearing joint and OA development is mostly due to trauma. In **chapter 3**, a selection of soluble mediators is measured in the wrist joint in the course of OA progression. In **chapter 4** we compare the osteoarthritic joint environment of the knee and wrist for multiple mediators.
To examine the involvement of synovial tissue and cartilage in osteoarthritis we studied the production of inflammatory mediators by osteoarthritic cartilage and synovial tissue explants in culture. To be able to study the role of synovial tissue in cartilage metabolism, we first validated a co-culture model with both joint tissues in **chapter 5**. To study the role of inflammatory mediators in cartilage metabolism and to see whether commonly used anti-inflammatory drugs could enhance repair of osteoarthritic cartilage we explored the effect of triamcinolone in chapter 5 and celecoxib, a specific COX-2 inhibitor in **chapter 6**. As IL-6 and OSM are both increased in OA synovial fluid and produced by OA synovial tissue, we studied the role of these cytokines in osteoarthritic cartilage repair and regeneration (**chapter 7 and 8**). To conclude, in **chapter 9** our findings are summarized and three key questions on inflammatory mediators in osteoarthritis are discussed.

Chapter 2

An explorative study comparing levels of soluble mediators in control and osteoarthritic synovial fluid

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Abstract

Objective

Soluble mediators in synovial fluid are acknowledged as key players in the pathophysiology of osteoarthritis (OA). However, a wide-spectrum screening of such mediators in synovial fluid is currently lacking. In this study, the levels of 47 mediators in the synovial fluid of control donors and osteoarthritic (OA) patients were compared.

Materials & Methods

Synovial fluid was collected from control donors (n=16) and end-stage knee OA patients (n=18) and analysed for 47 cytokines, chemokines and growth factors using several multiplex ELISAs. A Mann-Whitney U test was used to determine differences between OA and control controls. A principal component analysis (PCA) was performed to cluster the 47 mediators.

Results

The majority of the mediators could be detected in both control and OA synovial fluid. IL-6, IP-10, MDC, PDGF-AA and RANTES levels were found to be higher in OA compared to control synovial fluid ($p<0.001$). Leptin, IL-13, MIP-1β, sCD40L levels were higher and eotaxin and G-CSF levels were lower in OA synovial fluid than in control synovial fluid, albeit borderline significant ($p<0.05$). The PCA enabled identification of 6 clusters of mediators, which explained 76% of the variance.

Conclusions

The current study provides the first extensive profile of cytokines, chemokines and growth factors present in control and OA synovial fluid. Increased levels of mediators such as MDC and IL-6 imply involvement of inflammatory processes and might be associated with the influx of inflammatory cells in OA synovial tissue. Moreover, the performed cluster analysis indicated multiple clusters, which could indicate different pathophysiological pathways in the joint.

A control group of knee synovial fluid samples (n=16) were collected from *post mortem* donors within 24 hours after death. Control donors had no history of OA, other joint pathology and possessed macroscopic healthy cartilage. OA synovial fluid samples (n=18) were collected during total knee arthroplasty. All OA patients were diagnosed according to the ACR criteria for OA[31]. Exclusion criteria were rheumatoid arthritis (RA) or infection. Synovial fluid samples were centrifuged at 3000 rpm for 3 minutes to spin down any cells or debris. The supernatant was stored at -80 °C until further analysis. The control synovial fluid samples were stored for 1 to 10 years and OA synovial fluid samples were stored for 1 to 3 years. None of the samples had ever been thawed before. Collection of the synovial fluid was done according to the Medical Ethical regulations of the University Medical Centre Utrecht and according to the guideline 'good use of redundant tissue for clinical research' constructed by the Dutch Federation of Medical Research Societies on collection of redundant tissue for research. As according to these guidelines, no information about the patients' characteristics could be obtained. Gender and age information was available for limited donors. Control donors had an average age of 39.6 ± 9.3 and consisted of 55% female. OA donors had an average age of 69.9 ± 7.9 and consisted of 64 % female. Due to the limited availability these data could not be linked to any of the outcomes.

Two hundred µl of each of the OA synovial fluid samples was pre-treated with 20 µl of hyaluronidase (Sigma, St, Louis, MO, USA; 10 mg/ml) for 15 min at 37 °C. Samples were spun down in a X-column (Corning, Amsterdam, Netherlands; Costar 8169). Finally, 150 µl of the synovial fluid sample was dissolved in 300 µl HPE-0.1375% Tween (Sanquin, Amsterdam, Netherlands). The pre-treated synovial fluid samples were used for all Multiplex ELISA assays mentioned below. To determine a wide panel of soluble mediators the commercially available human inflammation 42-multiplex and the human adipokine 13-multiplex (Millipore, Bellirica, MA, USA) were used according to the manufacturer's protocol. Additionally, 12 different soluble mediators were measured with the Bio-Plex suspension system (Bio-Rad laboratories, Hercules CA, USA) as previously described[32]. The levels of cytokines in the synovial fluid samples were expressed as pg/ml. All samples were measured in the same plate and in duplo. Levels below the lower limit of quantification (LLOQ) were indicated as the value of the lowest point on the calibration curve divided by 2. The measured mediators are listed in table 1. Data are expressed as median ± interquartile range (IqR) as the data had a non-Gaussian distribution. IBM SPSS 20.0 software (IBM SPSS Inc. Chicago, IL, USA) was used for the statistical analysis.
For this study, a descriptive statistics approach was used for the interpretation of the data due to the limited sample size. A Mann Whitney-U test was used to assess differences between control and OA synovial fluid samples due to the predominantly non-Gaussian distribution of the data. As a descriptive statistic approach was used, values with a p-value less than 0.001 were considered significant. P values between 0.05 and 0.001 were considered borderline significant. No *post-hoc* correction for multiple testing was performed on the data, due to the descriptive approach.

PCA was performed to enable identification of clusters (i.e. components) of interrelated mediators within the complete dataset. Mediator levels were mean-centered to remove dependence on magnitude of levels. Control and OA patient data were combined in one single PCA analysis. Separate analysis of patients and controls was judged impossible due to the limited number of subjects. Only mediators with communalities > 0.3 were included. Loading factors were maximized using Direct oblimin rotation with Kaiser Normalisation. The optimum number of clusters was then decided based on the scree-plot and eigenvalues (> 1.0). Mediators were categorized per cluster when their loading scores were > 0.5. A Crohnbach's α was performed on each cluster to determine internal consistency.

This is one of the first studies in which such a comprehensive profile of soluble mediators was measured in synovial fluid from (end-stage) OA patients and control donors. With regard to previous studies, the pattern of the mediator levels was found to be compatible for OA donors. However, in these studies a small panel of mediators was measured and the inclusion of control donors was lacking [33]. Table 1 provides an overview of the measured levels of each mediator (median ± IqR). The majority of the soluble mediators could be detected in the synovial fluid samples of both control and OA donors. Of the 47 measured mediators, 5 mediators were present at significantly different levels in OA compared to control synovial fluid. The levels of the chemokines MDC, RANTES and IP-10, the growth factor PDGF-AA and the pro-inflammatory cytokine IL-6 were significantly higher in OA than in control synovial fluid ($p < 0.001$). In addition, levels of the adipokine leptin, the chemokine MIP-1β, and the pro-inflammatory cytokine sCD40L were higher and levels of the chemokine eotaxin and the growth factor G-CSF were lower in OA than in control synovial fluid, albeit all with borderline significance ($p < 0.05$).

Table 1. Overview of soluble mediators

	Healthy SF (Median ± IqR)	CV (%)	<LLOQ or 0 (n)	OA SF (Median ± IqR)	CV (%)	<LLOQ or 0 (n)	Detection limit	Mann-Whitney U (p-value)
EGF	4.8 ± 13.1	102.3	11	4.8 ± 0	189.3	14	5.3	0.65
Eotaxin	14.6 ± 39.6	96.0	6	0 ± 0	236.7	15	12.1	*0.02
FGF-2	37.2 ± 70.4	137.5	2	21.6 ± 231.4	134.1	4	16.0	0.67
Flt-3 ligand	135.9 ± 119.7	59.0	0	103.5 ± 76.4	53.1	0	6.1	0.23
Fractalkine	0 ± 0	387.7	14	0 ± 19	168.5	12	7.6	0.14
G-CSF	34.8 ± 151	105.9	1	16.9 ± 15	66.9	0	3.9	*0.03
GRO	59.2 ± 79.5	74.4	0	84.2 ± 96	81.6	0	11.4	0.38
IFNα2	7.2 ± 10.5	67.4	7	16.4 ± 46.8	89.3	7	27.2	0.09
IFNγ	40.7 ± 12.9	23.30	0	28.0 ± 21.0	136.74	2	2.14	0.1
IL-10	3.3 ± 6.3	127.6	6	2.1 ± 2.7	103.7	3	0.3	*0.04
IL-12 (p40)	4.8 ± 1.7	60.5	9	4.8 ± 11.9	137.7	10	12.4	0.30
IL-15	9.9 ± 6.0	37.1	0	12.8 ± 5.7	31.2	0	0.6	0.32
IL-1α	0 ± 1.8	217.8	9	0 ± 4.9	153.4	12	1.5	0.74
IL-1ra	0 ± 6.9	125.6	8	0 ± 0	288.4	14	2.3	0.14
IL-1ß	0 ± 1.5	205.4	11	4.8 ± 11.9	298.6	14	0.7	0.61
IL-3	0 ± 0	0.0	16	4.8 ± 0	179.3	16	9.8	0.18
IL-6	4.8 ± 0	196.9	13	135.8 ± 224.6	120.3	3	0.4	#0.001
IL-8	16.2 ± 43.5	92.2	0	30 ± 23.5	170.9	0	0.3	0.24
IP-10	302.1 ± 280.8	107.6	0	710.4 ± 597.1	93.1	0	1.3	#0.001
MCP-1	542.4 ± 839.2	102.1	0	824.8 ± 645.5	47.8	0	1.2	0.58
MCP-3	4.8 ± 36	123.5	10	4.8 ± 5	215.5	14	5.2	0.83
MDC	52.2 ± 38.4	43.9	0	189.5 ± 119.8	41.8	0	2.4	#0.001
MIP-1α	4.8 ± 0	26.7	16	4.8 ± 0	172.7	15	6.6	0.06
MIP-1ß	9.6 ± 24.0	83.7	5	21.8 ± 23.5	127.6	3	3.2	*0.04
PDGF-AA	0 ± 2.1	201.3	11	72.6 ± 116.9	72.8	0	0.3	#0.001
PDGF-AB/BB	43.2 ± 42.9	93.6	1	34.2 ± 69.7	137.8	0	12.2	0.44
RANTES	15.6 ± 28.1	152.7	0	408.2 ± 910.9	114.3	0	1.6	#0.001
sCD40L	0 ± 0	230.9	13	5.9 ± 45.1	144.6	7	5.2	*0.005
sIL-2ra	37.5 ± 76.8	90.8	1	63.0 ± 72.5	65.9	0	7.5	0.11
TGFα	0 ± 0.6	201.2	9	0 ± 0	401.5	17	1.4	0.4
HGF	2554.4 ± 3505.7	80.2	0	2303 ± 1695.2	38.12	0	1.6	0.32
Leptin	82.2 ± 1565.3	133.21	4	1637.5 ± 2414.1	125.33	0	27.4	*0.01
Resistin	2713.4 ± 1854.6	87.25	0	3824.8 ± 3550.7	72.71	0	4.5	0.12
Adiponectin	>250000	na	na	>250000	na	na	80.3	na
OSM	0 ± 0	447.83	16	0 ± 0	307.79	12	1.59	0.09

*Overview of the measured mediator concentration (pg/ml) in control and osteoarthritic (OA) synovial fluid (SF). Data are indicated as median ± Interquartile Range (IqR). The coefficient of variation (CV) in percentage and Lower Limited of Quantification (LLOQ) with the number of samples are given. Data are subjected to non-parametric statistical analysis Mann-Whitney U; *p<0.05 and # p<0.001. GM-CSF, IL-12(p70), IL-13, IL-17, IL-2, IL-4, IL-5, IL-7, IL-9, TNFα, TNFβ, VEGF and NGF could not be detected.*

The PCA was performed to gain insight into the associations between individual mediators that were assessed. The PCA showed communalities > 0.3, which as such, were included in the analysis. PCA enabled identification of 6 clusters of interrelated mediators among the spectrum of 47 mediators (Table 2). These clusters may reflect important pathophysiological pathways in the joint. In the pattern matrix, the mediators IFNγ, OSM, IL-7, IL-1β, IL-8 and TGFα were clustered in the first cluster, and together explained 30.2 % of the total variance with an eigenvalue of 10.5. Cluster 2 included IL-3, MIP-1α, EGF, leptin and IL-12, (variance explained 16.8%, eigenvalue 5.9). Cluster 3, contained MCP-1, IL-10, eotaxin and G-CSF (variance explained 10.8%; eigenvalue 3.8). Cluster 4 contained RANTES, PDGF-AB/BB, PDGF-AA, sCD40L, IP-10, IFNα (variance explained 6.4%, eigenvalue 2.2). Cluster 5 included Flt-3 ligand, HGF, IL-15 and sIL-2ra (variance explained 6.1%, eigenvalue 2.1). Cluster 6 included the adipokines IL-6 and resistin (variance explained 6.0%, eigenvalue 2.1). In total, these 6 clusters explained more than 76% of the variance. The first two clusters mainly contained pro-inflammatory mediators and cluster 3 included predominantly chemokines. Cluster 4 contained predominantly growth factors and cluster 5 factors associated with T-cell proliferation and maturation. Finally, cluster 6 contained only adipokines. Mediators in all these 6 clusters are known to be part of important processes in the joint homeostasis. However, distinguishing between potential clusters was difficult, since these mediators are involved in adjacent and interrelated pathways.

Cytokines, chemokines, adipokines and growth factors play a major role in inflammatory diseases, such as OA, and are currently intensively studied. The adipokines are associated with obesity, which is in itself associated with low-grade systemic inflammation, one of the risk factors for the development and progression of OA [10, 34, 35]. The different levels of IL-6, leptin and adiponectin between control and OA synovial fluid might indicate a role for certain adipokines in OA processes, which is in line with literature [36]. However, these data were not corrected for BMI, which might influence the outcomes. Another group of mediators consisted of chemokines. The measured chemokines such as the related Chemokines-Chemokines (CC) mediators RANTES, MDC, MIP-1α and MIP-1β were higher in OA than in control synovial fluid. This group of chemokines share the same receptor complexes, e.g. CCR1, CCR2 and CCR5 [37] and their major function is to attract inflammatory cells, e.g. T-cells, macrophages and other inflammatory cells to sites of inflammation. Chemokines, such as RANTES, are also capable of activating (inflammatory) cells in the production of inflammatory mediators. As shown in previous studies, RANTES promotes the production of IL-6 by synovial fibroblasts and enhances the inflammatory response in OA through the different CCR receptor in synovial tissue[38]. Moreover, CCR receptors are present in chondrocytes and treatment of cartilage explants with RANTES was demonstrated to increase release of proteoglycan [39]. The combination of multiple CC-chemokines in combination with IL-6 and leptin in the synovial fluid was not demonstrated earlier. Blocking these CC-chemokines or their receptors in OA might reduce IL-6 and leptin production

and, consequently, the infiltration of inflammatory cells and the production of catabolic factors in the joint. Future research is necessary to elucidate this.

Table 2. Principal component analysis

Action	Mediator	Cluster					
		1	2	3	4	5	6
Pro-inflammatory	IFNγ	0.988					
Pro-inflammatory	OSM	0.980					
Pro-inflammatory	IL-7	0.971					
Pro-inflammatory	IL-1β	0.947					
Anti-inflammatory	IL-1ra	0.851					
Chemokine	MCP-3	0.849					
Pro-inflammatory	IL-8	0.823					
Growth factor	TGFα	0.612					
Pro-inflammatory	IL-3		0.942				
Chemokine	MIP-1α		0.919				
Proliferation	EGF		0.888				
Adipokine	Leptin		0.871				
Proliferation	IL-12		0.702				
Chemokine	MCP-1			0.841			
Anti-inflammatory	IL-10			0.807			
Chemokine	Eotaxin			0.653			
Chemokine	G-CSF			0.622			
Chemokine	RANTES				-0.953		
Growth factor	PDGF-AB/BB				-0.935		
Growth factor	PDGF-AA				-0.914		
Chemokine	IP-10				-0.705		
Growth factor	sCD40L				-0.683		
Pro-inflammatory	IFNα				-0.589		
T-cell factor	Flt-3 ligand					-0.827	
Growth factor	HGF					-0.795	
T-cell factor	IL-15					-0.703	
T-cell factor	sIL-2ra					-0.565	
Adipokine	IL-6						0.922
Adipokine	Resistin						0.678
Eigenvalues		10.5	5.8	3.8	2.2	2.1	2.1
Variances explained (%)		30%	17%	11%	6%	6%	6%
Cronbach's alphas		0.835	0.059	0.114	0.615	0.070	0.115

Pattern matrix after Oblimin rotation with Kaiser Normalisation as obtained from PCA in the spectrum of synovial fluid mediators. Control and osteoarthritic samples were combined. Only loading factors > 0.5 are displayed.

In none of the synovial fluid samples GM-CSF, IL-12(p70), IL-13, IL-17, IL-2, IL-4, IL-5, IL-7, IL-9, TNFβ, VEGF and NGF and TNFα, could be detected. IL-1 was only detected at very low levels. TNFα and IL-1 are pro-inflammatory cytokines, which are associated with cartilage degeneration, synovial inflammation and bone change [40]. Although in some *in vivo* animal models of OA,

blocking IL-1 or TNFα gave promising results, this could not be validated in clinical studies. Our results likewise do not support a prominent role for IL-1 and TNFα in end-stage OA patients. Nonetheless, this does not exclude a role for IL-1 and TNFα in for example early OA, as OA has a heterogeneous character with multiple phenotypes.

For the interpretation of the data it should be mentioned that our results are based on a small sample size, due to donor availability, which were not paired on age, BMI and sex. Moreover, it should be taken into account that ex-vivo modifications cannot be excluded for the control donors as samples were taken after death. Also due to the small sample size, no further statistics were performed on the PCA to study differences between the clusters for OA and control synovial fluid samples. Therefore this pilot dataset should be regarded as a reference and more extensive profiling studies are necessary to confirm these data.

In summary, this is the first study measuring a wide panel of mediators in the synovial fluid of both control and end-stage OA donors. Increased levels of mediators such as MDC, IL-6 and RANTES once more confirm involvement of inflammatory processes and might be associated with the influx of inflammatory cells in OA synovial tissue. In addition, the PCA indicated 6 clusters, which reflect different processes in the joint. Due to the small samples size no hard conclusions can be drawn. Nonetheless, this pilot dataset provides a valuable reference for future experiments to study pathophysiological pathways, and to be useful in more extensive profiling studies for OA.

Summary

Soluble mediators, e.g. cytokines, chemokines and growth factors, are acknowledged as key players in the pathophysiology of osteoarthritis (OA) [10, 20]. However, a wide-spectrum screening of such mediators in the joint environment is currently lacking. In this study, synovial fluid was collected from control donors and end-stage knee OA patients and analysed for 47 cytokines, chemokines and growth factors using several multiplex ELISAs. In addition, a principal component analysis (PCA) was performed to cluster the measured mediators. IL-6, IP-10, MDC, PDGF-AA and RANTES levels were found to be higher in OA compared to control synovial fluid ($p<0.001$). Leptin, IL-13, MIP-1β, sCD40L levels were higher and eotaxin and G-CSF levels were lower in OA synovial fluid than in control synovial fluid, albeit at borderline significance ($p<0.05$). Increased levels of inflammatory mediators and chemokines, such as MDC and IL-6, imply involvement of inflammatory processes in OA and might be associated with the influx of inflammatory cells in OA synovial tissue. Additionally, the PCA enabled identification of 6 different clusters, which explained 76% of the variance, and in this way could indicate different pathophysiological pathways in the joint. This dataset is valuable as a reference for future experiments to study pathophysiological pathways, and useful in more extensive profiling studies for OA.

Chapter 3

Inflammatory mediators in posttraumatic radiocarpal osteoarthritis

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Abstract

Purpose

Inflammatory mediators play a major role in the pathogenesis of osteoarthritis (OA) and rheumatoid arthritis (RA). Yet, mediator profiles in the wrist joint are unknown. The aim of this study is identification of the mediator profile in healthy, pre-OA and end-stage OA radiocarpal joints. We hypothesize an increase in soluble mediators in post-traumatic wrist OA.

Methods

We obtained radiocarpal synovial fluid samples from three groups of patients: healthy control (n=12) samples were collected during wrist ganglion resection; pre-osteoarthritic (n=16) samples during a 3-ligament tenodesis (3LT) procedure for complete scapholunate dissociation; and end-stage OA (n=20) samples in patients with proven radiological OA joint changes. Using multiplex ELISA we measured twelve different mediators: interleukin (IL)-1β, tumor necrosis factor-α (TNFα), oncostatin-M (OSM), interferon-γ (IFNγ), IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-1RA and osteoprotegerin (OPG). Statistical analysis was performed using ANOVA, and Bonferroni corrected post-hoc tests.

Results

Mediators IL-6, IL-10 and IFNγ were increased in OA wrists compared to healthy and pre-OA samples ($P<0.001$). TNFα, OSM, OPG, IL-8, IL-1RA were detected but not at increased levels in OA wrists. We found no differences between healthy and pre-OA joints in all twelve mediators. Mediators IL-4, IL-7, IL-13 and IL-1β were detected in neither healthy nor (pre-)OA samples.

Conclusions

Measurement of soluble mediators in wrist synovial fluid is feasible. We identified no difference between healthy and pre-OA samples, suggesting no altered inflammatory status at the time of the 3LT procedure. Consequently, mechanical disturbance seems to be the driving force towards OA and OA associated inflammation in this stage of scaphoid lunate dissociation. Increased levels of IFNγ, IL-6 and IL-10 confirm inflammatory changes in the mechanically disturbed post-traumatic OA radiocarpal joint.

Introduction

Radiocarpal osteoarthritis (OA) is the most common form of wrist OA, encompassing 57% of all cases [41]. As it originates mostly after joint trauma, most common causes are joint malalignment after a distal radius fracture, nonunion of scaphoid fractures or tearing of the scapholunate ligament [42, 43]. OA after nonunion of a scaphoid fracture is known as scaphoid nonunion advanced collapse (SNAC). Wrist OA following tearing of the scapholunate ligament is referred to as scapholunate advanced collapse (SLAC). Both injuries can progress towards pan-carpal OA when left untreated [42, 44].

As illustrated by its etiology, wrist OA developts mainly following intra-articular mechanical disturbances. However, individual difference in the risk for development of post-traumatic wrist OA and pace of progression remain unexplained [45, 46]. Therefore, the exact pathogenesis of post-traumatic OA is not yet fully understood [47]. Currently, OA's inflammatory component is increasingly recognized in both primary and post-traumatic OA[2, 10, 48]. In particular the release of certain anabolic and catabolic soluble mediators, *e.g.* growth factors, cytokines and chemokines, is thought to play a role in the progression of OA [2, 10, 49]. In a healthy joint the cartilage turnover by chondrocytes is balanced. After the initial joint trauma and during the course of OA the poise of anabolic and catabolic mediators in the joint is disturbed, resulting in net loss of cartilage matrix [10]. These soluble mediators can be divided into functional subgroups: chondroprotective, chondrodestructive and modulatory. Chondrodestructive mediators are a driving force for destruction of cartilage and progression of OA.

We hypothesize an increase in soluble mediators in post-traumatic wrist OA, in conjunction with the intra-articular mechanical disturbance. To provide insight in the wrist's inflammatory status, we measured twelve mediators in synovial fluid of healthy, pre-OA and OA radiocarpal joints.

Materials and methods

Sample and patients characteristics

Radiocarpal synovial fluid was sampled from three patient groups: healthy controls (n=12), pre-stage OA (n=16) and end-stage OA (n=20). Healthy synovial fluid samples were collected from radiocarpal joints in patients treated for a dorsal or volar wrist ganglion. Patients with suspicion of associated carpal instability, clinical or radiological signs of OA, or recurrent ganglia were excluded. These samples were considered the best available healthy controls (table 1).

Pre-stage OA synovial fluid samples were collected during 3-ligament tenodesis (3LT) procedures, as described by Garcia-Elias *et al.* [50], performed for complete scapholunate dissociation. Since untreated scapholunate dissociation can progress to wrist OA/SLAC, we identified these patients as a possible pre-OA group[50]. The 3LT procedures were only performed before the onset of the first SLAC stage (without radiological signs of OA). Diagnosis of scapholunate dissociation was made based on history, clinical examination, and imaging studies (plain X-ray with or without additional MRI). During surgery, the diagnosis was confirmed by a ruptured (dorsal) scapholunate ligament.

End-stage OA synovial fluid samples were taken from patients undergoing various surgical procedures for end-stage OA. Patients in this group had clinical symptoms and radiological changes consistent with advanced OA of the radiocarpal joint; all patients had a history of wrist trauma. In addition, the American College of Rheumatology (ACR) criteria for OA were met by patients included in this group[51]. Baseline characteristics are shown in table 1.

Table 1. Baseline characteristics

	Healthy	Pre-OA	OA	p value*
Samples (n)	12	16	20	
mean age (yr, SD)	33.6 (±9.7)	36.1 (±13.7)	44.5 (±16.9)	0.11
Sex ratio ♂:♀	4:8	7:9	8:12	
BCA (µg/ml)	673.6 (±242.4)	602.5 (±202.8)	676.1 (±155.5)	0.63

**p value is based on one-way ANOVA; n=number, yr=years, SD=standard deviation*

Sample collection

Immediately after opening of the radiocarpal joint capsule, a sample of synovial fluid was absorbed into a pre-weighed, standard size, sterile gauze swab. The saturated swab was then placed in 500 µl HPE-0.1375% Tweenbuffer solution (Sanquin, Amsterdam, Netherlands). Samples and buffer were vortexed to dissolve the synovial fluid prior to a two minute 3000 rpm centrifuge cycle to spin down any cells or debris. The supernatant was stored at -80 °C until further analysis. This project was approved by the Medical Ethics Committee of our institution.

Protein assay

To confirm full saturation of each swab, a bicinchoninic acid (BCA) protein assay (Thermo scientific, #23227, Rockford, USA) was performed to quantify protein levels according to the manufacturer's protocol. In short, a standard curve was made using bovine serum albumin. Pre-treated synovial fluid samples were incubated for 30 min at 37 ˚C with color reagent A+B and measured at 540 nm. The protein concentration was calculated using the standard curve and expressed as µg/ml (table 1.).

Multiplex ELISA

In the present experiment, twelve mediators were measured: interleukin (IL)-1β, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-1RA, osteoprotegerin (OPG), tumor necrosis factor (TNF)-α, oncostatin-M and interferon (IFN)-γ (table 2). The selection of mediators was based on findings in rheumatoid arthritis, knee OA and the availability of mediator modulating treatment [20, 52]. Samples were analyzed using a multiplex ELISA as previously described by de Jager *et al.* [32, 53]. In short, two hundred µl of each of the OA synovial fluids with buffer solution samples was pre-treated with 10 µl of hyaluronidase (Sigma, St, Louis, MO, USA; 10 mg/ml) for 30 min at 37 °C. Samples were spun down in a X-column (Corning, Amsterdam, Netherlands; Costar 8169). Finally, 5 µl rat-mouse-serum was added to bind any residual interfering antibodies.

Pre-treated samples were incubated with pre-coated carboxylated beads (Luminex corporation, Austin TX, USA). Recombinant proteins were used to make a standard curve. Treated samples were incubated with the coupled beads. After incubation with the appropriate biotinylated antibodies, samples were thoroughly washed and incubated with streptavidin-PE for ten minutes. After washing, samples were measured and analyzed using the Bio-Plex suspension system (Bio-Rad laboratories, Hercules CA, USA) with Bio-Plex Manager software version 5.0. Concentrations of mediators in the conditioned medium were calculated using standard curves and are expressed as pg/ml.

Statistical analyses

Data are presented as mean ± standard deviation. An analysis of variance (ANOVA) between groups was performed followed by a post-hoc Bonferroni correction. A p-value <0.05 was considered significant.

Results

Baseline characteristics revealed no significant difference in patient age or protein levels between groups. Of the twelve measured soluble mediators, three showed significantly different inter-group concentrations. IL-6 ($p<0.001$), IL-10 ($p=0.001$) and IFNγ ($p=0.044$) were significantly increased in the OA synovial fluid compared to healthy and pre-OA samples. IL-8 showed a trend towards an increase ($p=0.074$). TNFα, OSM, OPG, IL-8, IL-1RA were not increased in the OA group. IL-4, IL-13, IL-7 and IL-1β were not detected. No significant differences between the healthy and pre-OA synovial fluid were found for all measured mediators (figure 1).

Table 2. Panel of measured mediators

		Westacott *et al* 1990	Van de Ham *et al* 2009	Beekhuizen *et al* 2013	Teunis *et al* 2013			
	Sampled joint	Knee	Various	Knee	Radiocarpal			
	Age	73 +/-12	57 +/- 38					
	Type	OA	OA	OA	OA	Pre-OA	healthy	
Mediator	Function	Concentration						P Value
destructive								
IL-1β	- ↑ MMP* - ↑ IL-8, IL-6	28 ± 5	1 ± 1	5 ± 12	ND	ND	ND	
IFN-γ	- ↑ production MMP	7 ± 1	1 ± 1	29 ± 21	70 ± 34	43 ± 31	44 ± 28	0.044
TNF-α	- ↑ MMP - ↑ IL-8, IL-6	0.5 ±0.04 ng/ml	5 ± 2	ND	1 ± 1	1 ± 4	0.1 ± 0.3	
OSM	- ↑ production MMP	-	0 ± 0	0 ± 0	24 ± 21	16 ± 12	17 ± 9	
IL-7	- ↑ increased survival of IFN-γ producing T-cells - ↑ aggrecanasesproduction	-	-	ND	ND	ND	ND	
IL-8	- ↑ IL-6, IL-1β, TNF-α	-	-	30 ± 24	98 ± 161	16 ± 15	20 ± 17	
protective								
OPG	- ↑ IL-1RA	-	2 ± 2	-	576 ± 726	1149 ± 1426	745 ± 875	
IL-4	- ↓ TNF- α - ↑ IL-1RA - neutralizesupregulation of ADAMTS-genes	-	0 ± 0	ND	ND	ND	ND	
IL-10	- ↓ TNF- α - ↑ IL-1RA	-	ND	2 ± 3	163 ± 78	75 ± 61	84 ± 67	0.001
IL-13	- ↓ TNF- α - ↑ IL-1RA	-	0.2 ± 0.1	ND	ND	ND	ND	
IL-1RA	- blocks IL-1 receptor, so inhibits effects of IL-1	-	-	0 ± 0	185 ± 241	197 ± 162	180 ± 121	
modulatory								
IL-6	- ↑ MMP's	-	201 ± 301	136 ± 225	272 ± 98	129 ± 81	148 ± 99	< 0.001

All value are expressed in pg/ml; ND = not detectable
** Beekhuizen et al is expressed median +/- interquantile range; others as mean +/- StD*
MMP = matrix metalloproteinases

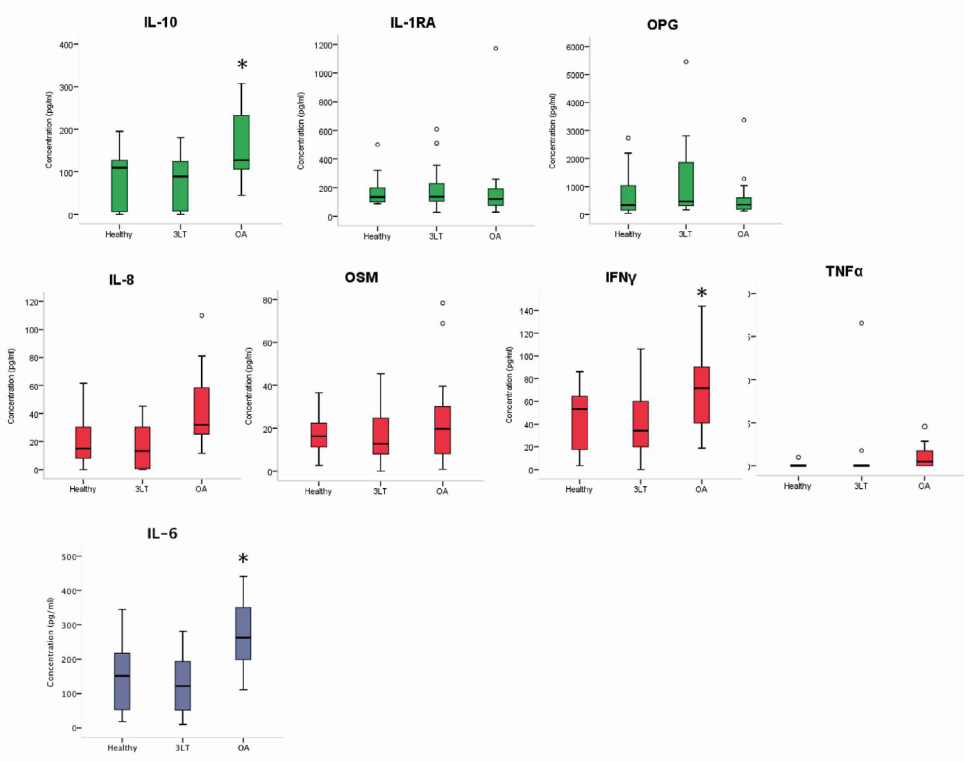

Figure 1: Concentration of cytokines in healthy, 3LT and OA synovial fluid
Results of the multiplex ELISA for healthy, 3LT and OA synovial fluid; Whiskers indicate minimum and maximum concentration (pg/ml); * indicate $P < 0.05$).

Discussion

Over the years, it has become evident that the inflammatory cytokine network contributes substantially to the pathogenesis of OA [8, 9, 10]. This study proves measurement of soluble mediators in wrist synovial fluid is feasible and confirms an inflammatory component in end-stage wrist OA, as we found increased levels of IFNγ, IL-6 and IL-10. In contrast to end-stage OA, inflammatory mediators are lacking in the scapholunate dissociation group. Possibly the initial rise in mediators immediately after the traumatic event is halted and normalizes over time; this suggests changed joint mechanics become the driving force towards wrist OA. We hypothesize that at a defined moment in a more advanced stage of SLAC, inflammatory changes arise and catalyze the development of OA. Several other studies have looked at cytokines in synovial fluid (table 2). In a comparable study Van den Ham *et al.* measured cytokines in the synovial fluid of various rheumatoid arthritic and OA joints, not including the radiocarpal joint [54]. Although direct comparison of results is not possible because of different joints involved and variance in

sample collection, their results are in line with our lack of IL-4, IL-13 and low levels of IL-1ß (1.1 ± 1.3 pg/ml vs. not detectable in our study) and TNFα (4.7 ±2.4 pg/ml vs. 1.0 ±1.3 pg/ml in our study) in OA joints. A study conducted by Westacott *et al [55].* found even lower levels of TNFα (0.5 ±0.04 ng/ml), but higher levels of IL-1ß (27.8 ±4.5 pg/ml). However, OA samples were acquired from older patients (mean age 72.9 years) with longstanding knee OA; average disease duration was 12.7 years. Mediator modulation has been a promising therapy in the treatment of OA[56, 57], as it proved beneficial in other diseases associated with a strong increase in mediators such as rheumatoid arthritis and anterior cruciate ligament tears [40, 58]. However, modulating therapies showed only short-term beneficiary effects in knee and hand OA[59-61].

In our opinion, despite the increase of soluble mediators found in wrist OA, successful modulation remains doubtful. Post-traumatic wrist OA primarily starts as a mechanical disturbance, followed by an increase in soluble mediators only in end-stage OA. Even if inflammation could be diminished by specific mediator targeted therapy, the primary mechanical disturbance causing OA remains unchanged. However, in conjuncture with surgery, mediator modulating therapies might improve varying surgical results for wrist OA. Possible weaknesses of the present study should be noted: healthy synovial fluid samples were collected from radiocarpal joints in patients treated for a dorsal or volar wrist ganglion. An array of reasons for the development of ganglia has been proposed, ranging from joint capsule herniation to ligament stress [62]. We took care to only select patients without definable underlying diseases (*e.g.* joint laxity or carpal instability). We consider these samples to be the best (ethically) available healthy controls. As no power analysis was performed, it is possible our study was unable to identify small differences in mediator levels. Nonetheless, despite relatively small sample seize we were able to identify a correlation between several mediators and wrist OA.

In summary, scapholunate dissociation is characterized by altered joint mechanics known to cause OA. We found no differences between healthy and pre-OA samples, therefore mediator profiles suggest no altered inflammatory status. We detected significant changes between healthy and advanced OA wrists as we found increased levels of IFNγ, IL-6 and IL-10. Hence, we confirm the concept of an altered inflammatory joint environment in the post-traumatic OA wrist.

Chapter 4

Soluble mediators in primary knee and posttraumatic wrist osteoarthritis

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Abstract

Background

New discoveries about the pathophysiology changed the concept that all forms of osteoarthritis are alike; this lead to the delineation of different phenotypes such as age, trauma or obese related forms.

Purposes

We aim to compare soluble mediator profiles in primary and posttraumatic osteoarthritis. Based on the general faster progression rate of posttraumatic osteoarthritis, we hypothesize a more inflammatory profile.

Methods

We collected synovial fluid from 20 primary osteoarthritic knee and 20 posttraumatic osteoarthritic wrist joints. By multiplex enzyme-linked immunosorbent assay we measured 17 mediators: chemokine ligand 5, interferon-γ, leukemia inhibitory factor, oncostatin-M, osteoprotegerin, tumor necrosis factor-α, vascular endothelial growth factor, interleukin (IL)-1α, IL-1β, IL-1 receptor antagonist, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13 and IL-17.

Results

Ten mediators were higher in posttraumatic osteoarthritic synovial fluid: tumor necrosis factor-α (TNFα), IL-1α, IL-1RA, IL-6, IL-10, IL-17, oncostatin-M, interferon-γ, chemokine ligand 5 and leukemia inhibitory factor ($p<0.001$). IL-1ß, IL-4, IL-7 were not detected, TNFα was not detected in primary osteoarthritic synovial fluid. IL-8, IL-13, osteoprotegerin and vascular endothelial growth factor levels did not differ between the synovial fluid types.

Conclusions

When compared to primary knee osteoarthritis, posttraumatic wrist osteoarthritis seems characterized by a stronger inflammatory response. Our results do not support a prominent role for IL-1ß and TNFα in end-stage osteoarthritis.

Introduction

Osteoarthritis is the most common joint disease and from middle age onward it is one of the most frequent and symptomatic health problems[63]. Its incidence is expected to rise because of an ageing population and the obesity epidemic. Osteoarthritis is a multifactorial disease causing articular cartilage failure induced by a complex interplay of genetic, metabolic, biochemical and biomechanical factors [64, 65]. New discoveries about the pathophysiology have changed the concept that all forms of osteoarthritis are alike and share the same clinical and structural characteristics[66]. This notions lead to the delineation of different clinical and structural phenotypes such as age, trauma or obesity dominated forms of the disease [2].
The posttraumatic subtype of osteoarthritis is characterized by faster progression at a younger age than the primary osteoarthritis subtype [67-69] and has a higher susceptibility of specific joints such as the ankle, shoulder and wrist [2, 42, 70]. Altered joint mechanics are recognized to be a driving force in posttraumatic osteoarthritis. However the concept of residual joint instability after joint trauma as the sole cause of osteoarthritis seems insufficient as osteoarthritis develops even if reconstructive surgery successfully stabilizes the joint [49, 71]. This suggests a role for anabolic and catabolic soluble mediators such as growth factors, cytokines, and chemokines from the time of the initial joint injury up to end stage osteoarthritis [49, 64, 72, 73].

The aim of the study was to compare the soluble mediator profiles of primary knee osteoarthritis and posttraumatic wrist osteoarthritis. Based on the general faster progression rate of posttraumatic osteoarthritis, we hypothesize a more inflammatory profile.

Materials & Methods

Patient characteristics
We collected synovial fluid from two groups of patients: posttraumatic wrist osteoarthritis samples (n=20) were obtained during various surgeries for radiocarpal osteoarthritis. Primary kmee osteoarthritis (n=20) synovial fluid was acquired during total knee replacement due to end stage osteoarthritis. The American College of Rheumatology criteria for osteoarthritis were met by patients included in both groups[51]. Exclusion criteria were rheumatoid arthritis or infection. In accordance with 'good use of redundant tissue for research' constructed by the Dutch Federation of Medical Research Societies, tissue samples were anonymized precluding use of patients' characteristics for data analysis. Therefore, synovial fluid samples could not be matched for age, BMI or sex. Collection of synovial fluid was approved by the Medical Ethics Committee of our institution (12-223/C).

Sample collection

Knee synovial fluid was aspirated directly after opening of the joint capsule. Due to the low amount of synovial fluid in the wrist joint, samples were collected by pre-weighed, standard size, sterile gauze swabs. This technique allows collection of synovial fluid when the available quantity is low[73]. Immediately after opening of the radiocarpal joint, a sample of synovial fluid was absorbed. The saturated swab was then placed in 500 µl HPE-0.1375% Tween buffer solution (Sanquin, Amsterdam, Netherlands). Both wrist and knee synovial fluid samples were vortexed prior to a two minute 3000 rounds per minute centrifuge cycle to spin down any cells or debris. Thereafter, the supernatant was stored at -80 °C until further analysis.

As we could not reliably determine the exact volume of the swabbed synovial fluid samples by their weight, all cytokine levels were normalized to their protein content. To quantify the protein levels, we performed a bicinchoninic acid protein assay (Thermo scientific, #23227,Rockford, USA) according to the manufacturer's protocol. In short, a standard curve was made using bovine serum albumin. Pretreated synovial fluid samples were incubated for 30 minutes at 37°C with color reagent A+B and measured at 540 nm. The protein concentration was calculated using the standard curve and expressed as micrograms per milliliter.

Multiplex enzyme-linked immunosorbent assay

We measured 17 mediators: interleukin (IL)-1α and β, IL-1 receptor antagonist (RA), IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, IL-17, chemokine ligand five (CCL5), interferon (IFN)-γ, leukemia inhibitory factor (LIF), oncostatin-M (OSM), osteoprotegerin (OPG), tumor necrosis factor (TNF)-α and vascular endothelial growth factor (VEGF). We based our selection on findings in rheumatoid arthritis, osteoarthritis and the availability of mediator modulating therapies [10, 20, 64]. Samples were analyzed using a multiplex enzyme-linked immunosorbent assay, as previously described by de Jager et al.[53, 74]. In short, we pretreated 200 µL of each of the synovial fluids with buffer solution samples with 10 µl hyaluronidase (Sigma, St, Louis, MO; 10 mg/mL) for 30 min at 37°C. Samples were spun down in a X-column (Costar 8169; Corning, Amsterdam, Netherlands). Finally, 5µL rat-mouse serum was added to bind any residual interfering antibodies. Pretreated samples were incubated with precoated carboxylated beads (Luminex Corp, Austin, TX). Recombinant proteins were used to make a standard curve. Treated samples were incubated with the coupled beads. After incubation with the appropriate biotinylated antibodies, samples were thoroughly washed and incubated with streptavidin-PE for 10 minutes. After washing, samples were measured and analyzed using the Bio-Plex suspension system (Bio-Rad Laboratories, Hercules, CA) with Bio-Plex Manager software, version 5.0. All samples were measured in duplicate in the same plate. Concentrations of mediators in the synovial fluid were calculated using standard curves.

Statistical Analysis

The Shapiro-Wilk test showed a predominantely non-Gaussian distribution of the data; therefore cytokine levels were compared by Mann-Whitney U test. We determined and adjusted the level of significance (P) using the Bonferroni correction for multiple testing. A P-value of <0.0038 was considered significant (i.e., adjusted P-value cutoff =.05/15=0.0033). All statistical analyses were conducted in Stata 13 (StataCorp LP, College Station, TX). As cytokine levels were normalized to their protein content, we report normalized concentrations as 10^{-3} picogram mediator/microgram protein/ml, median ± interquartile range.

Results

Of the 17 measured soluble mediators, 10 were higher in posttraumatic than in primary osteoarthritis: IL-1α, IL-1RA, IL-6, IL-10, IL-17, CCL5, IFNγ, LIF, OSM and TNFα ($p<0.001$). TNFα could not be detected in any of the primary osteoarthritis samples, whereas it was detectable in posttraumatic synovial fluid. IL-1ß, IL-4 and IL-7 were not detected in either group; IL-8, IL-13, OPG and VEGF did not differ between the two groups (Table 1).

Table 1. Concentration of mediators in synovial fluid of patients with primary osteoarthritis of the knee and posttraumatic osteoarthritis of the wrist

Mediator	Wrist OA (median ± IqR)	Knee OA (median ± IqR)	P value
IL-1α	27 (14-51)	0.91 (0-1.4)	< 0.001
IL-1β	ND	ND	
IL-1RA	249 (155-390)	18 (15-25)	< 0.001
IL-4	ND	ND	
IL-6	533 (404-713)	39 (27-83)	< 0.001
IL-7	ND	ND	
IL-8	85 (51-162)	52 (34-71)	0.0397
IL-10	259 (217-473)	39 (35-56)	< 0.001
IL-13	ND	0 (0-0.73)	0.009
IL-17	353 (213-570)	9.6 (3.8-16)	< 0.001
CCL5	3554 (2170-5535)	209 (86-395)	< 0.001
IFN-γ	146 (83-183)	5.6 (4.7-10)	0.0045
LIF	431 (183-661)	11 (5.9-21)	< 0.001
OSM	40 (17-61)	0.49 (0-5.0)	< 0.001
OPG	721 (391-1215)	2137 (802-6575)	< 0.001
TNFα	1.0 (0-3.6)	ND	0.0011
VEFG	131 (82-325)	216 (126-358)	0.099

Concentrations are in 10^{-3} picogram mediator / microgram protein / ml

Discussion

The aim of the study was to compare the soluble mediator profiles of primary knee osteoarthritis and posttraumatic wrist osteoarthritis. We confirmed our hypothesis of a more inflammatory profile in the posttraumatic osteoarthritis group. Several potential shortcomings of the study should be kept in mind. To allow comparison of samples we normalized mediator levels to their protein concentrations. Synovial fluids from different joints were previously shown to contain similar levels of serum proteins in rabbits[75]. Although it cannot be excluded that serum protein levels change when joints are inflamed or that the similarity between joints may not hold true for humans, this was the appropriate way to normalize between samples. Additionally, our correcting for multiple testing might have been too stringent, and, due to the low number of available samples this study may not have been adequately powered to detect smaller differences.

Distinct differences in concentration were noted between posttraumatic wrist osteoarthritic mediators and primary knee osteoarthritis. Although some of these are protective, such as Il-10 and IL-1RA, in general, posttraumatic wrist osteoarthritis seemed characterized by a stronger inflammatory response as proinflammatory mediator; IL-1α, IL-6, IL-17, CCL5, IFNγ, LIF, OSM and TNFα levels were higher. For this study we carefully selected two very different groups of patients with osteoarthritis. We were able to demonstrate very clear differences in cytokine levels. A comparison of cytokine levels expressed in joints with different types of osteoarthritis has never been made before. It could be argued that the differences found in the current study were related to joint type rather than differences in pathology, however, it was previously described that inflammation as characterized by macrophage and T-cell number and Il-6 production was not dependent on joint type in the human patient[76]. We therefore speculate that the more pronounced inflammatory response in the wrist samples is related to the posttraumatic origin and that increased inflammation might offer a paradigm for the faster progression of posttraumatic osteoarthritis compared to primary osteoarthritis.

This initial study contributes to our knowledge and vision on the role of cytokines in the pathogenesis of osteoarthritis and can help to direct further research to markers and treatments for osteoarthritis. Many new pharmacological approaches in the management of osteoarthritis are under development [77] and mediator modulation could be a promising therapy [59, 66]. Despite the promising protective effects of IL-13[20] and IL-4[78], our data confirm the existing literature[54, 79] indicating a limited role for those cytokines in end stage osteoarthritis as IL-4 could not be detected and only low levels of IL-13 were found in primary osteoarthritis. IL-1ß and TNFα have been indicated as prominent inflammatory mediators associated with cartilage degeneration, bone changes and synovial inflammation[40, 66]. IL-1 family cytokines seem to play an important role in the period directly following joint trauma [80-82] as increased IL-1

expression has been documented after mechanical joint injury and correlates with the severity of cartilage damage[80]. In *in vivo* animal models of osteoarthritis blocking IL-1ß and TNFα gave promising results; however, this could not be validated in clinical studies[58]. In both the literature and our study, IL-1ß and TNFα were not detectable or were detected at low levels in synovial fluid from patients with end stage posttraumatic as well as primary osteoarthritis [54, 55, 79].

Our results, likewise, do not support a prominent role for IL-1ß and TNFα in end stage osteoarthritis. Lack of in-depth understanding of phenotype pathogenesis restricts further development of diagnosis, treatment, and monitoring of the many forms of osteoarthritis. Our study is the first to compare cytokine levels and to show differences between subgroups of osteoarthritis. Growing knowledge of the pathogenetic mechanisms involved in osteoarthritis improves understanding and will allow specific targeted treatment in those with different subsets of the disease.

Chapter 5

Osteoarthritic synovial tissue inhibits proteoglycan production in human osteoarthritic cartilage; Establishment and characterisation of a long-term coculture

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Abstract

Objective

Although both cartilage and synovium are affected in osteoarthritis, no *in vitro* coculture models of human osteoarthritic tissues have been described. The aim of this study is to develop an *in vitro* model that includes both synovium and cartilage.

Methods

Cartilage and synovium explants were cultured alone or in coculture for 21 days. Histology, LDH release, matrix metalloproteinase (MMP) activity, glycosaminoglycan (GAG) content, release and synthesis and cytokine production were used to evaluate synovial tissue functionality and its effect on cartilage metabolism. To assess the possibility of intervention in the model system, the effect of triamcinolone was studied.

Results

Throughout the entire culture period, synovial tissue remained viable and produced cytokines. Monocultures of synovial and cartilage explants produced different cytokine subsets. Cytokine subsets in coculture were most similar to those previously described in osteoarthritis synovial fluid. MMP activity was only detectable in synovial explant monoculture and in coculture. Coculture with synovial tissue reduced final GAG content ($P < 0.02$) via inhibition of GAG production ($P < 0.001$) rather than through increase of GAG release. Addition of triamcinolone inhibited cytokine production, MMP activity in coculture and synovial tissue and counteracted the inhibition of GAG production induced by coculture. Triamcinolone in cartilage monoculture, however, reduced GAG production.

Conclusion

Synovium affects cartilage metabolism by reduction of GAG production. Triamcinolone relieved this inhibition, while being inhibitory when added to cartilage monoculture. These results clearly indicate the importance of tissue coculture, which may be a promising tool for studying osteoarthritis pathophysiology and evaluation of possible interventions.

Introduction

Osteoarthritis (OA) is a major health problem affecting more than 14% of the people aged 25-74 in the United States. With ageing of the population, an increasing incidence is anticipated in the near future [83]. At this time there is no cure for OA, only symptomatic relief and at end-stage OA, total joint arthroplasty. To this day, the exact pathogenesis and aetiology of OA remains unclear and is still a subject of much debate and research. Various mechanisms have been postulated, in which cartilage and synovial tissue are both proposed to play a crucial role [8, 84]. In particular synovial inflammation and subsequent release of specific catabolic and anabolic cytokines from the synovial tissue are thought to mediate the initiation and progression of OA[20, 85]. Synovial tissue from OA patients displayed increased expression of inflammatory mediators and production of matrix metalloproteinases (MMP) that are capable of degrading cartilage proteoglycans and collagen[13, 86].

Despite the widely acknowledged role of the synovial tissue in OA, most *in vitro* research mimics OA by the addition of inflammatory cytokines, such as interleukin-1 (IL) or tumor necrosis factor (TNF)–alpha, to cartilage explants or chondrocyte culture to study disease-related alterations in cartilage metabolism[87]. On a limited scale, cocultures of monolayer chondrocytes with synovial tissue or synovial tissue cells have been employed[88]. However, isolation and monolayer culture on tissue culture plastic is known to affect cell phenotype and hence some of these cocultures may have limited translational value [89, 90]. Although experience is available with long-term culture of cartilage explants, less is known about the viability and functionality of synovial tissue during longer term culture. Apart from some coculture models with animal tissue, cocultures of human synovial tissue and cartilage explants lasted up to a maximum of one week[91-94]. To date no long-term coculture models with human OA cartilage and synovial tissue have been described.

The aim of this study was to develop and characterise an *in vitro* model that includes both synovial tissue and cartilage. As cartilage explants are already known to survive long-term culture periods[95], the viability and functionality of synovial tissue, over a culture period of 21 days, was first evaluated by histology, lactate dehydrogenase (LDH) release and the secretion of cytokines. Subsequently, the effect of osteoarthritic synovial tissue on chondrocyte metabolism and matrix production of cartilage explants was studied. Finally, the possibility to modulate this interaction was tested by the use of the corticosteroid triamcinolone.

Materials & Methods

Sample collection

Osteoarthritic articular cartilage and synovial tissue were obtained from patients undergoing total knee arthroplasty (n=9). Cartilage explants were taken from the non-weight bearing part of the femoral condyles, as the weight bearing parts were commonly destroyed by the OA. Synovial tissue was stripped from fat and capsule tissue and showed, upon visual inspection, a generalised mild inflammation and fibrosis. The synovial tissue and cartilage were cut into pieces of approximately 1 by 1 mm, with an average wet weight of 24.5 ± 13.2 mg per cartilage explant and 28.4 ± 13.3 mg per synovial tissue explant. From each donor, 6 cartilage explants were fixed immediately in 4% formaldehyde for histological evaluation of degeneration stage (supplementary table 1) and 6 explants placed at -20 °C for biochemistry. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in the UMCU[96].

Viability of synovial tissue during culture

Synovial tissue explants of three donors were cultured in three different culture media to evaluate the most suitable medium for setting up the coculturing. The first culture medium consisted of Dulbecco's Modified Eagle Medium (DMEM) high glucose, 10% fetal calf serum (FCS, Gibco BRL Life Technologies, Germany) and 1% antibiotics (penicillin and streptomycin); the second culture medium was based on DMEM with 1% FCS and 1% antibiotics; the third culture medium was serum-free and consisted of DMEM, 1% ascorbate-2-phosphate, 1% insulin transferrin selenium (ITS)-X (Gibco BRL Life Technologies, Germany), 1% antibiotics and 50 μg/ml L-proline (Sigma, St Louis MO, USA). Synovial tissue explants (n=12 per culture medium) were cultured in 24 wells plates for 3, 7, 14 and 21 days in a humidified incubator in an environment of 5% CO_2 and 37 °C. Culture medium was renewed every 3-4 days. Part of the conditioned medium was immediately used for lactate dehydrogenase (LDH) activity measurement and a part was stored at -80 °C for cytokine analysis. Explants were harvested at day 3, 7, 14 and 21. Fresh explants were used for Live/Dead viability staining; Explants were fixed in 4% formaldehyde at room temperature or frozen in OCT compound (Sakura Finetek, Zoeterwoude, Netherlands) and stored at -80 °C for histochemical and immunohistochemical analysis.

Cartilage-synovial tissue coculture

Synovial tissue and cartilage of the same patient were used for the cocultures. 6 OA donors were used for the coculture experiments. At day zero, 6 cartilage samples were evaluated using the OARSI Osteoarthritis Cartilage Histopathology Assessment System [97]and scored between grade 1.5 and 3.8 (Supplementary Table 1) and 6 explants were digested to assess glycosaminoglycan (GAG) content at day zero (method described below). The degree of OA correlated with GAG content (Supplementary Table 1).

Cartilage explants (n=6) and synovial tissue (n=6) were cultured for 21 days alone or together (coculture; n=12) in 24-well plates (Corning, NY, USA). To prevent direct cell-cell contact between cartilage explants and synovial tissue, synovial tissue explants were cultured in Millicell filter inserts with a pore size of 0.4 μm (Millipore, Billerica, MA, USA). All explants were cultured in 1.0 ml of DMEM containing 1% ascorbate-2-phosphate, 1% ITS-X, 1% antibiotics and 50 μg/ml L-proline in a humidified incubator at 5% CO_2 and 37 °C for 21 days. Culture medium was renewed every 3-4 days and stored at -80 °C for analysis. At the end of the culture period, conditioned medium was collected and stored at -80 °C for analysis of cytokine concentration, NO concentration, GAG concentration and MMP activity. Explants were processed for paraffin histology or stored at -20 °C for biochemical analysis. In 3 experiments (3 donors), 0.1 mM triamcinolone acetonide (throughout the remainder of the manuscript referred to as triamcinolone (T6501, Sigma, St Louis MO, USA) was added[98, 99].

Cytotoxicity

Viability of the synovial tissue was determined using the spectrophotometric LDH cytoxicity assay (Roche, Mannheim, Germany) according to the manufacturer's protocol. 100 μl of medium sample were mixed with 100 μl dye solution and incubated for 15 minutes at room temperature. After 15 minutes, a stop solution was added and absorbance was read at 490 nm with a reference filter at 655 nm. Results were presented in arbitrary units (AU).

Live/Dead staining

Synovial explants were stained at day 0, 14 and 21 with a Live/Dead immunofluorescence staining (Invitrogen, Eugene Oregon, USA) for viability according to the manufacturer's protocol. In short, the explants were incubated in 1 μl Calcein AM diluted in 1000 μl DMEM for 30 minutes. After the incubation period the explants were washed in PBS and incubated in 1 μl Ethidium homodimer-1 diluted in 1000 μl DMEM for 5 minutes. The synovial explants were then placed on a glass slide, covered with a cover slip and evaluated under the fluorescence microscope. Green represents viable cells, red dead cells.

(Immuno)histochemical analysis

From paraffin-embedded cartilage and synovial tissue explants, 5 μm sections were cut. Cartilage sections were stained using Safranin-O/fast green [100] to evaluate morphology and GAG content and scored using the OARSI Osteoarthritis Cartilage Histopathology Assessment System with a maximum score of 6 for severe osteoarthritic damage. Synovial tissue sections were stained with haematoxylin-eosin to assess overall morphology.

To specifically evaluate the presence of macrophages, immunohistochemical staining for CD68 was performed and to evaluate the presence of T-lymphocytes, immunohistochemical staining for CD3 was performed. Sections were deparaffinised and rehydrated to distilled water. 0.3% H_2O_2

was used for 10 minutes to block endogenous peroxidase activity. Antigen retrieval was done by heating for 1 hour in citrate buffer. For the CD68 and CD3 staining, sections were incubated with mouse monoclonal antibody (mAB) CD68 (Leica biosystems Newcastle, Novocastra, working dilution 1:400) and mouse mAB CD3 (DAKO, Hamburg, Germany, 1:50), respectively. After washing with PBS-Tween buffer (0.1% Tween), CD68 sections were incubated with a biotinylated goat anti-mouse (DAKO, Hamburg, Germany, 1:200) and then incubated with peroxidase-conjugated streptavidin (DAKO, Hamburg, Germany, 1:500). After a final wash step in PBS-Tween, sections were placed in 3,3'di-amino-benzidine (DAB) for 30 minutes and counterstained with Mayer's hematoxylin. For the CD3 staining, sections were incubated with Link and Label (DAKO, Denmark, 1:100) and sections were placed in Fast Red staining and counterstained with Mayer's hematoxylin. As positive control a tonsil section was used, as negative control an isotype matched control antibody (Monoclonal Mouse IgG1, Dako, Denmark, 1:200) was used.

GAG analysis

GAG release and total GAG content was quantified using the di-methylmethylene-blue (DMMB) spectrophotometric analysis described by Farndale[101]. Cartilage explants were digested in 200 µl 2% papain (Sigma, St. Louis, MO, USA) in 50 mM phosphate buffer, 2 mM N-acetylcysteine, and 2 mM Na_2-EDTA (pH 6.5) at 60 °C overnight. Two hundred µl of DMMB solution and 100 µl of medium sample or papain digest were mixed and absorbance read at 540 nm and 595 nm using the spectophotometer (Bio-Rad laboratories, Hercules CA, USA). As reference, chondroitin sulfate C (Sigma, St Louis MO, USA) diluted with culture medium or PBS-EDTA was used. GAG concentrations in the media and papain digests are given in µg GAG/g wet weight of cartilage. Calculation of GAG production was done with the following formula: $GAG_{production} = (GAG_{content\ day\ 21} - GAG_{content\ day\ zero}) + GAG_{cumulative\ release}$ given in µg GAG/g wet weight of cartilage.

NO analysis

Medium concentration of nitric oxide (NO) was determined by quantifying its derived product nitrite using the spectrophotometric Griess reagent (Sigma, St Louis MO, USA) according to the manufacturer's protocol. In short, nitrite concentrations of the cultured media sample were measured by mixing 70 µl of conditioned medium with 70 µl Griess reagent. Absorbance was read at 540 nm (Bio-Rad laboratories, Hercules CA, USA). As reference, $NaNO_2$ dissolved in PBS was used to construct a standard curve for calculating the NO release of each sample. NO concentrations are reported in µmol.

Multiplex ELISA

To determine the cytokine levels in conditioned medium, a multiplex ELISA was used as previously described[32, 53]. Validation of the Multiplex ELISA was previously done by de Jager et al., showing excellent sensitivity and specificity. The panel of cytokines measured is largely based

on the recent reviews by Rutgers *et al*.[48], Fernandes *et al[20].* and Goldring *et al[10]*. and two research papers describing the presence and effect of these cytokines in OA[102, 103]. The cytokines measured were IL-1β, IL-1RA, IL-4, IL-6, IL-7, IL-8, IL-10, IL-13, osteoprotegerin (OPG), oncostatin M (OSM), TNF-α and IFN-γ. In short, specific antibodies were coupled to carboxylated beads (Luminex corporation, Austin TX, USA). Recombinant proteins were used to make a standard curve. Medium samples were incubated with the coupled beads. After incubation with the appropriate biotinylated antibodies, samples were thoroughly washed and incubated with streptavidin-PE for 10 minutes. After washing, the samples were measured and analysed using the Bio-Plex suspension system (Bio-Rad laboratories, Hercules CA, USA) with the Bio-Plex Manager software version 3.0. The concentrations of cytokines in the conditioned medium were calculated using the standard curves and are expressed as pg/ml. Using specific ELISA's for determination of IL-6 (Cytoset Invitrogen, Camarillo CA, USA) and IL-8 (Cytoset, Biosource, Nivelles, Belgium) in a randomly selected subset of conditioned media were used to compare the Multiplex ELISA data.

MMP activity

For assessing matrix metalloproteinase (MMP) activity, the FS-6 substrate assay was used[104]. The FS-6 substrate is known to be converted by MMP-1, 2, 3, 7, 8, 9, 13 and 14. In short, 50 μl FS-6 substrate (10 μM) dissolved in MMP buffer was added to 50 μl of culture medium in a 96-wells black plate with clear bottom (Greiner Bio-one, Alphen a/d Rijn, The Netherlands). Extinction wavelength was 320 nm and emission was measured at 405 nm during 6 hours, every 30 minutes using the Gemini SpectraMax (Molecular devices, Sunnyvale CA, USA). MMP activity is presented as Relative Fluorescence Units (RFU) emitted per hour.

Statistical analysis

SPSS version 16.0 (SPSS Inc., Chicago, IL, USA) was used for data analysis. Data are expressed as mean ± SD. An univariate analysis of variance was performed for GAG content after 21 days of culture, GAG release, GAG production and NO release. To correct for inter-donor variability a so-called randomized block design was used. P values less than 0.05 were considered statistically significant.

Results

Viability synovial tissue, macrophage and T-cell content and cytokine production

OA synovial tissue explants were cultured for 21 days in three different culture media. LDH release of the synovial tissue explants was highest in the first days of culture and remained low during the rest of the culture (figure 1a), indicating limited cell death, without clear differences between the three media. To confirm the presence of viable cells at the end of the culture period, cells in

the explants were lysed for maximum LDH release from the cultured synovial tissue. Maximum LDH release upon devitalisation reached > 50 AU, which was far beyond the concentration during culture indicating the presence of viable cells during culture (data not shown). Furthermore, viability was confirmed by a live/dead assay after 21 days of culture (figure 1b).

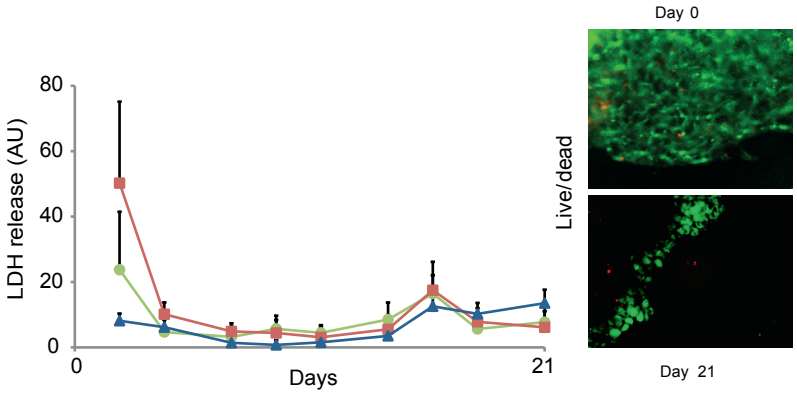

Figure 1. Viability of synovial tissue during 21 days of culture in 3 different media.
A. LDH release from synovial tissue for 3 different culturing conditions (10% FCS green line; 1% FCS red line; serum free medium blue line). Results are an average of 3 different donors, per donor n=6. Mean ± SD in arbitrary units (AU) of n = 18 given for each time point. B. Live/dead assay for day zero and after 21 days of culture. Day 14 and day 21 showed viable cells (only day 21 shown; green cells present viable cells; red the dead cells, magnification 40x).

Macroscopically, all synovial tissue explants displayed fibrosis and hyperaemia and signs of hyperplasia as demonstrated by H&E staining (data not shown). Hyperplasia was determined by cell number increase and thickening of the synovial layer. CD68+ and CD3+ cells were present at day zero, 3, 7, 14 and 21 days of culture (Figure 2a), indicating the presence of synovial macrophages and T-lymphocytes, respectively, during culture in all culture media. As viability was not compromised in either culture medium, serum-free culture medium was chosen for further experiments to avoid interference of serum with cytokine analysis and have better control over the culture conditions. Multiplex ELISA on the conditioned medium confirmed the release of pro- and anti-inflammatory cytokines by synovial tissue during the entire culture. Cytokine profiles of synovial tissue for 3 different synovial tissue donors showed that the synovial tissue produced cytokines during the entire culture period (Figure 2b). In particular the synovial tissue secreted high concentrations of IL-1RA, IL-6, OPG and IL-8.

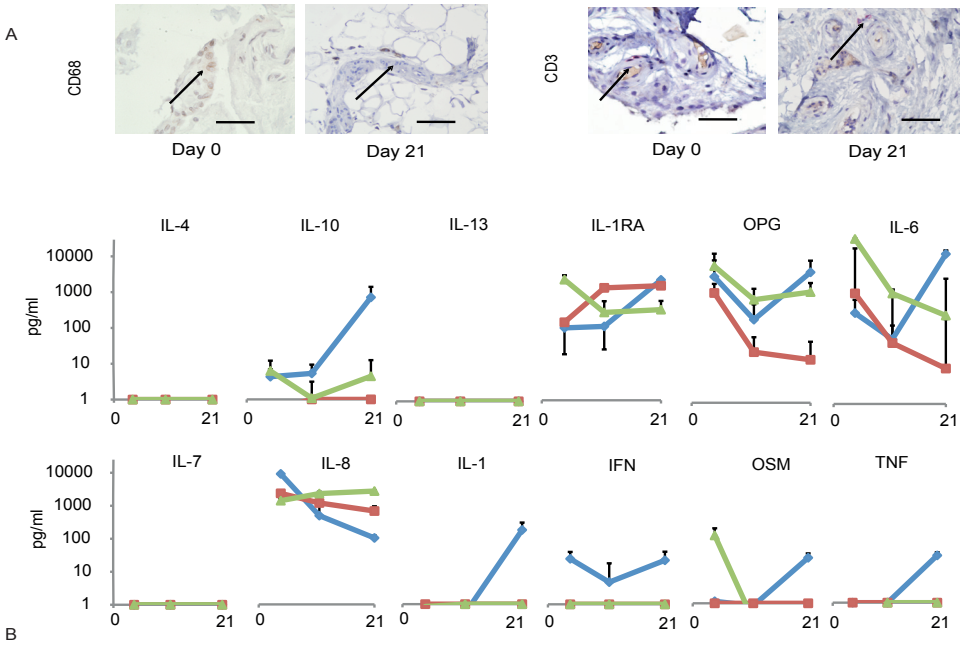

Figure 2. Viability of synovial tissue during 21 days in culture.
A. CD3 and CD68 immunohistochemical staining day 0 and day 21 (scale bar represents 20 μm; magnification 100x). Cells positive for CD3 are shown in red, cells positive for CD68 in brown (positive cells indicated by arrow). B. Cytokine profile for 12 cytokines at day 4, 11 and 21 of the conditioned media from synovial tissue for 3 different donors; each line represents the average of six sample of one donor. Days of culture on X-axis (mean ± SD n=6; concentration in pg/ml). Cytokine profile showed production of IL-1ra, OPG, IL-6 and IL-8 for all 3 donors during the entire culture period.

GAG content, release and production in cocultures of synovial tissue and cartilage

Neither cartilage nor synovial tissue explants showed outgrowth of cells after culturing. During the entire culture period, GAG release was seen from all the cartilage explants. However, GAG release through time (Figure 3a) and cumulative GAG release (Figure 3b) were not different between cartilage explants cocultured with synovial tissue explants or cartilage cultured alone. GAG release from synovial tissue was negligible (data not shown). GAG content in cartilage cocultured with synovial tissue was significantly lower after 21 days of culture than in cartilage cultured alone ($P < 0.02$, figure 3c). GAG production in cartilage explants cocultured with synovial tissue was reduced ($P < 0.001$, figure 3d). NO production and the histological aspect of the tissue after culture was not significantly different between cartilage cocultured with synovium and cartilage cultured alone (data not shown).

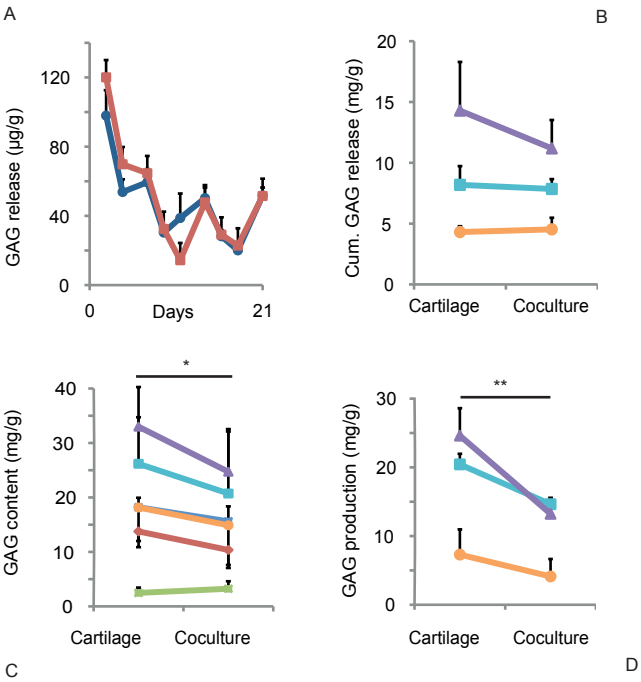

Figure 3. Glycosaminoglycan content, release and production in cartilage cultured alone or cocultered with synovial tissue.
A. GAG release over time from cartilage cultured alone (blue line) or cocultured with synovial tissue (red line); 1 representative donor (mean ± SD in µg GAG/g cartilage; n=6 per condition). B. Cumulative GAG release for 3 different donors; each line represents one donor (mean ± SD in mg GAG/g cartilage; n=6 explants per condition). C. GAG content after 21 days of culture; each line represents a donor (n = 5 per donor, mean ± SD given in mg GAG/g cartilage,$P < 0.02$). D. GAG production in 21 days for 3 different donors; each line represents a donor (n = 5 per donor; mean ± SD given in mg GAG/g cartilage, ** $P < 0.001$).

Cytokine and MMP production in coculture vs monocultures

Several pro- and anti-inflammatory cytokines were consistently produced by cartilage and synovial tissue during the entire culture period. Although the absolute cytokine concentrations were variable between cultures, the subsets produced by each type of tissue were consistent over the donors (data not shown). Cartilage explants produced IL-1β, IL-4, IL-7, IL-10 and IL-13 but did not secrete IL-6, IL-8 and IL-1RA (Figure 4). Synovial tissue explants did secrete IL-6, IL-8 and IL-1RA, however IL-1β, IL-4, IL-7, IL-10 and IL-13 were not detected in the medium. OPG was produced by both tissues, although at much higher levels by the cartilage explants. OSM and IFN-γ showed an irregular patern of production in both tissues while TNF-α was not detected in any of the cultures. Cytokines previously described in OA synovial fluid are IL-1β (+/-), IL-1RA (+), IL-6 (+/-), IL-7 (+), IL-8 (+), IL-10 (+/-), IL-13 (+), OPG (+), OSM (+), IFN-γ (+), TNF-α (-) and IL-4 (-), (present: (+), irregularly present: (+/-) and not present: (-) (27) and thus the subsets found in coculture were most similar to those found in vivo compared to the monocultures. In the comparison between the Multiplex ELISA data and the standard ELISA no clear difference was (data not shown).

In the cartilage explant cultures alone no MMP activity was detected in the culture medium, whereas in medium of synovial tissue cultured alone or cocultured with cartilage, MMP activity was detected during the entire culture period (Figure 5).

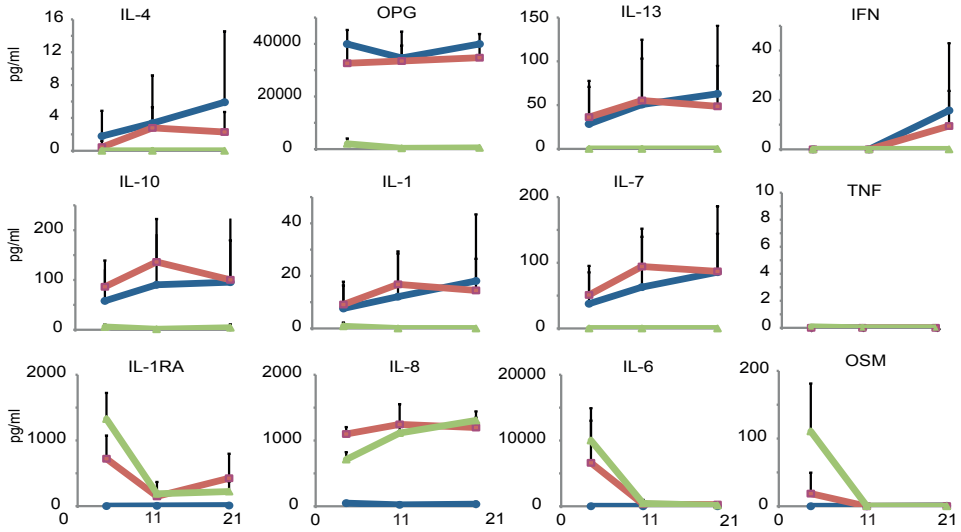

Figure 4. Profile of cytokines secreted in medium of cartilage, synovial tissue or coculture of cartilage with synovial tissue.
Twelve pro- and anti-inflammatory cytokines were measured at day 4, 11 and 21 in conditioned media using the multiplex ELISA. Cartilage cultured alone (blue line; dot); Synovial tissue (green line; triangle) cultured alone; Coculture of cartilage and synovial tissue (red line; square); one representative donor during 21 days in culture (mean ± SD n=6; concentration in pg/ml).

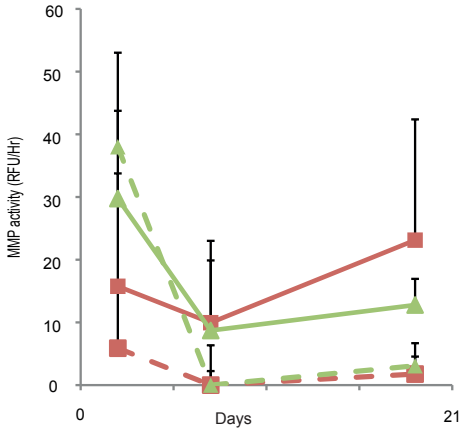

Figure 5. MMP activity in medium of cartilage and synovial tissuein the presence or absence of triamcinolone.
Activity shown as relative fluorescence unit (RFU) per hour (n=6, one representative donor) at day 2, 7 and 19 in the conditioned media. Cartilage cultured alone showed no detectable MMP activity, cartilage coculture with synovial tissue (solid red line; squares) and synovial tissue alone (solid green line; triangles) showed MMP activity. MMP activity was completely abolished by triamcinolone after 7 days of culture (dashed green line; triangles and red line; squares).

Effect of triamcinolone on GAG content, release and production

Triamcinolone has been reported to inhibit the production of pro-inflammatory cytokines and decrease MMP activity[105]. To evaluate this in our model, MMP activity and cytokine profiles were measured in the conditioned medium. MMP activity in the synovial tissue and in coculture decreased upon addition of triamcinolone (Figure 5). Furthermore, multiplex ELISA demonstrated a decrease of cytokines released by both cartilage and synovial tissue (Figure 6). Although the production of most cytokines was completely abolished by the addition of triamcinolone, OPG and IL-8 levels were only affected to a limited extent.

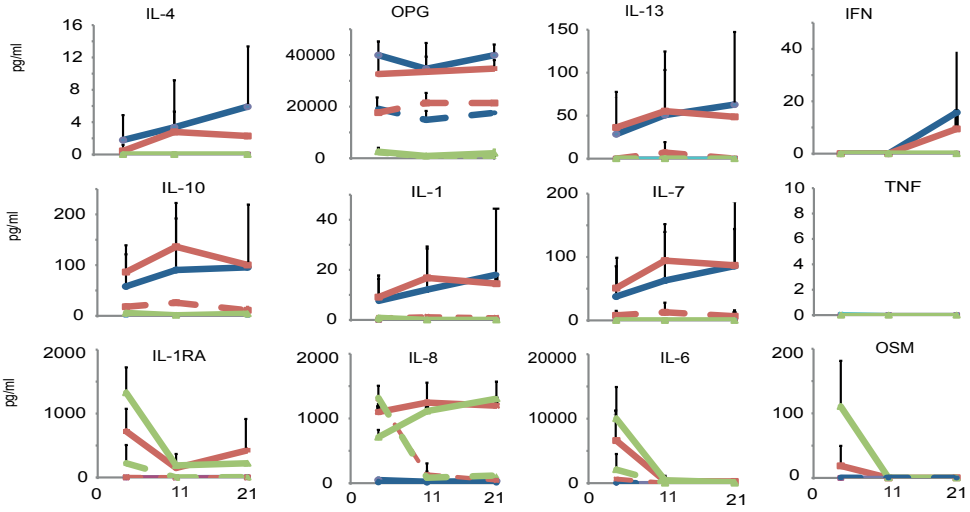

Figure 6. The effect of triamcinolone on profile of secreted cytokines.
Cytokine profiles at day 4, 11 and 21 in the conditioned media of cartilage, synovial tissue and coculture in the presence or absence of triamcinolone. Cartilage (blue line; dot), cartilage with triamcinolone (blue dashed line; dot), coculture of cartilage and synovium (red line; square), coculture of cartilage and synovium with triamcinolone (red dashed line; square), synovial tissue (green line; triangle) and synovial tissue with triamcinolone (green dashed line; triangle) during 21 days of culture. 1 representative donor (mean ± SD n=6; concentration in pg/ml), showing for multiple cytokines a complete decrease of cytokine production when triamcinolone is added. OPG and IL-8 were only partially affected.

Addition of triamcinolone influenced GAG turnover in the cartilage explants (Figure 7). In cultures with cartilage explants only GAG release ($P < 0.001$, figure 7b) and GAG production decreased ($P < 0.001$, figure 7d), with no effect on production and final content. In cocultures of synovial tissue and cartilage explants, GAG release in coculture was inhibited by triamcinolone ($P < 0.042$, figure 7b). For two of the three donors, triamcinolone also prevented the inhibition of GAG production in cartilage cocultured with synovium (each donor $P < 0.001$). Final GAG content in coculture was restored to the level of cartilage monoculture, suggesting that the effect of synovial tissue was counteracted by triamcinolone (Figure. 7c).

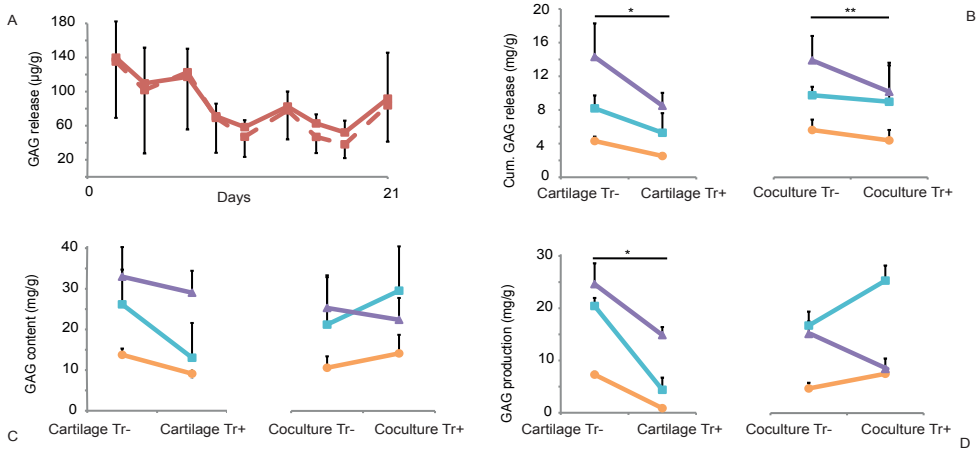

Figure 7. The effect of triamcinolone on GAG content, release and production
Cartilage cultured alone or cocultured with synovial tissue in the presence of absence of 0.1 mM triamcinolone (TR-/+). A. GAG release over time from cartilage cocultured with synovial tissue with (dashed lines) or without triamcinolone (solid lines); Results from 1 representative experiment are shown (mean ± SD in µg GAG/g cartilage; n=6 per condition). B. Cumulative GAG release of cartilage cultured alone or cocultured with synovium, supplemented with triamcinolone or unsupplemented; three lines represent three donors (mean ± SD in mg GAG/g cartilage; n=5 per donor per condition, * $P < 0.001$, ** $P < 0.042$). C. GAG content for cartilage alone or cocultured with synovium, supplemented with triamcinolone or unsupplemented; three lines represent three donors (mean ± SD in mg GAG/g cartilage; n=6 per donor per condition). D. GAG production for cartilage alone or cocultured with synovium; three lines represent three donors (mean ± SD given in mg GAG/g cartilage; n=5 per donor per condition).

Discussion

The current study is the first to describe an *in vitro* model that takes the role of both synovial and cartilage tissue in human OA-related cartilage degeneration into account. Cultured OA synovial tissue remained viable and contained macrophages and T-lymphocytes even after 3 weeks of culture. Most importantly, the synovial tissue was capable of producing cytokines throughout the entire culture period. The presence of synovial tissue in coculture with cartilage decreased the final GAG content of cartilage by decreasing GAG production of the chondrocytes, without a clear effect on GAG release. Interestingly, many cytokines appeared to be derived from the cartilage rather than the synovial tissue, namely IL-4, IL-7, IL-10, IL-13 and OPG. However, synovial tissue in turn secreted cytokines that were not produced by cartilage, such as IL-6, IL-8 and IL-1RA. Only OPG was produced in both cultures, albeit at higher concentrations in cartilage cultures. Comparison with literature-based data on the cytokine content and profiles of OA synovial fluid furthermore showed that the cytokine profiles in the cocultures of cartilage and synovial tissue were most similar to profiles found in OA synovial fluid[106], suggesting the current model mimics the OA joint more closely. Finally, addition of triamcinolone demonstrated the possibility

to intervene in the process by changing the cytokine production, counteracting the anti-anabolic effect of synovial tissue on cartilage and decreasing the release of GAGs.

Most *in vitro* OA models are based on simulation of the OA intra-articular environment by the addition of high levels of pro-inflammatory compounds, in particular IL-1 and TNF-α, to cartilage explants or chondrocytes. Usually these cytokines are added at concentrations 10-1000 times higher than the levels usually found in OA synovial fluids[48], usually below 3.0 pg/m and 5.0 pg/ml for TNF-α and Il-1 respectively, in line with the levels found in the current coculture. Although synovial tissue is generally assumed to play a major role in OA-related cartilage degeneration, very few coculture models have been described. Most of these models depart from healthy, often nonhuman or rheumatoid arthritis (RA) tissues and will therefore not accurately represent the OA environment[107, 108].

In the current study, coculturing cartilage with synovial tissue did not increase GAG release, even though active MMPs were secreted by synovial tissue and not by cartilage. However, coculturing did reduce GAG production, leading to a net decrease in GAG content of cartilage. This is in line with recent data on cocultures of healthy bovine cartilage with healthy human synovial tissue, where at 3 days of culture a strong reduction in sulphate incorporation was found. Inhibition of IL-1 and TNF-α signalling did not abolish this effect, which may be explained by a lack of production of these factors by synovial tissue and cartilage. This is in accordance with the current study where no TNF-α production and limited production of IL-1β was found. The predominant effect of synovial tissue on cartilage matrix synthesis rather than on release once more shows that chondrocyte function in terms of matrix production is affected in OA and that cartilage degeneration seen in OA is not only a result of matrix degradation. However, as no sulfate incorporation assays have been carried out, it cannot be excluded that a variable part of the GAG release actually represented a failure to retain newly synthesised GAGs in the matrix.

It is unclear which of the factors is responsible for the inhibition of GAG synthesis in the cartilage by synovial tissue. IL-6, IL-8 and OSM secreted by the synovial tissue are all three known to have an effect on cartilage[109, 110]. Interference with these factors may demonstrate whether they actually play a role in the found inhibition of GAG production. However, in cartilage degeneration and OA, many mediators are likely to play a role. The panel of factors measured in this study is limited to the most common pro- and anti-inflammatory cytokines, whereas the role of other factors cannot be ruled out, such as leptin or IL-17, both recently shown to be involved in cartilage degeneration[111, 112].

The current model showed a clear effect of a corticosteroid on cytokine secretion and MMP production by cartilage and synovial tissue, which is in accordance with previous *in vitro* studies[113]. Also the inhibition of GAG production in single cartilage explant culture by triamcinolone was similar to the effects of corticosteroids found previously on chondrocytes and cartilage explants[114, 115], suggesting that corticosteroids may not be given without restraint for the treatment of OA. However, in coculture, triamcinolone actually counteracted the inhibitory effect of synovial tissue on cartilage matrix production. This again stresses the importance of combining both tissues in OA in vitro models. In addition to the positive effect of triamcinolone on GAG production in coculture, triamcinolone also partially inhibited GAG release. Although MMP activity in coculture was inhibited by triamcinolone, the role of MMPs in proteoglycan degradation in this model is debatable. As the higher MMP activity in cocultures compared to cartilage monoculture was not accompanied by an increase in GAG release, a role of the set of MMPs assayed in this study seems unlikely. It would be of high interest to look at the presences of TIMPs as they may have obscured the activity of the MMPs. Also other matrix degrading enzymes such as ADAMTS4 and -5 may play a role in this coculture model and the GAG release found. In addition, collagen turnover was not determined in this coculture model and may have been affected at later time points by the MMPs released from the synovial tissue.

Finally, a donor-related variability was noted in the current model, in particular in absolute cytokine levels and also in chondrocyte metabolism. Differences is tissue response may be related to differences in grades of OA and hence in baseline metabolic state. OA Stage-dependent difference can account for differences found in absolute values, whereas specific mediators are present in different stages of the disease. Differentiation between OA grades in the analysis of effects may actually enable tailoring of new treatments for the various stages of OA.

We propose the first long-term *in vitro* model that is capable to evaluate the effect of various soluble factors on both synovial and cartilage tissue, better mimicking the situation in a diseased joint. Multiple outcome parameters can be used in the model; we evaluated GAG release and content, MMP activity and cytokine production. Also other parameters may be considered, especially disease markers such as CTX2 for collagen type II breakdown[116]. Altogether the model can be used to evaluate the effect of a potential OA therapy on both cartilage and synovial tissue and give insight in the complex interplay between synovial tissue and cartilage in the maintenance and progression of OA.

Chapter 6

Celecoxib suppresses inflammation without affecting cartilage repair in osteoarthritic cartilage explants but fails to inhibit the production of inflammatory mediators during regeneration of cartilage

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Submitted

Abstract

Aims

To elucidate whether suppression of inflammation by COX-2-inhibition affects cartilage repair and regeneration.

Methods

The production of inflammatory mediators was determined by ELISA. Glycosaminoglycan content and release were determined in cultured osteoarthritic cartilage explants, a co-culture of osteoarthritic cartilage explants with synovium and a tissue regeneration culture using isolated chondrocytes.

Results

Celecoxib decreased the production of multiple inflammatory factors by osteoarthritic joint tissues. However, it did not affect glycosaminoglycan metabolism. In chondrocyte regeneration cultures, celecoxib partly inhibited PGE2 release but had no effect on inflammatory factors nor on glycosaminoglycan turnover.

Conclusions

We found no indications for a positive effect of celecoxib on osteoarthritic cartilage repair, despite an inhibition of the secretion of several inflammatory factors. Moreover, during cartilage regeneration the inflammatory cytokine production by chondrocytes seems largely COX-2-independent. Our data suggest that inflammation may not always be inhibitory in cartilage repair, nor does it necessarily induce degradation.

Introduction

Osteoarthritis (OA) is a chronic joint disorder characterized by joint pain and disabilities. Key features of OA are degeneration of the cartilage, changes of the subchondral bone and synovial inflammation [1]. Inflammation is assumed to play a crucial role in osteoarthritic cartilage degeneration by the induction of various extracellular matrix degrading enzymes and inhibition of matrix protein production [85]. Celecoxib, a specific cyclooxygenase (COX)-2 inhibitor, is commonly used in the treatment of pain in OA and has fewer gastro-intestinal side effects than the commonly used non-specific non-steroidal anti-inflammatory drugs (NSAIDS) [117]. COX-2 expression can be induced by several cytokines in inflammatory conditions such as (rheumatoid) arthritis or acute tissue damage [118, 119]. In turn COX-2 inhibition was postulated to positively affect tissue integrity through inhibition of inflammation, thereby reducing ensuing breakdown of tissue and repressive effects on repair and regeneration [29, 30, 120, 121]. Inhibition of inflammation by COX-inhibitors has been suggested to be mediated through both COX-dependent and independent pathways [122].

Nonetheless, the described effects of celecoxib on cartilage regeneration and repair are conflicting [123]. A recent study in COX-2 knockout mice showed no protective effect on cartilage degeneration in a surgically induced model of OA [124]. Furthermore, celecoxib did not influence proteoglycan synthesis [13] or collagen type II synthesis [14] in different models for chondrogenic differentiation. Positive effects of celecoxib include inhibition of chondrocyte hypertrophic differentiation [125] and chondroprotection in OA in an *in vivo*/*ex vivo* study with human patients [30]. In addition, celecoxib decreased cartilage degeneration in an OA cartilage explant model [29, 120]. However, most of these studies measured short-term effects, not the result of longer-term exposure on final tissue integrity. In addition, despite the role of COX-2 in inflammation and its postulated role in cartilage matrix breakdown, until now little attention has been paid to the effect of celecoxib on the production of inflammatory mediators [126] and to what extent this is related to changes in cartilage metabolism.

In the current study we investigated the effect of celecoxib on the production of inflammatory factors in cartilage repair and regeneration using a range of *in vitro* models, focussing on long-term effects and measuring glycosaminoglycans (GAGs) as chondrogenic readout.

Materials & Methods

Collection of cartilage and synovial tissue

Healthy cartilage (macroscopically intact cartilage, from a knee without signs of cartilage degeneraton or synovial inflammation) was collected from post-mortem donors and osteoarthritic cartilage and synovial tissue was collected from end-stage OA patients undergoing total knee arthroplasty. Cartilage explants were harvested from the femoral condyles, cut into square pieces of approximately one by one mm and the wet weight was determined (mean 12 +/- 5 mg). Synovial tissue was cut into small pieces and wet weight was determined (mean 21 +/- 9 mg). From each donor, six cartilage explants and one synovial tissue explant were fixed immediately in 10 % buffered formalin for (immuno)histological evaluation and six cartilage explants were stored at -20 °C for biochemical analysis at t=0. The rest of the cartilage and synovial tissue explants were used for culturing. This study does not meet the definition of human subjects' research or require informed consent as anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our university hospital. The material was used according to the national guidelines 'code of conduct for the proper secondary use of human tissue' [96, 127] and approved by the institutional review board (protocolnumber 01/163).

Culture model with osteoarthritic cartilage

Osteoarthritic cartilage explants were cultured in the presence or absence of celecoxib (Biovision) dissolved in DMSO. Celecoxib was added at different concentrations (0.1 μM, 1.0 μM and 10 μM) with as control condition 0.1 % DMSO. Explants were cultured in a total of 1 ml of culture medium. Culture medium (DMEM, 2 % Human Serum Albumin, 1 % penicillin/streptomycin, 1 % ITS-X, 0.4 mM ascorbic acid) was renewed three times a week and stored at -80 °C for further analysis. After 21 days of culture the explants were stored at -20 °C for biochemical analysis. In total 6 different OA cartilage donors were used for the cultures (4 explants per condition).

Co-culture model with osteoarthritic cartilage and synovial tissue

A co-culture of cartilage and synovial tissue was used to study the effect of celecoxib in an inflammatory osteoarthritic environment, as previous described [57]. In short, osteoarthritic cartilage explants were co-cultured with osteoarthritic synovial tissue in the presence or absence of celecoxib. Just as in the cartilage explants alone, a total of 1 ml of culture medium was used and a dose response was performed (0.1 μM, 1.0 μM and 10 μM). Total culture period was 21 days and medium was renewed three times a week and stored at -80 °C for further analysis. In total 6 OA cartilage donors with 4 cartilage/synovium explants per condition were used for the cultures.

Tissue regeneration culture by isolated chondrocytes

For the regeneration culture both healthy and osteoarthritic articular cartilage was cut and digested in collagenase 0.15 % (w/v) (Worthington, Lakewood, USA) in Dulbecco's Modified Eagle Medium (DMEM) overnight at 37 °C. Subsequently, the chondrocytes were seeded in a monolayer at a cell density of 5000 cells/cm^2 and cultured in expansion medium containing DMEM, 10 % Fetal Bovine Serum (FBS), 1 % penicillin/streptomycin (100 U/100 µg/ml) and 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, USA). Chondrocytes were cultured until passage 2 and then used in a cartilage regeneration model as described previously [128, 129]. In short, 720.000 cells were seeded at high density on collagen type II (chicken sternal cartilage, #C9301, Sigma-Aldrich, St louis, MO, USA) coated Millicell filters (Millipore Co., Bedford, MA, USA) and cultured in redifferentiation medium containing DMEM, 2 % Human Serum Albumin, 1 % penicillin/streptomycin, 1 % ITS-X (Gibco; #51500, Bleiswijk, The Netherlands), 0.4 mM ascorbic acid and 5 ng/ml TGF-β2 (R&D Systems, Minneapolis, USA). The constructs were cultured in a total of 1 ml of culture medium and in the presence or absence of 1.0 µM celecoxib (Biovision, Milpitas CA, USA) dissolved in DMSO (final concentration of DMSO for all cultures was 0.1 %). Control conditions included the same concentration of DMSO as the celecoxib cultures. The cultures were incubated at 37 °C in 5 % CO_2. Culture medium was renewed three times a week for total culture period of 28 days. Conditioned culture medium was stored in -80 °C for further analysis. In total 6 OA and 6 healthy chondrocyte donors with 5 constructs per condition were used for the cultures.

(Immuno)histochemistry

For (immuno)histochemistry, fresh cartilage and synovial tissue explants and filters after 28 days were fixed overnight in 10 % buffered formalin, embedded in paraffin and cut into 5 µm sections. To show the cartilage degeneration in cartilage explants and neo-cartilage formation in the filters, a safranin-O/Fast-green staining was used [100]. For COX-2 immunolocalisation, the sections were blocked in 0.3% H_2O_2 in methanol for 20 minutes. For antigen retrieval, sections were boiled in citrate buffer (0.01M; pH 6.0) for 10 minutes and placed in TBS-BSA for 60 minutes, after which the sections were incubated with mouse COX-2 antibody (Cayman chemical; CX229; 1:200; Michigan, USA [130]) overnight. The samples were washed with TBS-BSA and incubated with biotinylated goat anti-mouse IgG (Dako, Denmark, 1:200 in TBS-BSA) for 1 hour. Samples were washed and incubated with Streptavidin (Dako, 1:500 in TBS-BSA) for also 1 hour. Finally, samples were developed with DAB or Powervision (Leica Microsystems, Wetzlar, Germany). As an isotype-specific negative control mouse IgG1 (Dako, 1:200 in TBS-BSA) was used in the same concentration as the COX-2 antibody.

COX activity

To determine COX activity in tissue and cell-conditioned medium, a prostaglandin E_2 (PGE_2) ELISA was performed (KGE004B, R&D; Minneapolis, USA) according to the manufacturer's protocol. COX activity was expressed as PGE_2 concentration in pg/ml.

Multiplex ELISA

The presence of interleukin (IL)-1α, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-13, oncostatin M (OSM), monocyte chemotactic protein (MCP)-1, adiponectin, leptin and neurotrophic growth factor (NGF) in conditioned medium was determined using multiplex ELISA as described previously [32, 53]. The mediators in the above mentioned panel has been shown to play a role in joint inflammation, cartilage repair or regeneration [17]. Validation of the Multiplex ELISA was previously done by de Jager *et al*., showing excellent sensitivity and specificity [32]. The samples were measured and analysed using the Bio-Plex suspension system (Bio-Rad laboratories). The concentration of all measured mediators in the conditioned medium was calculated using the standard curves and are expressed as pg/ml. Only the conditioned media of day 7 and day 28 were measured.

Glycosaminoglycan (GAG) content and GAG release

GAG release and total GAG content was quantified using the di-methylmethylene-blue (DMMB) spectrophotometric analysis described by Farndale *et al* [101]. Cartilage explants and filters were digested in 400 µl 2 % papain (Sigma-Aldrich) in 50 mM phosphate buffer, 2 mM N-acetylcysteine, and 2 mM Na_2-EDTA (pH 6.5) at 60 °C overnight. Two hundred microliter DMMB solution and 100 µl of medium sample or papain digest were mixed and absorbance read at 540 nm and 595 nm using a spectrophotometer (Bio-Rad Laboratories, Hercules CA, USA). As reference, chondroitin sulfate C (Sigma-Aldrich) was used. GAG release and GAG content for the explants are given in mg GAG/g cartilage and for the filters in µg GAG/µg DNA.

DNA assay

The DNA content per sample was determined from the papain digest using a Picogreen DNA assay (Invitrogen, Life Technologies, USA) in accordance with the manufacturer's instructions.

Statistical analysis

Data are expressed as mean with standard deviation (SD). SPSS 16.0 software (SPSS Inc. Chicago, Illinois, USA) was used for the statistical analysis. Univariate analysis of variance (ANOVA), with a randomized block design and a post-hoc Bonferroni was used to test for differences between different conditions. Normal distribution of the residuals was verified by Kolmogorov-Smirnov test and homogeneity of variances by Levene's test. Differences with a *p*-value less than 0.05 were considered statistically significant. No *post hoc* correction for multiple testing was determined in the multiplex ELISA data.

Results

Celecoxib has no effect on GAG metabolism in an osteoarthritic cartilage explant model

To study if COX-2 inhibition could support the repair of an osteoarthritic cartilage matrix, OA cartilage explants were cultured in the presence of celecoxib. Immunohistochemical detection of COX-2 confirmed the presence of the COX-2 enzyme in osteoarthritic cartilage (Fig. 1A). Osteoarthritic cartilage explants produced low amounts of PGE_2 and celecoxib decreased the production of PGE_2 (Fig. 1B; $p = 0.01$). However, celecoxib had no effect on GAG content or GAG release (Fig. 1C/D).

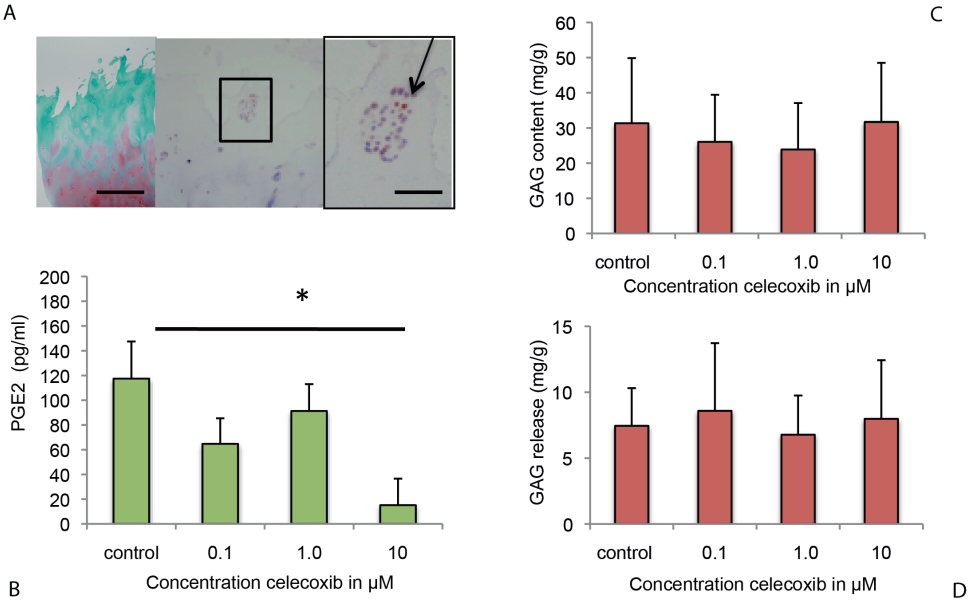

Figure 1. Effect of celecoxib on OA cartilage explants.
The safranin-O staining showed osteoarthritic cartilage changes (A). The presence of COX-2 in OA cartilage explants is visible with IHC (A; pink staining, positive cell indicated by the arrow in magnification). Celecoxib in a concentration of 10 µM decreases PGE_2 production by OA cartilage explants (B; day = 4; mean +/- SD of 3 donors, 4 explants per condition; * $p < 0.001$). In cartilage explants cultured alone no effect of celecoxib was seen on GAG content (C) or total GAG release (D) during the entire culture (mean +/- SD of 6 donors, 4 explants per condition).

Celecoxib suppresses inflammation in coculture model of osteoarthritic cartilage and synovial tissue without influencing GAG metabolism

To better simulate the condition of increased inflammation in OA, cartilage explants were co-cultured with patients' own synovial tissue that is known to release inflammatory factors [17]. Immunohistochemical detection of COX-2 showed the presence of the COX-2 enzyme in both

osteoarthritic cartilage and osteoarthritic synovial tissue (Fig. 2A). PGE_2 was clearly secreted in these co-cultures (Fig. 2B) and in addition, IL-1α, IL-1β, NGF, IL-10, leptin, MCP, adiponectin, IL-8, OSM and IL-6 were detected as well at day 7. IL-4 and IL-13 were not detectable in medium conditioned by osteoarthritic cartilage and synovial tissue. Celecoxib dose-dependently decreased PGE_2 production in co-culture (Fig. 2B; * $p < 0.001$). At 10 μM the production of the inflammatory cytokines IL-1α, IL-1β, IL-6 and OSM decreased, but also of IL-10 and NGF (* $p < 0.05$, Fig. 3). No effect of celecoxib was seen on the production of leptin, adiponectin, IL-8 and MCP-1. At day 28 in co-culture the same mediators were produced, albeit at lower levels than at day 7, with the same effects of exposure to celecoxib (data not shown). Irrespective of the concentration used, celecoxib did not influence GAG content or GAG release in cartilage explants / synovial tissue co-cultures (Fig. 2C/D).

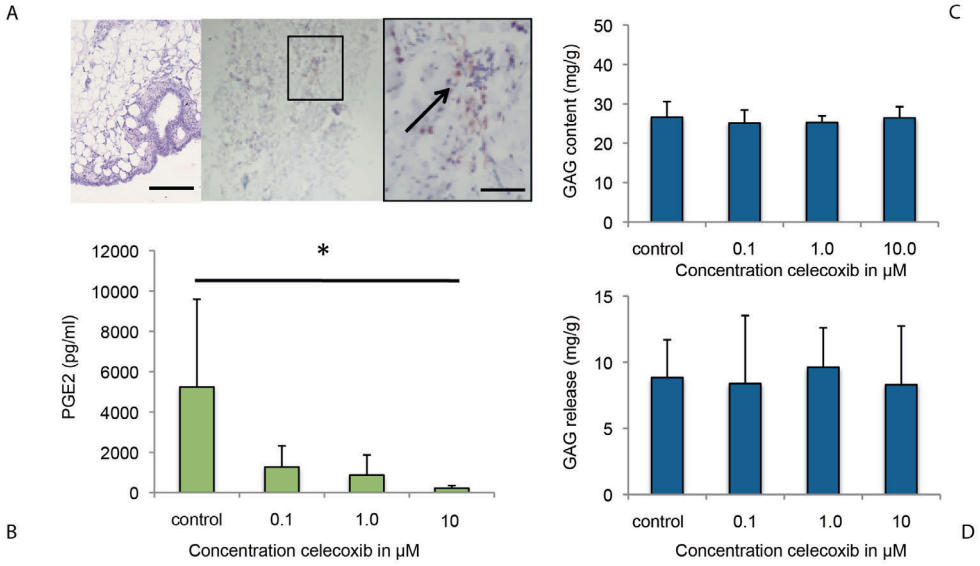

Figure 2. Effect of celecoxib on OA cartilage explants co-cultured with OA synovial tissue.
The H&E staining showed osteoarthritic synovial changes (A). IHC showed the presence of COX-2 in OA synovial tissue explants (red staining, indicated by arrow in magnification). Celecoxib decreases PGE_2 production in a dose dependent manner (B; day = 4; mean +/- SD of 3 donors, 4 cartilage/synovium explants per condition; * $p < 0.001$). In cartilage explants cultured with synovial tissue no effect of celecoxib was seen on GAG content (C) after 21 days of culture or total GAG release (D; mean +/- SD of 6 donors; 4 cartilage/synovium explants per condition).

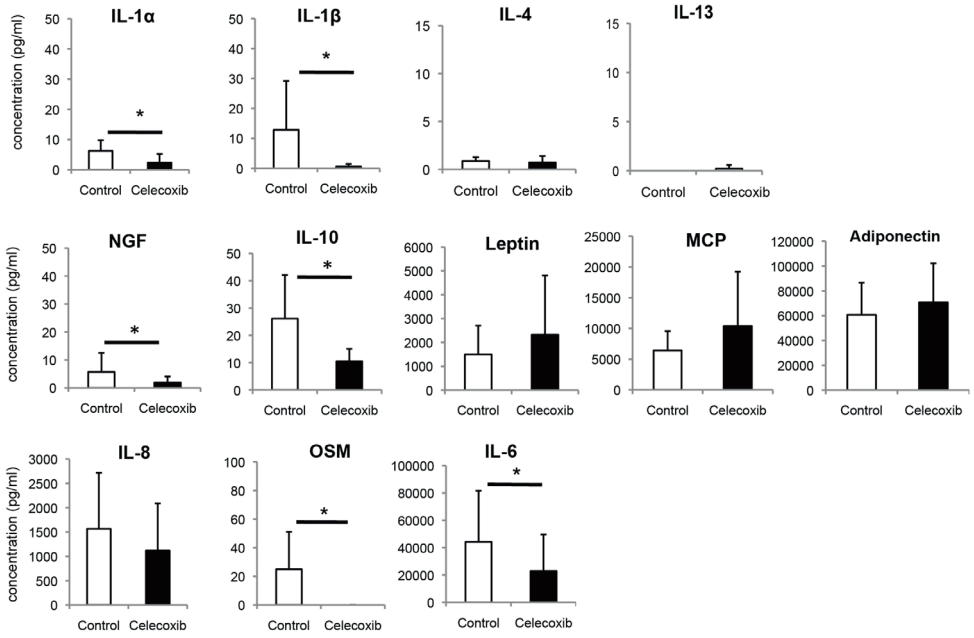

Figure 3. Production of mediators in the co-culture model at day 7.
Celecoxib at 10 μM decreases production of IL-1α, IL-1β, IL-6, IL-10, OSM and NGF. No effect is seen on the production of leptin, adiponectin, IL-8 and MCP-1 (mean +/- SD of 3 donors, 4 cartilage/synovium explants per condition; * $p = 0.05$; no *post hoc* correction for multiple testing).

COX-2 independent production of inflammatory mediators during tissue regeneration culture by isolated chondrocytes

We then used isolated healthy and osteoarthritic chondrocytes in a cartilage regeneration model to investigate the effect of celecoxib on cartilage regeneration. Safranin-O staining showed the formation of neo-cartilage by both chondrocyte sources after 28 days of culture (Figs. 4A and 5A). Expression of the COX-2 enzyme in healthy and osteoarthritic chondrocytes after culturing of 28 days was confirmed (Figs. 4B and 5A). PGE_2 was released by osteoarthritic chondrocytes, however, no release was measured by healthy chondrocytes (50 pg/ml vs 0 pg/ml), indicating increased inflammatory reactions in OA (Fig. 5B). PGE_2 production by osteoarthritic chondrocytes could be inhibited by celecoxib, albeit not completely (Fig. 5B; $p = 0.03$). Celecoxib was unable to decrease the production of IL-6, IL-8, IL-10, Leptin, MCP-1 and NGF at day 7 of high-density culture with OA chondrocytes (Fig. 6). There was no production of IL-1α, IL-1β, IL-4, IL-13, OSM and adiponectin in the high-density culture (not shown). Again, healthy chondrocytes produced IL-6, IL-8, IL-10, MCP-1, NGF and leptin at day 7 of regeneration culture and celecoxib had no effect on the production of the produced mediators (data not shown). At day 28 the same mediators were produced, albeit lower than at day 7, with a similar lack of effect of celecoxib (data not shown). Addition of celecoxib failed to affect GAG content (Figs. 4C and 5C), GAG

release (Figs. 4D and 5D) or DNA content (data not shown) in the high-density regeneration culture.

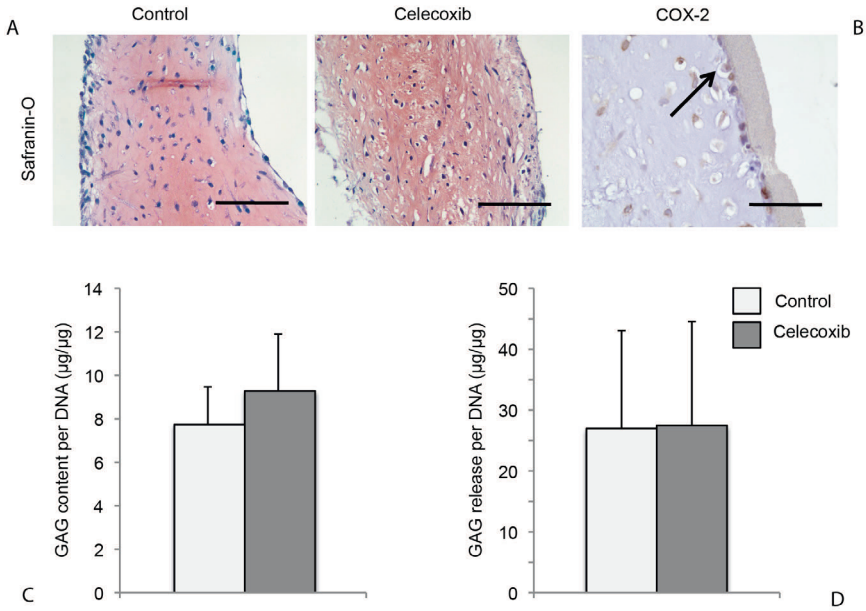

Figure 4. Effect of celecoxib on healthy chondrocytes.
Cartilage formation after 28 days of culture is visible as safranin-O positive staining for both cultures with and without celecoxib (A). The presence of COX-2 in healthy chondrocytes is demonstrated with immunohistochemistry (B) (day = 28, brown staining; indicated by the arrow, bar represents 100 µm). Celecoxib had no effect on total GAG content at day 28 (C) or release during the 28 days of culture (D; mean +/- SD of 6 donors, 5 constructs per condition).

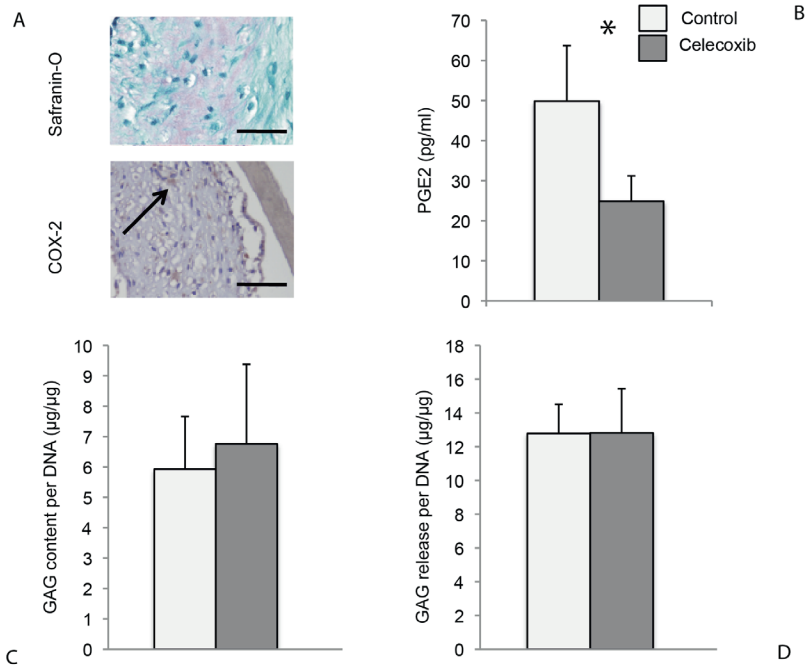

Figure 5. Effect of celecoxib on OA chondrocytes.
Neo-cartilage formation after 28 days of culture is visible as safranin-O positive staining (A). The presence of COX-2 in OA chondrocytes is demonstrated with immunohistochemistry for COX-2 (B; brown staining; indicated by the arrow, scalebar represents 100 µm). Celecoxib decreased PGE_2 production by chondrocytes (day =10), however, still some PGE_2 production was present (* $p = 0.03$; mean +/- SD, average of 3 donors 4 constructs per condition). Celecoxib had no effect on total GAG content at day 28 (C) or release during the 28 days of culture (D; mean +/- SD of 6 donors, 5 constructs per condition).

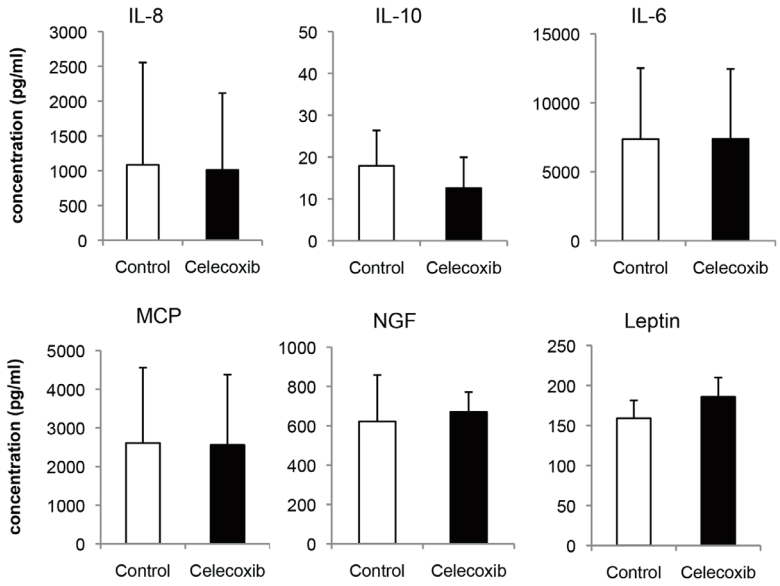

Figure 6. Production of mediators by OA chondrocytes.
OA chondrocytes produced IL-6, IL-8, IL-10, MCP-1, NGF and leptin at day 7 of regeneration culture. There was no production of IL-1α, IL-1β, IL-4, IL-13, OSM and adiponectin by the OA chondrocytes (not shown). Celecoxib had no effect on the production of the produced mediators (mean +/- SD of 3 donors, 4 constructs per condition; no *post hoc* correction for multiple testing).

Discussion

In the current study, inhibition of inflammation by celecoxib was found not to affect cartilage meatbolism in osteoarthritic explants or a co-culture of osteoarthritic synovial tissue with osteoarthritic cartilage, despite the clearly decreased secretion of many inflammatory mediators including PGE_2. Furthermore, activation of inflammatory pathways during tissue regeneration by isolated chondrocytes seemed to be largely COX-2 independent nor did celecoxib affect cartilage regeneration otherwise.

In previous studies where cartilage explants were cultured in the presence of, a generally high concentration of a pro-inflammatory stimulus, e.g. IL-1 or tumor necrosis factor (TNF), celecoxib did inhibit the induced degeneration[131]. The lack of effect on cartilage degradation in the current study could be related to the type of OA tissues studied. In this study, cartilage and synovial tissue were obtained from patients with end-stage OA undergoing total knee replacement. This 'late' OA tissue may have a less 'inflammatory' character than early OA tissue[13, 132]. COX-2 expression was found to be significantly lower in synovial tissue obtained from joints with greater OA severity than with lower OA severity[133]. Furthermore, it was very recently shown that levels

of inflammatory mediators secreted by the synovium in end-stage osteoarthritis were not different to those from healthy synovium, indicating that in end-stage OA inflammation may play a lesser role[134]. However, recently we showed that factors produced by OA synovial tissue did decrease matrix content of cocultured cartilage. This effect consisted of a decrease in matrix production rather than an increase of degradation [57]. This may indicate that at the relatively low levels found in OA, pro-inflammatory factors may still be capable of inhibiting regeneration without induction of degradation. However, inhibition of the inflammatory response by celecoxib did not affect production of inflammatory mediators in the current in vitro models either. Possibly the lack of effect of COX-2 inhibition on cartilage repair can be explained by the concomitant decrease in anti-inflammatory factors, such as IL-10, which may have counteracted the effects of inhibiting inflammatory mediator production. Moreover, inhibition of the COX-2 enzyme will not only inhibit the production of PGE_2, but also of other prostaglandin mediators, e.g. $PGF_{2\alpha}$, PGD_2 and thromboxane. Where PGE_2 has been associated with cartilage destruction in a cartilage explant model [135], other prostaglandins have been associated with enhanced cartilage production. In particular PGD_2 and $PGF_{2\alpha}$ have been shown to increase collagen type II and aggrecan synthesis [136, 137].

Alternatively, the effects of inflammatory mediators on cartilage matrix production may not always be repressive. We recently showed that IL-6, a classic inflammatory cytokine, in fact modestly stimulated cartilage matrix production during tissue regeneration culture and its inhibition in OA synovial fluid cocultured with OA explants decreased GAG content[138]. In addition, despite the increased production of PGE_2 and other inflammatory mediators, GAG production by osteoarthritic chondrocytes is not lower than that of healthy chondrocytes[139]. Recent evidence even suggests that inflammation may, in addition to causing degeneration, also have a positive role in regeneration[140]. Transient activation of NF-κB was shown to facilitate chondrogenesis through the induction of SOX9 [141-143]. Our data also support a role of inflammation in regeneration, as high concentrations of inflammatory mediators were found to be released during tissue regeneration culture by isolated chondrocytes. However, contrary to the production of inflammatory mediators by osteoarthritic cartilage explants, production of these factors by osteoarthritic chondrocytes during regeneration culture could not be inhibited by celecoxib indicating that the production of inflammatory cytokines was not COX-2 dependent. Surprisingly, healthy cells did not produce any PGE_2 at all, whereas pro-inflammatory cytokine production was not different from osteoarthritic cells. Various other mechanisms may have been involved in the production of inflammatory cytokines, including NFκB activation and STATs pathway, which were not studied here[144].

PGE_2 production in regeneration culture by osteoarthritic chondrocytes was only partially decreased by COX-2 inhibition, possible indicating involvement of COX-1. This may implicate that strategies to improve cartilage regeneration by cell-based procedures such as autologous chondrocyte implantation, may require a very different approach than strategies to develop disease-modifying treatments for OA.
Celecoxib has been suggested to have both COX-dependent and independent potential as a disease-modifying drug[122, 145], but we found no decrease in inflammatory mediators and no effect on matrix production in regeneration cultures.

Our study is the first to evaluate the effect of celecoxib on inflammation measuring a broad panel of cytokines, chemokines and growth factors produced by osteoarthritic cartilage and synovial tissue. In previous studies only IL-6 and IL-8 production were evaluated [126]. Although addition of celecoxib resulted in a clear reduction of inflammatory mediators, such as PGE_2, IL-6, NGF and IL-1 in the co-culture model, it did not alter GAG production or GAG release. Synovial inflammation and the produced inflammatory mediators are associated with pain sensation in OA [146, 147] and this could explain the pain reduction achieved by treatment with celecoxib [148]. GAG metabolism showed clear differences in the response to celecoxib between individual donors (not shown). Although the number of donors used for the current study was too low to draw any statistical conclusions, we cannot exclude the possibility that patients may be divided in responder and non-responder categories. This could also explain why in multiple studies using OA cartilage and chondrocytes in *in vitro* models, differential effects on cartilage metabolism were seen.

Conclusion

To study the effect of celecoxib on cartilage we have used a variety of different long-term culture models with human tissues and cells. So far no studies were based on the comparison of multiple culture models focussing on cartilage repair in cartilage explants and regeneration in chondrocytes. We found indications that inflammation is regulated differently in osteoarthritic tissues and cells. Cartilage repair, however, was never affected by celecoxib. A true potential of celecoxib as a disease modifying osteoarthritic drug did not emerge from this study, although of course its application as pain treatment is still relevant

Chapter 7

Interleukin 6 is elevated in synovial fluid of patients with focal cartilage defects and stimulates cartilage matrix production during regeneration

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Abstract

Aim

This study aimed to determine whether, like in osteoarthritis, increased levels of IL-6 are present in the synovial fluid of patients with symptomatic cartilage defects and whether this IL-6 affects cartilage regeneration as well as the resident cartilage in the osteoarthritic knee.

Materials & Methods

IL-6 concentrations were determined by ELISA in synovial fluid and conditioned media of regenerating chondrocytes of symptomatic cartilage defect, healthy and osteoarthritic donors. The effect of IL-6 on cartilage regeneration and on metabolism of the resident cartilage in the knee was studied by both inhibition of endogenous IL-6 and addition of IL-6, in a regeneration model and in OA explants in the presence of synovial fluid respectively. Readout parameters were DNA and glycosaminoglycan (GAG) content and release.

Results

Synovial fluid of patients with symptomatic cartilage defects contained more IL-6 than of healthy donors ($p=001$) and did not differ from osteoarthritic donors. IL-6 production of osteoarthritic chondrocytes was higher than of healthy and defect chondrocytes ($p<0.001$). Adding IL-6 increased GAG production in healthy chondrocytes and decreased GAG release in osteoarthritic chondrocytes ($p<0.05$). Inhibition of IL-6 present in osteoarthritic synovial fluid showed a trend towards decreased GAG content of the explants ($p=0.06$).

Conclusion

Our results support a modest anabolic role for IL-6 in cartilage matrix production. Targeting multiple cytokines including IL-6 may be effective in improving cartilage repair in symptomatic cartilage defects and osteoarthritis.

Introduction

Cytokines are thought to play an important role in articular cartilage degeneration[65]. In rheumatoid arthritis (RA), the pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) are known to have pivotal roles in its pathofysiology[149]. In addition to IL-1 and TNF-α, also interleukin-6 (IL-6) has been demonstrated to play a role in cartilage degeneration in RA. In mice models of RA, cartilage destruction was shown to be dependent on IL-6[150, 151]. Furthermore, tocilizumab, a humanized monoclonal antibody against the IL-6 receptor, now has an established role in the treatment of RA[152]. Besides efficacy in the amelioration of clinical signs and symptoms, tocilizumab has also demonstrated to reduce joint space narrowing and levels of cartilage degradation biomarkers [153-155].

Although not as outspoken as in RA, mild and intermittent inflammation is frequently observed in symptomatic focal cartilage lesions, a condition thought to predispose to the development of osteoarthritis (OA), and in OA. Elevated concentrations of inflammatory mediators, including IL-6, have been found in the serum and synovial fluid of OA patients[12, 156-162] and correlated to radiographic knee OA[163, 164]. However, the presence of IL-6 in joints with symptomatic cartilage defects has not been evaluated until now. In other joint injuries known to predispose to OA, such as anterior cruciate ligament (ACL) injuries[165-167] and meniscal tears[158, 168], increased levels of IL-6 have been detected in the synovial fluid. High levels of intra-articular inflammatory cytokines may, additionally to causing degeneration, also hamper tissue regeneration as cartilage repair was previously shown to be affected by the composition of the intra-articular synovial fluid[128, 169, 170].

In OA most of the IL-6 present in the knee originates from the synovium[57]. However, chondrocytes in culture are capable of producing IL-6, albeit under most conditions at low amounts[171-173]. Various stimuli, such as inflammatory molecules[174, 175] and binding of (fragmented) matrix components through binding of discoidin domain receptor 2 (DDR2)[176, 177] have been reported to induce IL-6, all mechanisms proposed to play a role in OA. Chondrocytes can be stimulated by IL-6 either by binding directly to the gp80 receptor, but mostly through trans-signaling, in which IL-6 binds first to the soluble IL-6Rα present in the synovial fluid, and then forms a heterodimeric association with the membrane-bound gp130 receptor[178].

Despite its possible role in OA, studies investigating the role of IL-6 in OA models have provided inconsistent results. *In vitro* stimulation of chondrocytes with IL-6 has revealed anabolic effects, such as up-regulation of tissue inhibitor of metalloproteinases (TIMP)-1[179] and type II collagen[180], as well as catabolic effects, such as down-regulation of cartilage matrix genes[181, 182], inhibition of proteoglycan synthesis[183] and stimulation of aggrecanase production[184,

185]. *In vivo* models have also revealed both chondroprotective and chondrodegenerative properties of IL-6. A protective role of IL-6 in a spontaneous OA model was reported in ageing male mice[186], but both mechanically induced OA and OA induced by hypoxia-inducible factor (HIF)-2α, identified IL-6 as the mediator of cartilage destruction[187]. However, many studies have failed to demonstrate a direct effect of IL-6 on cartilage matrix metabolism[188-190]. One of the explanations for this lack of effect may lie in the simplified set up of many studies in which IL-6 has been added, whereas the action of IL-6 may depend on other factors in the joint, in particular in the synovial fluid.

This study evaluated the presence of IL-6 in the synovial fluid of patients with symptomatic cartilage lesions and patients with late stage OA, its production by chondrocytes isolated from these patients, and its role in cartilage regeneration. In addition, to evaluate possible effects of high levels of IL-6 in the synovial fluid on resident cartilage in the knee, we cultured OA cartilage explants in the presence of OA synovial fluid in which IL-6 was selectively inhibited.

Materials and Methods

Synovial fluid and cartilage sample collection and cell isolation
The use of material from healthy donors was approved by the institutional ethical committee. The anonymous use of redundant material for research purposes from OA donors and donors with symptomatic focal cartilage defects undergoing surgery is part of the standard treatment agreement with patients in the University Medical Center Utrecht.

Macroscopically healthy articular cartilage (n=6) and synovial fluid (n=20, age 25 to 47, average 40 years) was obtained from donors within 24 hours postmortem. Defect cartilage (n=3) and synovial fluid (n=22, age 20 to 48, average 33 years) was obtained from donors undergoing either microfracture or autologous chondrocyte implantation (ACI) for focal grade III and IV cartilage defects. During those procedures, the cartilage defect is debrided to remove all cartilage remnants down to the subchondral bone and create a stable cartilage rim. The debrided cartilage was used for chondrocyte isolation. Chondrocytes from this location were recently shown to have good regenerative capacities compared to cells harvested from non-weight bearing cartilage normally used for ACI. Of the 22 patients with symptomatic cartilage defects, one had associated ACL injury and a history of partial menisectomy, another three had received previous partial menisectomies and one an ACL reconstruction. OA cartilage (n=12) and synovial fluid (n=27, age 53 to 81, average 70 years) was obtained from donors undergoing total knee arthroplasty. Synovial fluid was spun down at 300g to remove debris, and stored at -80 until use or analysis.

Cartilage samples were rinsed in phosphate buffered saline (PBS), cut into small pieces and enzymatically digested overnight at 37 °C in a 0.15% collagenase type II (Worthington, Lakewood, NJ, USA) in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Bleiswijk, The Netherlands) with penicillin/streptomycin (100 U/ml/100 µg/ml; Invitrogen, Life Technologies). After digestion, the cell suspension was filtered through a 70 µm cell strainer (BD Biosciences, San Diego CA, USA), and the chondrocytes were spun down by 10 minutes centrifugation at 300g.

Measurement of IL-6 levels

To determine the IL-6 levels in the synovial fluids of healthy, defect and OA donors and in the conditioned media of healthy, defect and OA regenerating chondrocytes, a multiplex ELISA was performed as previously described[32, 53]. Briefly, specific antibodies were coupled to carboxylated beads (Luminex Corporation, Austin TX, USA). Recombinant IL-6 was used to make a standard curve. Synovial fluid samples were first treated with hyaluronidase for 30 min at 37 °C. Medium samples were directly incubated with the coupled beads. After incubation with the appropriate biotinylated antibodies, samples were thoroughly washed and incubated with streptavidin–phycoerythrin (BD Biosciences) for 10 minutes. After washing, the samples were measured and analyzed using the Bio-Plex suspension system (Bio-Rad Laboratories, Hercules CA, USA) with Bio-Plex Manager software, version 3.0. The concentration of IL-6 in the media and synovial fluid was expressed as pg/ml using the standard curves. Results of specific ELISAs for determination of IL-6 levels have previously been shown to be comparable to multiplex ELISA.[57]

Regeneration culture

Isolated chondrocytes from healthy, defect and OA cartilage were expanded in monolayer at 37 °C and 5% CO_2 at a seeding density of 5000 cells per cm^2 in expansion medium consisting of DMEM (Invitrogen), 10% fetal bovine serum (Hyclone, Thermo Scientific, Etten-Leur, the Netherlands), penicillin/streptomycin (100 U/100 µg/ml; Invitrogen) and 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, MN, USA). After two passages, the chondrocytes were seeded on collagen type II-coated (Chicken sternal cartilage; Sigma-Aldrich, Zwijndrecht, The Netherlands) Millicell filters (Millipore Co., Bedford MA, USA), at 1.6x10^6 cells per cm^2 and redifferentiated during 28 days in redifferentiation medium consisting of DMEM (Invitrogen), 0.2 mM l-ascorbic acid-2-phosphate (AsAp; Sigma-Aldrich), 2% human serum albumin (Sanquin, the Netherlands), penicillin/streptomycin (100 U/100 µg/ml; Invitrogen), 2% ITS-X (Invitrogen) and 5 ng/ml TGF-β_2 (R&D systems). Since fibrillar type II collagen was previously shown to induce IL-6 release from chondrocytes[176, 177], we measured the release of IL-6 from P2 chondrocytes (n=3 healthy donors) cultured on filters coated with type I collagen (rat tail; BD Biosciences), and denatured type I and II collagen. Collagen was denatured by heating for 45 minutes at 70°C. Levels of IL-6 in the supernatant were determined by specific ELISA for IL-6 (Cytoset; Invitrogen) according to the manufacturer's instructions.

Endogenous IL-6 production of defect chondrocytes proved to be not significantly different from healthy chondrocytes, but much lower than of OA chondrocytes and therefore the role of IL-6 endogenously produced by defect chondrocytes (n=3) and OA chondrocytes (n=3) was studied through blockage of IL-6 with an activity-inhibiting antibody. To this end, the medium was supplemented with either 1 or 4 ug/ml anti-hIL-6 (purified mouse monoclonal IgG_1; R&D systems) or IgG_1 isotype control (R&D systems). Furthermore, IL-6 activity was blocked with 100 ng/ml tocilizumab (RoActemra®, Roche, Woerden, the Netherlands), a humanized monoclonal antibody directed against the IL-6 receptor. The dose of anti-hIL-6 was chosen based on an IL-6-dependent murine plasmacytoma proliferation assay, as described earlier[191]. The concentration of tocilizumab was chosen based on previously observed average concentrations of IL-6 receptor in the synovial fluid of patients with OA of approximately 10-40 ng/ml[161, 162]. Since the effects of IL-6 inhibition were limited in expanded cells, we also verified the effects of Il-6 inhibition in freshly isolated OA cells (P0; n=3).

Since endogenous IL-6 production was relatively low in healthy chondrocytes, possible effects of high concentrations of IL-6 were further investigated by the addition of 10 ng/ml rhIL-6 with 25 ng/ml rhIL-6Rα (R&D Systems) to both healthy (n=3) and OA (n=3) chondrocytes. Medium was changed three times a week and supernatant was collected and stored at -80° C until later analysis. Per condition, six filters were seeded, five for biochemical analysis and one for histological evaluation

Osteoarthritic cartilage explant culture

OA cartilage from three donors was cut into explants of ~1 mm by 1 mm with mean SD wet weight of 7.8 ± 2.8 mg per cartilage explant. Cartilage explants were cultured for 14 days in explant medium consisting of DMEM (Invitrogen), penicillin/streptomycin (100 U/100 µg/ml; Invitrogen), 1% ITS-X (Invitrogen), 0.1 mM AsAp (Sigma-Aldrich) and 0.2% proline (Sigma-Aldrich), which was either or not supplemented with 25% pooled OA synovial fluid from eight donors. Six explants per condition were used, five for biochemical analysis and one for histological evaluation. To study the role of IL-6 present in the synovial fluid, 4 ug/ml anti-hIL-6 or IgG_1 isotype control and/or 100 ng/ml tocilizumab, were added to the medium. Medium was changed three times per week and collected and stored at -20°C until later analysis.

The role of IL-6 was also studied by the addition of IL-6. Explants from 8 OA donors (11.3 ± 3.8 mg, minimum of 3 explants per condition) were pre-cultured for 24 hours in culture medium after which either or not rhIL-6 (50 ng/ml) with IL-6 receptor (rhIL-6Rα; 200 ng/ml)[192] was added. Explants were cultured for an additional 10 days with medium renewal every other day and the supernatant was collected and stored at -20°C until later analysis.

Glycosaminoglycan and DNA analysis

After culture, the explants and the regenerated tissue were digested overnight in a papain buffer (250 µg/ml papain (Sigma-Aldrich) in 50 mM EDTA and 5 mM L-cysteine) at 56ºC, followed by determination of the glycosaminoglycan (GAG) content using the dimethylmethylene blue (DMMB) assay[193]. The ratio of absorption at 540 nm to 595 nm was used to calculate the GAG content, using chondroitin sulphate (shark; Sigma-Aldrich) as a standard. Supernatant was also collected and analysed for GAGs released into the medium.

The DNA content was determined from the papain digest using a Picogreen DNA assay (Invitrogen) in accordance with the manufacturer's instructions.

Histological evaluation

Both regenerated tissue and explants were fixed in 10% buffered formalin, dehydrated in alcohol, rinsed in xylene and infiltrated and embedded with paraffin. For histology, 5 µm sections were stained with safranin-O (Merck, Darmstadt, Germany) for GAG and counterstained with Weigert's haematoxylin (Klinipath, Duiven, the Netherlands) and 0.4% fast green (Merck) for nuclei and cytoplasm, respectively.

Statistical analysis

All statistical analyses were performed using SPSS 18.0. Results are displayed as mean ± SD. Differences between controls and IL-6 blocked samples were determined by univariate analysis of variance using a randomized block design and posthoc LSD-test when four or more conditions were compared to each other. Differences in Il-6 concentration were determined by the Kruskal-Wallis test, using posthoc Mann-Whitney U test and Bonferroni correction for synovial fluids, and using nested ANOVA with posthoc t-test and Bonferroni correction for conditioned media. Differences between the various collagen coatings were determined by univariate analysis of variance with posthoc t-test with Bonferroni correction.

Results

IL-6 in synovial fluid

Synovial fluid of donors with symptomatic cartilage lesions contained significantly more IL-6 than that of healthy donors (261±385 pg/ml versus 64±120 pg/ml, p=0.001), and was slightly lower but not significantly different from OA patients (396±508 pg/ml, Figure 1). IL-6 levels in the five patients with symptomatic focal cartilage defects and associated or previous ACL injury and/or partial menisectomy were not significantly different from the group as a whole (154±70 pg/ml, p=0.6).

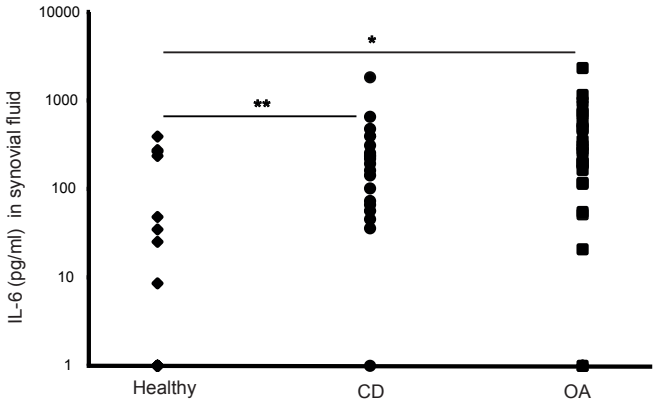

Figure 1. Increased concentration of IL-6 in the synovial fluid of patients with cartilage damage.
Concentration of IL-6 in the synovial fluid of healthy (H), symptomatic cartilage defect (CD) and osteoarthritic (OA) donors. Note the logarithmic scale of the Y-axis; *p<0.001, **p=0.001.

IL6 production in cell culture

Regenerating chondrocytes of the various origins produced IL-6 reaching concentrations that were at least tenfold higher than the concentrations present in the synovial fluid of the corresponding donor category (Figure 2A). OA chondrocytes (9368±3284 pg/ml) produced significantly more IL-6 than both healthy (2814±995 pg/ml) and defect chondrocytes (3246±2089 pg/ml, p<0.001). There was no significant difference in IL-6 production between healthy and defect chondrocytes.

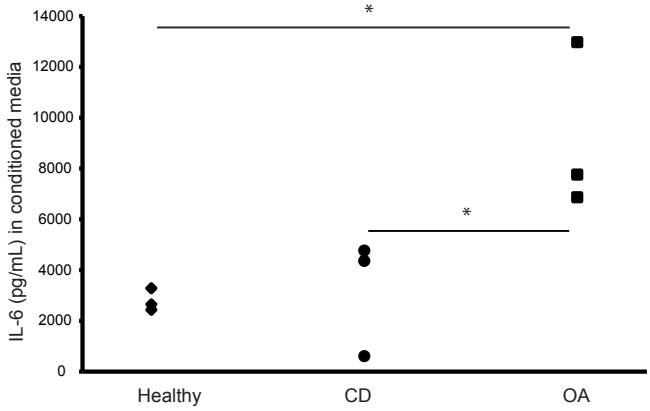

Figure 2. Cartilage regeneration culture: IL-6 production and histology.
Passage 2 expanded chondrocytes from healthy (H), defect (CD) and osteoarthritic (OA) cartilage were seeded in high density on type II collagen-coated filters and cultured during 28 days. Concentration of IL-6 in conditioned media of regenerating chondrocytes on day 7; *p<0.001.

To verify whether IL-6 production during regeneration was induced by the fibrillar type II collagen used for filter coating in this model, we measured IL-6 production of regenerating chondrocytes on filters coated with various collagens. There was no difference in IL-6 production between type I and II collagen-coated filters and also not between native or denatured collagen-coated filters (Table 1). Also GAG and DNA content was not different between the various coatings.

Table 1. Effect of collagen coating on IL-6 and cartilage matrix production

	Col I	Col II	Col ID	Col IID	p-value
IL-6 (pg/ml)	4294±2152	4604±1661	4268±1446	5463±1991	0.543
GAG (µg)	80±24	85±30	77±25	85±34	0.361
GAG release (µg/ml)	408±29	410±35	400±38	498±157	0.412
DNA (µg)	10±1	9±1	10±2	10±1	0.818

No significant differences were observed between type I collagen (Col I), type II collagen (Col II), denatured type I collagen (Col ID) and denatured type II collagen (Col IID) coating on IL-6 production at day 7, GAG content, GAG release and DNA content after 28 days of culture.

Regeneration culture

To evaluate whether the high levels of IL-6 produced by the chondrocytes during regeneration play a direct role in cartilage regeneration, IL-6 was inhibited using an activity-inhibiting antibody during regeneration of P2-expanded defect- and OA chondrocytes. As no difference was found in IL-6 production between healthy and defect chondrocytes, only defect and osteoarthritic chondrocytes were studied. No effects were found on cartilage matrix production, although an increase in DNA content was found exclusively in OA chondrocytes ($p=0.009$, Figure 3). Verification of these results in non-expanded osteoarthritic chondrocytes showed similar lack of effect on cartilage matrix production and also the effect on DNA was lost (data not shown). Antagonism of the IL-6 receptor with tocilizumab in osteoarthritic chondrocytes failed to influence GAG and DNA content (data not shown).

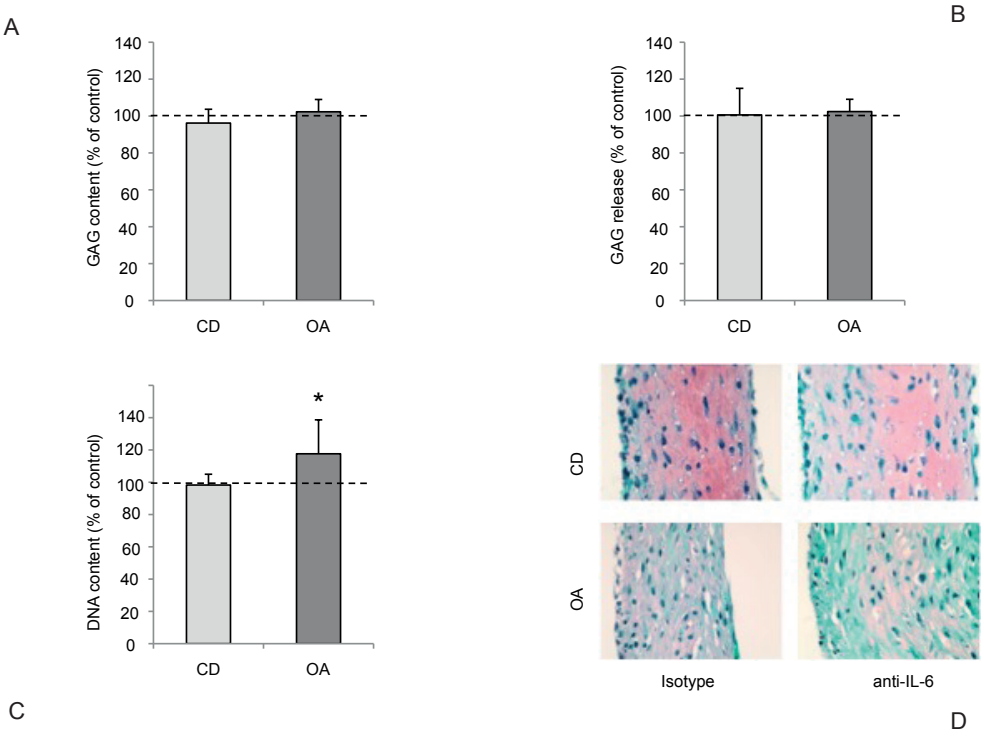

Figure 3. Cartilage regeneration after inhibition of endogenously produced IL-6.
Cartilage regeneration cultures of three defect (CD) and three osteoarthritic (OA) donors with blockage of endogenous IL-6 with an activity-inhibiting antibody. (**A-C**) GAG content, GAG release and DNA content after 28 days of culture in IL-6 blocked samples depicted as % of control samples (mean ± SD); *p=0.009. (**D**) Safranin O staining of representative CD and OA chondrocyte donors regenerating either without (Control, IgG isotype added) or with inhibition (anti-IL-6) of endogenous IL-6.

In healthy and defect chondrocytes endogenous IL-6 production was much lower than in OA chondrocytes. We therefore hypothesized that these cells could be more responsive to stimulation with exogenous IL-6 than OA chondrocytes. To examine whether exogenously added IL-6 could affect regeneration, 10 ng/ml rhIL-6 with 25 ng/ml rhIL-6Rα was added during regeneration culture of healthy and OA chondrocytes. In healthy chondrocytes, exogenous rhIL-6 increased GAG production in the neocartilage and a higher GAG/DNA ratio was found (p=0.002, Figure 4A). In OA chondrocytes, IL-6 decreased GAG release (p<0.001, Figure 4B) without affecting final GAG content in the neocartilage.

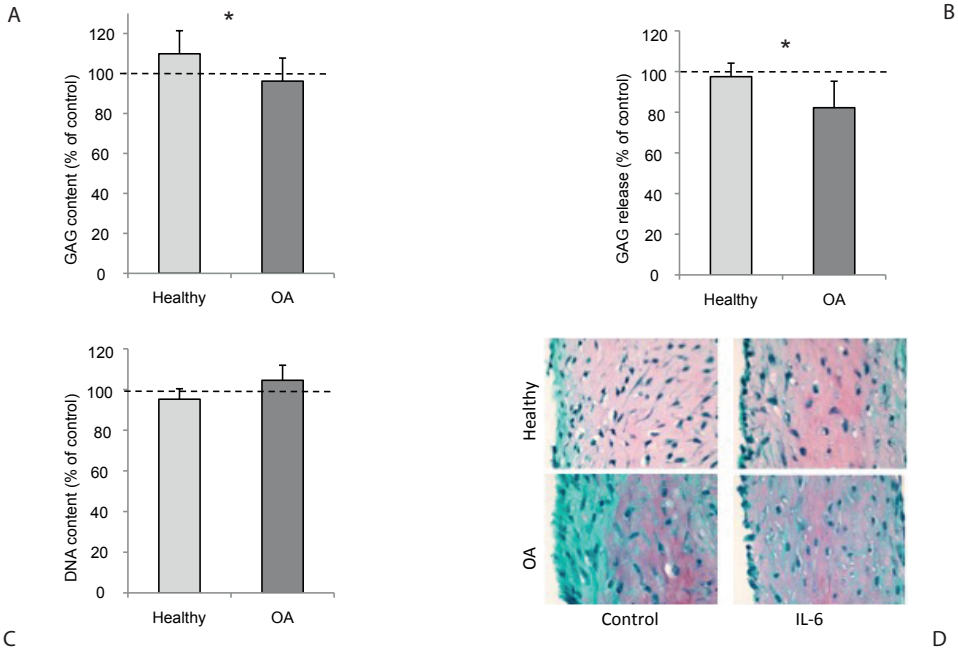

Figure 4. Cartilage regeneration in the presence of IL-6.
Cartilage regeneration cultures of three healthy (H) and three osteoarthritic (OA) donors with addition of rhIL-6 (10 ng/ml) and rhIL-6Rα (25 ng/ml). (**A-C**) GAG content, GAG release and DNA content of IL-6 supplemented samples depicted as % of control samples (mean ± SD); *p<0.01. (**D**) Safranin O staining of neocartilage generated by chondrocytes from healthy and osteoarthritic donors without or with addition of rhIL-6.

Osteoarthritic explant culture

To study the effect of high levels of IL-6 present in the synovial fluid on resident cartilage in the knee, we performed OA cartilage explant studies in the presence of OA synovial fluid in which IL-6 was inhibited. Ideally we would have also liked to perform these experiments with synovial fluid from defect donors and explants from defect cartilage, but due to the very limited amount of material that can be obtained from these patients this was not feasible. In the absence of synovial fluid, inhibition of IL-6, by antibodies inhibiting cytokine or receptor activity, did not alter GAG and DNA content of the cartilage explants, nor was GAG release affected (data not shown). However, when L-6 was inhibited in the presence of synovial fluid a trend towards a decrease in GAG content of the explants was observed (p=0.06, Figure 5A). Addition of synovial fluid increased the DNA content of explants (54.0±28.0 μg/gr cartilage versus 33.6±20.9 μg/gr cartilage, p=0.002), which was abolished by blocking IL-6 (42±20 versus 54±28 μg/gr cartilage). Exogenous IL-6 in combination with soluble IL-6 receptor in the absence of synovial fluid did not alter the GAG (Figure 5B) or DNA content of the explants and also did not modulate GAG release (Figure 5D), indicating that IL-6 in the synovial fluid exerts its effects with help of or by interaction with other factors.

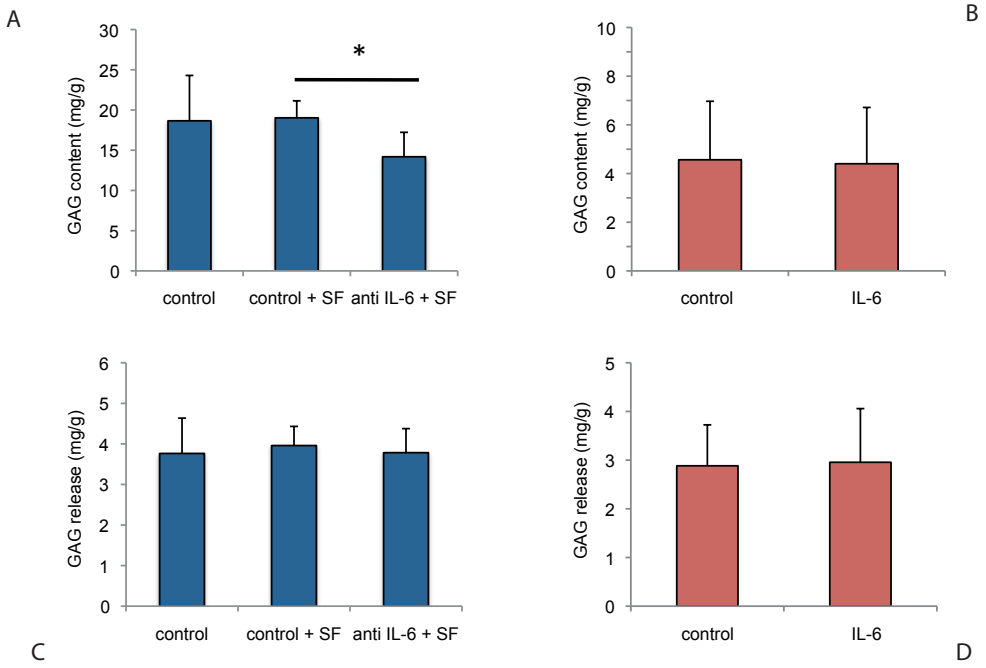

Figure 5. OA cartilage explant culture in the presence synovial fluid with or without blocking of IL-6 or with or without exogenous IL-6.
(**A, C**) GAG content and GAG release in OA cartilage explants from three donors cultured either in medium only, medium supplemented with 25% OA synovial fluid or medium with 25% OA synovial fluid in which IL-6 is blocked with an activity-inhibiting antibody (mean ± SD in mg/ gr); *p=0.06. (**B,D**) GAG content and GAG release in OA cartilage explants from eight donors cultured either or not with the addition of rhIL-6 (50 ng/ml) with rhIL-6Rα (200 ng/ml).

Discussion

In this study we show increased IL-6 levels in the synovial fluid of patients with symptomatic cartilage defects compared to normal subjects. The levels in patients with symptomatic cartilage defects were comparable to levels of IL-6 in patients with OA. Furthermore, we demonstrated for the first time that chondrocytes, especially OA chondrocytes, produce high concentrations of IL-6 during regeneration. Inhibition of this endogenously produced IL-6 did not affect cartilage matrix turnover, but addition of extra IL-6 increased GAG content of neocartilage formed by healthy chondrocytes and decreased GAG release by osteoarthritic chondrocytes. Furthermore, inhibition of IL-6 present in the synovial fluid showed a trend towards decreased matrix production in OA explants. All of these results point towards an anabolic role of IL-6 in cartilage repair, albeit with limited effects.

Levels of IL-6 similar to those reported here were previously shown in healthy[157] and OA[157, 158, 194] synovial fluid, however, only limited data were available on IL-6 levels in joints with symptomatic focal cartilage defects. These are typically the joints that will be treated to stimulate regeneration of cartilage with techniques such as ACI. Only one study has reported on the levels of IL-6 in cartilage lesions of variable depth, but did not specify whether the damage was focal or whether more generalized OA-like cartilage degeneration was present in the knee[194], which is an important parameter for the indication of ACI. IL-6 levels seemed to coincide with the grade of cartilage damage. In our study, only symptomatic focal grade III and IV cartilage lesions in otherwise healthy knees were included, which is more clinically relevant for cartilage regeneration, although no distinction was made between grade III and grade IV defects.

Most likely, IL-6 present in the synovial fluid originates from cells in the synovial membrane[161]. In addition, adipose tissue, including that of the fat pad in the knee, is an important source of inflammatory mediators, including IL-6[195, 196], at least partly explaining the association of OA with obesity. Although we did not collect information regarding the BMI of the donors, it is likely that the OA donors were more obese, at least partly explaining the higher levels of IL-6 found here. Furthermore cartilage can produce IL-6, although chondrocytes embedded in their original matrix produce very little IL-6[57]. However, during regeneration, chondrocytes produced high levels of IL-6, which when implanted intra-articularly, in theory, could cause high local concentrations. The production of IL-6 appeared to be characteristic of regeneration and not of the type II collagen used in this cartilage regeneration model, as filter coating with type I collagen, which is not capable of inducing IL-6[176], resulted in the same IL-6 production.

Little is known on the role of IL-6 during regeneration, but inhibition of IL-6 did not influence GAG or DNA content in the newly formed cartilage. However, addition of IL-6 decreased GAG release by OA chondrocytes and in healthy chondrocytes, which produce much less IL-6, addition of IL-6 resulted in an increase of GAGs deposited into the newly formed matrix. Furthermore, in osteoarthritic explant culture, a trend towards a decrease of matrix production upon inhibition of IL-6 was observed. The limited availability of synovial fluid restricted its presence in culture to 25% and therefore higher percentages may have yielded more outspoken effects. The limited effect of IL-6 inhibition on cartilage matrix turnover is unlikely to be due to a lack of inhibitory activity of the antibody, as this and other similar antibodies have demonstrated effectiveness in inhibiting IL-6 bioactivity in other models, including models with chondrocytes as target cells[183, 197]. However, in the cartilage explants, penetration of the inhibiting antibodies into the cartilage matrix may have been limited, still enabling paracrine signaling, although this is probably limited considering the fact that chondrocytes in their native extracellular matrix hardly produce IL-6.

The role of IL-6 in cartilage metabolism has been the subject of much debate. IL-6 is often described as a modulatory factor, because it can induce both anabolic and catabolic mechanisms. Recently, IL-6 injection into the joint of mice was described to cause cartilage destruction, but in that study, like in many others, supra physiological concentrations of IL-6 were used[187]. Concentrations of IL-6 similar to those found in the synovial fluid usually do not have effects on the expression of cartilage matrix proteins in cartilage[188-190]. To our knowledge, this is the first study to demonstrate effect of physiological concentrations of IL-6 on cartilage matrix production during regeneration, albeit modestly. IL-6 is known to be induced by various catabolic stimuli present in OA such as interleukin 1β (IL-1β)[174], prostaglandin E_2 (PGE_2)[175], increased shear stress[198] and extracellular matrix components such as hyaluronan fragments[199] and matrillin-3[176]. IL-6 in turn, is capable of inducing factors such as metalloproteinases (MMPs) [200], transforming growth factor β (TGFβ)[201], vascular growth factor (VEGF)[202], and many others which are important for tissue remodeling. In bone IL-6 also induces remodeling through increased osteoclastogenesis[203], which is thought to be important in the observed inhibition of radiographic disease progression in RA patients treated with tocilizumab[153, 204]. IL-6 has also been shown to have anabolic effects on cartilage, both indirectly through the up-regulation of factors such as tissue inhibitor of metalloproteases-1 (TIMP-1)[205], bone morphogenetic protein-7 (BMP-7)[180] and TGFβ[201], as well as directly through the up-regulation of cartilage matrix proteins[180]. Injection of IL6 into the joint cavity of mice stimulated PG synthesis in cartilage[206] and IL-6 knockout mice showed more extensive naturally occurring cartilage loss[186] and reduced PG synthesis[186, 207]. The current study indicates that IL-6 has a mainly anabolic role in cartilage regeneration, although the effects are not strong, with increased GAG production in healthy chondrocytes and decreased GAG release in OA chondrocytes. Possibly the IL-6 in the synovial fluid of patients with symptomatic cartilage defects is induced in the course of regeneration and plays a role in tissue regeneration after cartilage damage.

Inflammatory mediators secreted by synovium and present in the synovial fluid have been demonstrated to affect cartilage repair in vitro[57, 128, 169, 170, 208]. Therefore, it is essential to characterize the mediators present in synovial fluid of symptomatic cartilage defects and osteoarthritis and determine their role in cartilage metabolism to verify whether modulation of the intra-articular environment, in particular immediately prior to, or during cartilage repair procedures such as ACI, may hold promise for the improvement of their outcome. The increased levels of IL-6 in joints with symptomatic cartilage defects or OA may positively affect matrix metabolism of the resident cartilage, and seems beneficial for generation of neocartilage as occurs during ACI or microfracturing. Targeting multiple cytokines or pathways, including IL-6, may be effective in improving cartilage matrix production in symptomatic cartilage defects and OA.

Chapter 8

Inhibition of oncostatin M in osteoarthritic synovial fluid enhances GAG production in osteoarthritic cartilage repair

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Abstract

Introduction

Mediators in the synovial fluid are thought to play a major role in osteoarthritic cartilage turnover. The purpose of the current study was to investigate the role of oncostatin M (OSM) in osteoarthritis (OA) by evaluating the presence of the cytokine and its receptors in the OA joint and interfering with its activity in synovial fluid cocultured with cartilage explants.

Results

OSM levels were increased in the synovial fluid of osteoarthritic patients compared to healthy donors. Immunohistochemistry confirmed the presence of both the LIF and OSM receptors for OSM throughout the whole depth of osteoarthritic cartilage and synovial tissue, whereas in healthy cartilage their presence seemed more restricted to the superficial zone. Blocking OSM activity, using an activity inhibiting antibody, in 25% osteoarthritic synovial fluid added to OA cartilage explants culture increased glycosaminoglycan content from 18.6 mg/g to 24.3 mg/g ($P < 0.03$) and total production from 24.3 mg/g to 30.9 mg/g ($P < 0.003$). However, OSM exogenously added to cartilage explant cultures reflecting low and high concentrations in the synovial fluid (5 and 50 pg/ml) did not affect cartilage matrix turnover, suggesting that factors present in the synovial fluid act in concert with OSM to inhibit GAG production.

Conclusion

The current study indicates the potential to enhance cartilage repair in osteoarthritis by modulating the joint environment, by interfering with OSM activity.

Introduction

One of the key processes in osteoarthritis (OA) is loss of the articular cartilage extracellular matrix due to a shift of the balance between production and degradation. Increased synthetic activity is evident by clonal expansion of resident chondrocytes and increased intensity of the territorial matrix surrounding them. However, this cannot compensate for the generalised loss of proteoglycans in the extraterritorial matrix, leading to a net loss of cartilage matrix [4]. Soluble factors secreted from cartilage, synovial tissue and bone are known to play a role in the turnover of cartilage matrix and many have been shown to both increase cartilage degeneration and inhibit the production of the extracellular matrix, which has been documented most extensively for Interleukin-1 (IL-1) and tumor necrosis factor-α (TNFα) [10, 19, 20, 85]. Another soluble factor that may be important in affecting cartilage matrix integrity in OA is the pro-inflammatory cytokine oncostatin M (OSM). OSM, a cytokine from the interleukin-6 (IL-6) family, is secreted by osteoarthritic synovial tissue [57] and is found in rheumatoid synovial fluid [209]. OSM is associated with multiple processes in the body, e.g. haematopoiesis, neural and liver development and cell proliferation [210-212] and in rheumatoid arthritis (RA), OSM is associated with bone erosion, synovial inflammation and fibrosis and cartilage degeneration [178, 213]. The potential of OSM to degenerate cartilage and induce catabolic processes in chondrocytes was shown in different studies [36, 214], although nothing is known about its effect on cartilage synthesis. In addition, OSM is known to synergise with catabolic cytokines such as IL-1 and TNFα in the induction of cartilage degeneration in vitro [214-218]. However, most pro-inflammatory cytokines, including OSM, have been demonstrated only at low levels in osteoarthritic synovial fluid [48, 209, 219, 220], and it is not clear whether such interactions are relevant in OA-associated cartilage degeneration in vivo. Moreover, therapies targeting IL-1 and TNFα were shown to be ineffective in inhibiting osteoarthritic cartilage loss, in contrast to their application in rheumatoid arthritis.

OSM signals through two different receptor complexes, the GP130/LIFRβ (LIFR) or the GP130/OSMRβ (OSMR) complex [28, 221, 222]. The OSMR was demonstrated in a chondrocyte cell line and in synovial fibroblasts. Also OSM signalling is mediated through the proteinase kinase R signalling pathway in bovine chondrocytes [218]. Although both receptors for OSM have been shown in multiple tissues, e.g. liver and prostate with different expression in healthy and pathologic conditions [223, 224], the presence of the different receptors for OSM has not been studied in healthy or in osteoarthritic cartilage tissue *in vivo* [192].

The purpose of this study was to elucidate the role of OSM in OA and more specifically the effect of inhibiting OSM on cartilage integrity in OA. To this end, the levels of OSM in the synovial fluid of osteoarthritic patients were compared to healthy subjects, and the presence of receptors for OSM was evaluated in healthy and osteoarthritic joint tissue. Furthermore, the role of OSM in cartilage repair was studied by culture of OA cartilage explants with OA synovial fluid in the presence or absence of a blocking antibody against OSM. Finally, to determine whether OSM

could directly affect cartilage repair, the effect of recombinant OSM on cartilage explants at concentrations found in OA synovial fluid was studied.

Materials & Methods

Harvesting of cartilage, synovial tissue and synovial fluid

Osteoarthritic cartilage was collected under sterile conditions from patients undergoing total knee arthroplasty for OA. Cartilage explants were harvested from the femoral condyles, cut into square pieces of approximately 1x1 mm and the wet weight was determined so GAG release, content and production could be corrected for cartilage weight [29]. No difference was made between damaged or more intact cartilage, for histology, tissue analysis nor explant cultures. Only the almost completely denuded bone areas were not used. From each donor, 6 cartilage explants were fixed immediately in 4% formaldehyde for histological evaluation and 6 were wet weighted and stored at -20 °C for biochemical analysis at t=0. The rest of the explants were used for culturing or cell isolation.

Osteoarthritic synovial fluid and synovial tissue was obtained during total knee arthroplasty, healthy synovial fluid and tissue were collected from *post mortem* donors within 24 hours after death, only if the knee cartilage was macroscopically healthy. The synovial tissue was immediately fixed in 4% formaldehyde for immunohistochemical analysis. Synovial fluid was stored at -80 °C. To obtain OA chondrocytes articular cartilage was cut and digested in collagenase 0.15 % (Worthington, Lakewood, USA) overnight at 37 °C. Subsequently, the chondrocytes were seeded in a monolayer at a cell density of 5000 cells/cm^2 and cultured in expansion medium containing Dulbecco's Modified Eagle Medium (DMEM), 10 % Fetal Bovine Serum (FBS), 1 % penicillin/streptomycin (pen/strep) and 10 ng/ml basic fibroblast growth factor (bFGF; R&D Systems, Minneapolis, USA). Chondrocytes were cultured until passage 2. Anonymous use of redundant tissue for research purposes is part of the standard treatment agreement with patients in our university hospital and according to the national guidelines 'code of conduct for the proper secondary use of human tissue [96].

Multiplex ELISA

The presence of OSM in synovial fluid was determined using multiplex ELISA, as part of a multiplex ELISA for several cytokines, as previous described [57]. Validation of the Multiplex ELISA was previously done by de Jager *et al*., showing excellent sensitivity and specificity [32, 53]. Synovial fluid was pre-treated with hyaluronidase for 15 min at 37 °C. Samples were spun down in a X-column (Corning, Costar 8169, NY, USA) and dissolved in 0.1375% HPE-Tween-20. The pre-treated synovial fluid was incubated with the coupled beads. After incubation with the biotinylated antibodies, samples were incubated with streptavidin-PE. The samples were

measured and analysed using the Bio-Plex suspension system (Bio-Rad laboratories, Hercules CA, USA). The concentration of OSM in the synovial fluid was calculated using the standard curve and is expressed as pg/ml.

(Immuno)histochemistry

For (immuno)histochemistry, the cartilage and synovial tissue explants were fixed overnight in 4% formaldehyde, embedded in paraffin and cut into 5 µm sections. To evaluate the cartilage degeneration grade [97], a safranin-O and fast-green staining was used [100]. For LIFR staining (sc-659, C-19; Santa Cruz Biotechnologies, CA, USA), sections were incubated with polyclonal α-rabbit LIFRβ (both cartilage and synovial tissue; concentration 0.67 µg/ml) for 1 hour at room temperature [223, 224]. The sections were incubated with α-rabbit – HRP (1.25 µg/ml; Dako, Denmark). Sections were developed by incubations with 3,3'di-amino-benzidine (DAB) for 5 minutes. For OSMR staining, sections were incubated with polyclonal α-goat OSMRβ (sc-8496, C-20; SantaCruz; for cartilage sections at 2.0 µg/ml and synovial tissue at 4.0 µg/ml) overnight. Sections were incubated with α-goat – HRP (2.5 µg/ml, Dako) for 1 hour. Finally, sections were developed by incubations with DAB. For the negative controls, adjacent sections of both healthy and osteoarthritic cartilage and synovial tissue were incubated with an isotype α-rabbit IgG (Dako) for the LIFR or isotype α-goat IgG (Santa Cruz Biotechnologies) for OSMR. As a positive control human thymus sections were used.

To quantify the LIFR and OSMR staining, the percentage of positive cells in both cartilage and synovial tissue sections was determined using semi-automatic software (Leica Microsystems, Rijswijk, The Netherlands). The cells were counted with a grid distance of 17.6 µm. All sections were blinded and at least 100 cells were counted per slide. In total at least 5 healthy and 5 OA donors were used for both cartilage and synovial tissue. For the cartilage explants only the positive cells in the middle and deep layer were counted, due to the loss of the superficial layer in OA cartilage explants. For synovial tissue all cells throughout the entire tissue were counted.

Culture of cartilage explants in OA synovial fluid

To evaluate the effect of OSM present in synovial fluid on cartilage metabolism, the activity of OSM in OA synovial fluid was blocked with an activity-inhibiting antibody. For these experiments the synovial fluid from multiple (+/- 8) OA donors were pooled to obtain sufficient amounts of synovial fluid for a total culture period of 14 days. In total 3 different pooled synovial fluids were used, at OSM concentrations between 25 and 40 pg/ml. The pooled synovial fluid was aliquoted and stored at -80 °C until use. Cartilage explants from 5 different donors (n=6 per donor/per condition) were cultured in the presence or absence of 25% pooled OA synovial fluid, at a final OSM concentration of 6.25 or 11.25 pg/ml (25 % OA synovial fluid dissolved in medium), in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL Life Technologies, Germany) containing penicillin and streptomycin (Gibco), ascorbate-2-phosphate (Sigma-Aldrich, St Louis MO, USA),

Insulin Transferrin Selenium-X (ITS-X) (Gibco) and 50 μg/ml L-proline (Sigma) for 14 days. To block the effect of OSM, an activity-inhibiting antibody against OSM was used at a concentration of 10 μg/ml (R&D; Minneapolis, USA, MAB295). Isotype IgG antibody (R&D; MAB002) and cultures with anti-OSM antibody only served as controls. The culture medium was renewed 3 times a week. For each medium change, fresh synovial fluid (25%) and anti-OSM or isotype antibody was added to the culture medium. The supernatant was stored at -80 °C for further analysis. After 14 days of culture, one explant was fixed for histochemical analysis. The rest of the explants stored at -20 °C for biochemical analysis.

Effect of OSM inhibition on PGE2 production by OSM stimulated OA chondrocytes
To assess whether the anti-OSM antibody was effectively blocking OSM activity, OA chondrocytes were cultured in the presence or absence of 2 ng/ml of recombinant OSM, or 10 μg/ml OSM activity inhibiting antibody (anti-OSM). As a control unstimulated cells and an isotype IgG was used. The OA chondrocytes were seeded in a density of 42.000 cells per well for a total cultured period of 3 days. After 3 days the conditioned medium was stored. The inhibiting effect of anti-OSM was determined using a prostaglandin E2 (PGE2) ELISA. In total 1 OA donor was used with n=4 per condition.

PGE2 analysis
PGE2 concentrations in the conditioned medium of OSM stimulated OA chondrocytes was determined using a competitive PGE2 ELISA (ENZO life sciences, Antwerpen, Belgium), according to the manufacturer's protocol.

Culture of cartilage explants in the presence of recombinant OSM
OA cartilage explants from 4 different donors (n=6 per donor/per condition) were cultured for 14 days in the presence or absence of recombinant OSM (R&D, 295-OM) at 5 and 50 pg/ml, representing the lowest and the average concentration of OSM found in OA synovial fluid. As a positive control a concentration of 10 ng/ml, previously shown to have an effect on cartilage metabolism [109, 225], was also included. Culture medium was renewed 3 times a week and stored at -80 °C for further analysis. One explant was fixed for further histochemical analysis. The remaining explants (n=5) were stored at -20 °C for biochemical analysis.

Biochemistry
GAG release and total GAG content was quantified using the di-methylmethylene-blue (DMMB) spectrophotometric analysis described by Farndale *et al [101]*. Cartilage explants were digested in 400 μl 2% papain (Sigma) in 50 mM phosphate buffer, 2 mM N-acetylcysteine, and 2 mM Na_2-EDTA (pH 6.5) at 60 °C overnight. Two hundred μl of DMMB solution and 100 μl of medium sample or papain digest were mixed and absorbance read at 540 nm and 595 nm using the

spectophotometer (Bio-Rad laboratories). As reference, chondroitin sulfate C (Sigma) was used. Calculation of GAG production was done with the following formula: $GAG_{production} = (GAG_{content\ day\ 21} - GAG_{content\ day\ zero}) + GAG_{cumulative\ release}$ given in mg GAG/g cartilage.

Statistical analysis

Data are expressed as mean ± SD. SPSS 16.0 software (SPSS Inc. Chicago, Illinois, USA) was used for the statistical analysis. The data was tested for normality and Gaussian distribution. A Student's t-test was used to assess difference between the healthy and osteoarthritic synovial fluid samples. Univariate analysis of variance (ANOVA), with a randomized block design and a post-hoc Bonferroni was used to assess differences in the glycosaminoglycan parameters. Differences with a *P*-value less than 0.05 were considered statistically significant. Differences in the percentage of receptor-positive cells in cartilage and synovial tissue scored after immunohistochemistry were evaluated by a Mann-Whitney U test.

Results

Presence of OSM in OA synovial fluid

In the OA synovial fluid samples OSM was detected in 10 of the 32 samples (range 0-570 pg/ml; mean 39 pg/ml), whereas in the healthy samples only one of the 23 samples contained OSM (range 0-25 pg/ml; mean 1 pg/ml; Fig. 1; $P < 0.001$).

Presence of receptors for OSM in joint tissue

The LIFR was present throughout the entire cartilage dept in both osteoarthritic and healthy cartilage (Fig. 2). In healthy cartilage the OSMR was mainly expressed in chondrocytes in the superficial layer and some chondrocytes in the middle and deep layer. However, in osteoarthritic cartilage, positive chondrocytes were found throughout the entire cartilage (Fig. 3). As the superficial layer in OA cartilage was lost, only the middle and deep zones of healthy and OA tissues were quantified for the presence of LIF and OSM receptor-positive cells. No statistically significant difference between healthy and OA cartilage for LIFR nor OSMR staining was found (table 1).

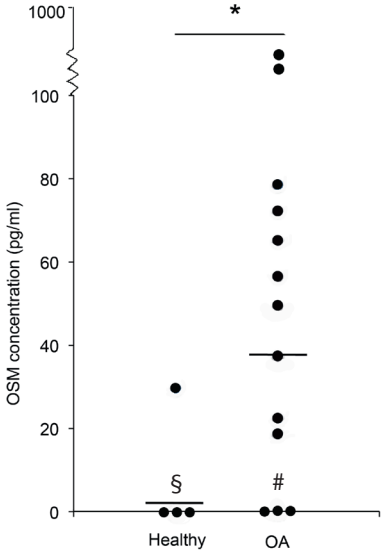

Figure 1. OSM concentration in healthy (n=24) and osteoarthritic (n=32) synovial fluid samples.
In healthy synovial fluid OSM was detected in only one sample (1.5 ± 6.8 pg/ml). In osteoarthritic synovial fluids OSM was detected in 10 out of 32 samples (39.7 ± 108.0 pg/ml; $*P < 0.001$). § = 24 samples, # = 22 samples.

Table 1. Results of LIFR and OSMR staining in osteoarthritic and healthy cartilage and synovial tissue

	Healthy (% ± SD)	Osteoarthritic (% ± SD)	P value
Cartilage			
LIFR	73.0 ± 10.4	72.3 ± 21.9	1.00
OSMR	19.4 ± 16.5	24.4 ± 16.8	0.73
Synovial tissue			
LIFR	71.2 ± 4.3	86.3 ± 9.0	* 0.04
OSMR	0 ± 0	10.3 ± 12.9	* 0.04

Per condition 5 samples were scored using semi-automatic software. At least 100 cells were counted per section. Data is given in % ± SD of positive cells.

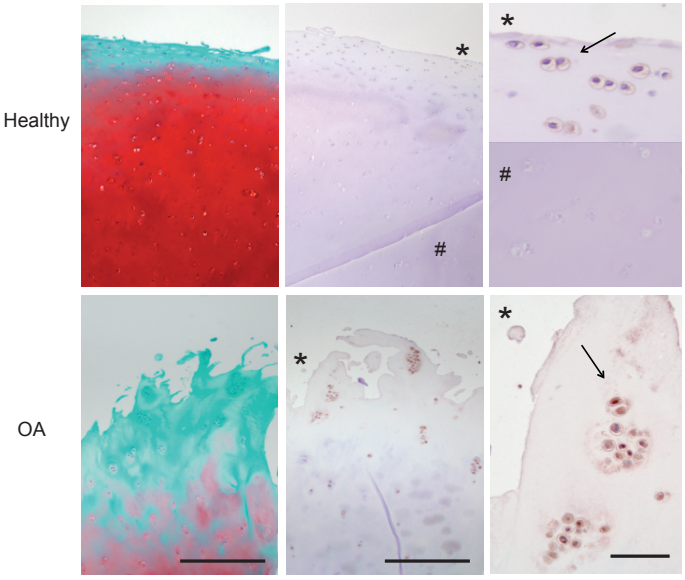

Figure 2. Immunohistochemistry for LIFR in healthy and osteoarthritic cartilage.
LIFR is present in osteoarthritic cartilage throughout the entire cartilage (below). In the healthy cartilage only some cells in the superficial layer showed positive staining (positive cells stained brown by DAB and indicated by the arrow; scale bar represents 500 μm, magnification 40 μm).

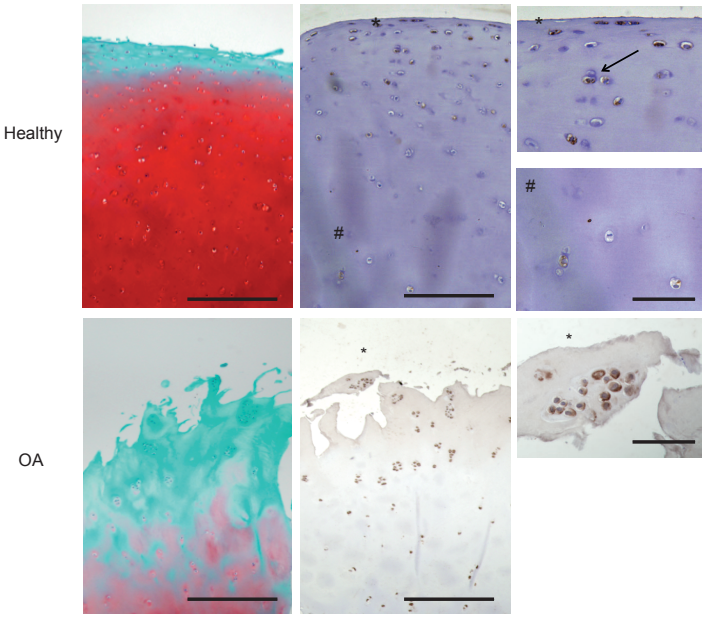

Figure 3. Immunohistochemistry for OSMR in healthy and osteoarthritic cartilage.
OSMR is present in osteoarthritic cartilage throughout the entire cartilage (below). In the healthy cartilage cells in the superficial layer showed positive staining and some cells in the middle/deep layer (positive cells stained brown by DAB and indicated by the arrow; scale bar represents 500 μm, magnification 40 μm).

Synovial tissue of both healthy and osteoarthritic joints expressed the LIFR (Fig. 4), with positive cells mainly expressed in the synovial tissue lining. The expression of LIFR in OA synovial tissue was slightly higher compared to healthy synovial tissue (table 1; $P < 0.04$). Furthermore, the OSMR was not expressed in any of the healthy synovial tissue donors (Fig. 4 and table 1), whereas in the osteoarthritic synovial tissue multiple cells in the synovial lining expressed the OSMR ($P < 0.04$; Fig. 4 and table 1).

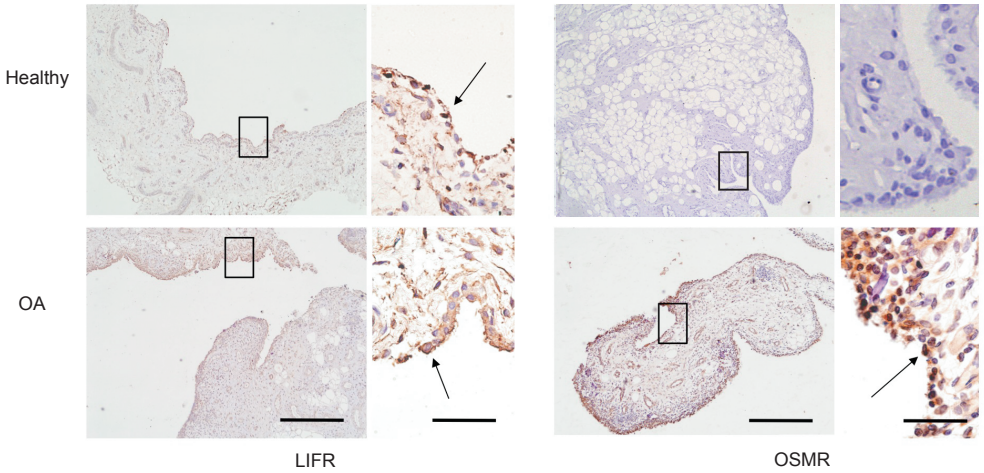

Figure 4. Immunohistochemistry for LIFR and OSMR in healthy and osteoarthritic synovial tissue. LIFR is present in both healthy and osteoarthric synovial tissue (left pictures). OSMR was abundantly expressed in osteoarthritic synovial tissue, however not in healthy synovial tissue (right pictures; positive cells stained brown by DAB and indicated by the arrow; scale bar represents 500 μm; scale bar in magnification represents 20 μm).

Effect of blocking OSM in synovial fluid on cartilage

To study the role of OSM in osteoarthritic cartilage degeneration, OA cartilage explants were cultured in the presence of OA synovial fluid (the OSM concentration in the pooled synovial fluids ranged between 30 and 40 pg/ml; data not shown), in the presence or absence of an activity-inhibiting antibody against OSM (anti-OSM). Although addition of OA synovial fluid to cartilage explants for 14 days did not affect GAG content, release or production in cartilage explants (Fig. 5a/b), blocking OSM in OA synovial fluid increased GAG content from 18.6 mg/g to 24.3 mg/g ($P < 0.03$) and production from 24.3 mg/g to 30.9 mg/g ($P < 0.003$) in cartilage explants (Fig. 5). Blocking OSM in synovial fluid did not have an effect on GAG release (Fig. 5c). Anti-OSM did not affect any of the turnover parameters in cartilage explants in the absence of synovial fluid. The effectiveness of the antibody was shown in OSM-stimulated OA chondrocytes, where the effect of recombinant OSM on PGE2 production was completely abrogated by the anti-OSM antibody but not by the isotype control (Fig. 5d; $P < 0.001$). There was a basal production of PGE2 by the OA chondrocytes as shown by the unstimulated control.

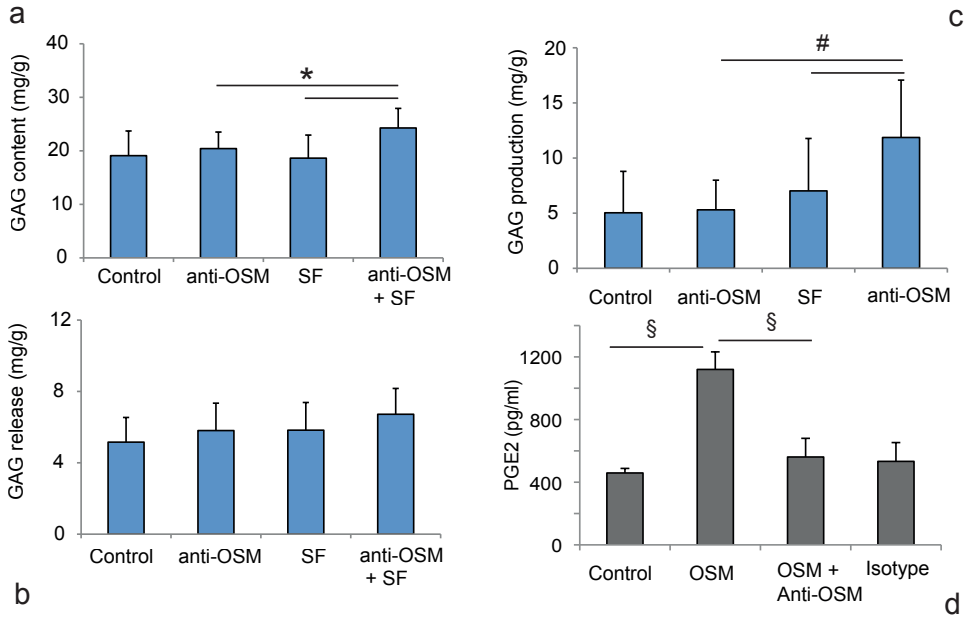

Figure 5. Effect of blocking OSM in synovial fluid on osteoarthritic cartilage explants cultured for 14 days. Blocking OSM in osteoarthritic synovial fluid increased GAG content in cartilage (Fig. 5a; * $P < 0.03$) and increased GAG production (Fig. 5b; # $P < 0.003$), however, did not have an effect on GAG release (Fig. 5c). Only addition of synovial fluid did not have an effect on GAG content, release or production in cartilage osteoarthritic cartilage (mean +/- SD of 5 different OA donors; 5 explants per donor/condition in mg GAG/g cartilage). Anti-OSM, at a concentration of 10 μg/ml, completely decreased the production of PGE2 induced by 2 ng/ml of recombinant OSM by OA chondrocytes (§ $P < 0.001$; Fig. 5d; mean +/- SD of 1 donor; n = 4 per condition).

Effect of recombinant OSM on OA cartilage

The effect of OSM alone on OA cartilage explants was studied by addition of recombinant OSM to OA cartilage explants. Only at a supraphysiological concentration of 10 ng/ml, but not at 5 or 50 pg/ml, GAG content decreased and GAG release increased (Fig. 6a/c; $P < 0.05$ and $P < 0.001$). No effect on total GAG production was noted irrespective of the concentration used (Fig. 6b).

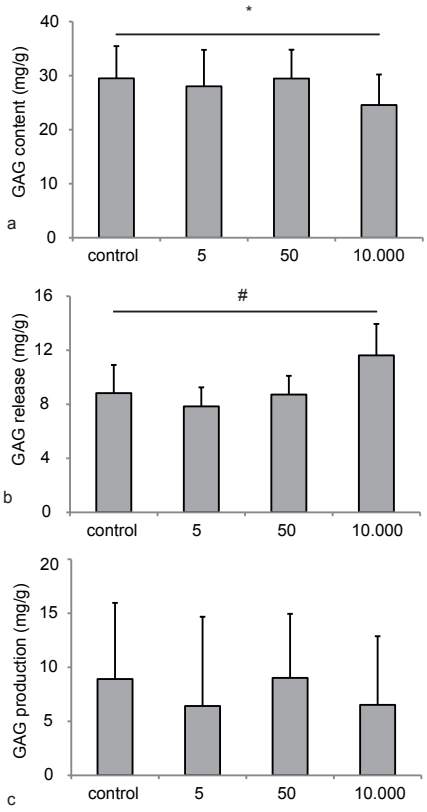

Figure 6. Effect of recombinant OSM on osteoarthritic cartilage explants cultured for 14 days.
At 10 ng/ml, recombinant OSM increased GAG release and decreased GAG content (Fig. 6a/c; * $P < 0.05$, # $P < 0.001$). However, no effect was seen at concentrations in the range of those found in OA synovial fluid (5 and 50 pg/ml). None of the concentrations of OSM had an effect on GAG production (Fig. 6b; mean +/- SD of 4 different OA donors; 5 explants/donor per condition in mg GAG/g cartilage).

Discussion

The current study showed increased levels of OSM in osteoarthritic synovial fluid and expression of both receptors for OSM in cartilage and synovial tissue. Although the receptors were found both in healthy and OA joint tissue, their expression seemed to be more widespread in OA tissue. Addition of OA synovial fluid to cartilage explant cultures did not affect matrix turnover. However, when OSM was blocked in OA synovial fluid, cartilage repair was clearly enhanced. In contrast, recombinant OSM added to cartilage explants at the concentrations found in OA synovial fluid did not affect chondrocyte metabolism.

The role of OSM in rheumatoid arthritis and joint tissues other than cartilage has been extensively studied [226, 227] [213, 228]. OSM in combination with IL-1 or TNFα induced bone destruction via the RANK/RANKL pathway [227]. Moreover, OSM triggered angiogenesis and cell migration in synovial tissue [108]. These changes are also known to occur in OA in synovial tissue [1]. In RA,

however, the levels of the pro-inflammatory cytokines IL-1, TNFα and OSM are high, in contrast to OA where almost no IL-1 or TNFα is present. Possibly this might cause different effects of OSM in RA compared to OA.

Receptors for OSM are found in multiple cell types in the joint, such as osteoblasts, synovial fibroblasts and chondrocytes, indicating that OSM could exert an effect on all tissues in the joint. Rowan *et al.* showed the presence of GP130/OSMRß complex, but failed to detect the GP130/LIFRß complex, in an immortalized chondrocyte cell line [192]. Other studies showed the presence of the OSMR and LIFR in both osteoblasts [213, 229] and synovial fibroblasts [228]. However, OSM receptor expression in healthy and diseased joint tissue has never been reported before, let alone the existence of differences in expression between healthy and diseased tissue. Although OSM receptor expression was not evidently different in cartilage tissue, in synovial tissue the expression of the receptors for OSM was up-regulated in osteoarthritic samples, in contrast to healthy tissue where almost no cells were positive. This indicates an increased expression of the OSMR in diseased joint tissue, which may further enhance the effects induced by OSM. Also the LIFR was upregulated in osteoarthritic synovial tissue.

Studies have showed that gene activation through the LIFR and the OSMR are different. Whereas OSM normally signals through the LIFR and activates JAK/STAT transcriptional activators, OSM can also act via OSMR. However, the OSMR can also activate specific pathways through activation of the MAPK signaling cascade [28, 230], suggesting that dependent on receptor expression, different responses, e.g. MMP activity and chondrocyte metabolism, could be generated in osteoarthritis. OSM is the only cytokine from the IL-6 family that can induce cartilage degeneration, albeit at non-physiologically high concentrations [215, 231]. This is most likely via the induction of matrix degrading proteases, e.g. MMPs and ADAMTS, which are known to be increased in OSM-stimulated cartilage explants or chondrocyte cultures [36, 109, 218, 225]. Although the proteases involved downstream of OSM signaling were not further identified in the current study, stimulation of GAG release by the addition of a high concentration of OSM is in line with previous data.

In RA, OSM levels are high and in concert with high concentrations of IL-1 and TNFα known to induce degeneration and GAG release [220]. The use of neutralizing OSM antibodies in two different models of murine RA was shown to partially prevent cartilage destruction [232]. In addition, blockers of several cytokines binding the GP130 family of receptors, such as IL-6, are currently used in the clinic [233, 234]. In particular, tocilizumab, a monoclonal antibody against the IL-6 receptor, was found to have a positive effect on disease symptoms and to decrease the levels of markers of bone and cartilage degeneration in patients with RA [154, 155].

Despite the clear degenerative effect of OSM at high levels, its mode of action in osteoarthritis seems to be somewhat different. Levels of OSM, but also of TNFα and IL-1 are low in OA synovial fluid [235]. Moreover, at the levels found in OA synovial fluid, OSM does not induce cartilage breakdown, which is in line with the observation that inhibition of the OSM present in OA synovial fluid does not inhibit GAG release. In contrast, the increased production and deposition of extracellular matrix found upon inhibition of OSM activity rather suggests an inhibitory effect of OSM on an anabolic factor(s) in the synovial fluid. In other studies the precise role and effect of OSM on extracellular matrix production should be further investigated.
As this is an artificial in vitro model with only cartilage explants we cannot exclude that the culture method (e.g. culture medium with high concentration insuline, no bone or synovial tissue present) may have partly masked the effect of inhibiting OSM. Future in vivo experiments will show if inhibiting OSM could stop OA degeneration in a more challenging and less controlled environment.

It should be borne in mind that in almost 70% of the OA synovial fluid samples OSM was undetectable, and hence its role may be limited to those patients in which OSM is produced. The presence of OSM in some patients may suggest different (stages of) pathology. We have recently shown that OSM clusters with several other cytokines such as IL-1β, IL-8 and IFNγ suggesting it is part of a common pathway[236], which should be further investigated. We observed however no association of OSM production with other aspects of pathology, such as synovial inflammation. However, we did not have access to the patient data and did not obtain synovial tissue of all OA patients, so we cannot state with certainty whether this association did not exist. Still, as inhibition of these low concentrations already resulted in such clear effects on cartilage repair, it cannot be excluded that very low, but undetectable OSM levels were present in all donors and would still have resulted in increased matrix production upon inhibition. Even if this would not hold true, a 30% reduction in OA disease morbidity would already have a huge impact on society in terms of disease burden. It is important to note that the synovial fluid could only be used at 25%, due to its limited availability, suggesting that the enhancement of cartilage matrix production may have been even more pronounced with higher concentrations of synovial fluid.
This study shows that OSM plays a role in cartilage matrix turnover during osteoarthritis. By blocking OSM in the synovial fluid of OA patients, cartilage repair is improved in osteoarthritic cartilage. Supported by the lack of effect found of exogenously added OSM, these data indicate that OSM indirectly inhibits repair mechanisms in osteoarthritis. Moreover, the presence of receptors for OSM in both cartilage and synovial tissue suggests that blocking OSM could have an effect on multiple joint tissues and not just cartilage. The current study is one of the first that show that cartilage repair can be improved by altering the joint environment in osteoarthritis.

Chapter 9

Summary and discussion

Summary

Osteoarthritis (OA) is a degenerative joint disorder involving cartilage destruction and joint inflammation. Despite extensive research, still much is unknown on the pathogenesis and aetiology of OA. Inflammation and inflammatory mediators (both local and systemic) are key in the pathogenesis of OA. For rheumatoid arthritis, multiple therapies are based on targeting the mediators that are associated with inflammation and joint destruction [237]. Motivated by these findings we set off to identify which inflammatory mediators play a role in OA and whether interfering with these inflammatory mediators could stop OA progression or aid in cartilage repair and regeneration.

We presented a comprehensive overview of 47 soluble mediators (cytokines, chemokines and growth factors) in synovial fluid from knees of healthy controls and OA patients (**chapter 2**). We showed an increase of multiple inflammatory mediators, such as interleukin (IL)-6, Monocyte Derived Chemokine (MDC) and 'regulated on activation, normal T cell expressed and secreted' (RANTES) compared to healthy controls, which is in line with the 'inflammatory process', as postulated over a decade ago [10, 19]. Principal component analysis (PCA) indeed showed clear pro- and anti-inflammatory clusters in synovial fluid of OA patients, associated with processes in the OA joint.
The knee is one of the most studied joints for OA, even though OA occurs in all joints. Since no studies had looked at the presence of soluble mediators in the wrist joint, we analysed soluble mediators in the wrist for three different subsets of patients (healthy, pre-OA and end-stage OA) using multiplex ELISA (**chapter 3**). The increased presence of IL-6, IL-10 and Interferon (IFN)-γ in the OA wrist patients compared to healthy and pre-OA indicates a catabolic and inflammatory environment in wrist OA as well. Also, we compared knee OA and wrist OA synovial fluid samples to study the differences between different joints (**chapter 4**). Nine of the measured mediators were higher in the wrist samples compared to the knee samples. The predominantly inflammatory environment in the post-traumatic wrist OA group may explain the faster and more destructive course in wrist OA [42].

Synovial tissue is crucial in maintaining joint homeostasis. However, the presence of synovitis in OA leads to the production of inflammatory mediators, which is thought to lead to cartilage degeneration. To study the effect of synovial tissue on cartilage metabolism we measured the production of inflammatory mediators by the different joint tissues and evaluated the effect of OA synovial tissue on cartilage metabolism in a co-culture model (**chapter 5**). The mediators produced by the co-culture model (synovial tissue and cartilage) showed the same profile as in the synovial fluid, indicating that the co-culture model used closely mimics the OA joint environment. Surprisingly, OA synovial tissue did not cause cartilage degeneration, however it inhibited cartilage

repair by the chondrocytes. This was visible as a decrease of glycosaminoglycan [5] production by OA chondrocytes and GAG content in the cultured cartilage explants. To prove the presence of synovitis in the OA synovial tissues used, the presence of lymphocytes and production of inflammatory mediators was demonstrated. Also, to investigate whether the produced mediators were responsible for the decreased cartilage repair, we blocked inflammatory cytokine production by a corticosteroid (triamcinolone). When the production of these soluble mediators was blocked by triamcinolone, both the production of the inflammatory mediators and the inhibiting effect of synovial tissue on cartilage repair were diminished.

As the corticosteroid triamcinolone used in chapter 5 could restore cartilage repair in the coculture model, we postulated that blocking the cyclooxygenase (COX)-2-mediated inflammatory pathway could also aid in cartilage repair. This had already been studied in different ex-vivo and in- vivo studies [29, 30], however, with ambiguous results. By using three different culture models (cartilage alone, the coculture model and chondrocyte regeneration model) the effect of COX-2 inhibition on the production of soluble mediators and cartilage metabolism was studied (**chapter 6**). Our focus on cartilage metabolism was both on cartilage regeneration (formation of new cartilage by chondrocytes) and cartilage repair (the potential of the chondrocytes to repair the damaged cartilage). In the co-culture of osteoarthritic cartilage and synovial tissue, soluble mediator production, such as prostaglandin-E2 (PGE2), IL-6 and oncostatin M [28], was decreased by celecoxib. However, no effect on cartilage repair was seen. Furthermore, we did not observe an effect of celecoxib on inflammatory mediator production or on cartilage regeneration in 3D chondrocyte culture. In our studies blocking COX-2 in 3 different models in-vitro does not indicate a clear effect on cartilage metabolism. As blocking complete inflammatory pathways (by triamcinolone and celecoxib) shows ambiguous results, and to further clarify the specific role of individual cytokines, studies were performed in which only one specific mediator was blocked, based on the profiling results of chapter 2.

Both IL-6 and OSM were elevated in OA synovial fluid (chapter 2) and produced by osteoarthritic joint tissue (chapter 5). The roles of these two specific mediators from the IL-6 family, which are involved in cartilage degeneration in rheumatoid arthritis [192], were further clarified (**chapter 7**). Because the production of IL-6 is increased in joint pathology (rheumatoid arthritis, OA, trauma) we postulated that decreasing IL-6 activity by an inhibiting antibody or blocking the IL-6 receptor would enhance cartilage regeneration and repair (chapter 7). Inhibition of IL-6 in osteoarthritic synovial fluid had no effect on cartilage repair or regeneration. However, to our surprise, when we added IL-6 an increased GAG production in healthy chondrocytes and decreased GAG release in osteoarthritic chondrocytes was observed. These data support an anabolic role for IL-6 in cartilage repair and regeneration, thus IL-6 may be effective in improving cartilage repair in osteoarthritis. Finally, the role of OSM, another cytokine from the IL-6 family, in osteoarthritis and cartilage

repair was studied (**chapter 8**). OSM was present in OA synovial fluid and tissue for multiple OA donors. Moreover, the synovial tissue of OA patients showed an increased presence of Leukemia Inhibitory Factor receptor (LIFR) and OSMR, both receptors binding OSM and associated with inflammation. *In vitro* blocking of OSM in OA synovial fluid clearly increased cartilage repair by the OA chondrocytes in cartilage explants, without affecting GAG release. Only when OSM was added to cartilage explants at high concentration, GAG release and cartilage degeneration were observed. We recommend that the potential role of OSM as a DMOAD should be further investigated *in vivo*.

In this thesis we identified multiple inflammatory mediators and clusters associated with joint inflammation. In a co-culture the mediators produced by OA synovial tissue and cartilage closely resemble the joint environment as measured in OA synovial fluid. Moreover, specific inflammatory mediators produced by the synovial tissue, such as OSM, are capable of slowing down cartilage repair. When the production of inflammatory mediators was decreased by anti-inflammatory drugs or by interference with specific mediators (e.g. OSM), chondrocyte metabolism was affected and cartilage repair improved. We showed that intervention with the production or activity of inflammatory mediators by synovial tissue plays an import role in cartilage metabolism and maybe could stop OA progression.

Discussion

Which soluble mediators characterize the OA joint environment?

Soluble inflammatory mediators such as cytokines, prostaglandins and matrix degrading enzymes have been described to play a role in the pathogenesis of OA [10, 19, 20]. As changes in cartilage and synovial tissue metabolism can be observed in the synovial fluid, and in addition the factors present are likely to directly affect cartilage metabolism, the synovial fluid is both indicative and instrumental in the processes occurring in the joint. Thus, soluble mediators present in the synovial fluid are a snapshot of the joint environment. Inflammatory mediators classically thought to have a causal role in cartilage degeneration are IL-1 and TNFα, levels of which are clearly increased in rheumatoid arthritis. Both IL1 and TNFα are potent catabolic mediators and capable of inducing cartilage degeneration [9]. Although they have also been postulated to be present in synovial fluid of OA patients, in our studies their synovial fluid levels were below the detection limit (chapter 2-5). Also the production of TNFα and Il-1 by OA synoviocytes has been taken as proof for their involvement, whereas most likely this is a general response of cells that are being removed from their natural environment. Healthy tissue commonly has been lacking as a control to prove that this production is really specific for OA cells [12, 238, 239]. However, the absence

of Il-1 and TNFα in OA synovial fluid does not exclude the role of other soluble mediators in cartilage destruction in OA. Principal component analysis (PCA) indeed showed clear pro- and anti-inflammatory clusters in synovial fluid of OA patients (chapter 2). Also in serum, cluster analysis has been shown to identify distinct clusters in serum mediators for bone metabolism, synovial tissue and inflammation [240]. Examples of pro-inflammatory mediators elevated in OA synovial fluid were IL-6, MDC and RANTES, indicating inflammation in the end-stage OA joint environment (chapter 2, 3).

The challenge in using synovial fluid as a marker for disease activity and cartilage metabolism is the large heterogeneity of the OA population. There is still much unknown concerning the different OA phenotypes and which factors (e.g. inflammatory mediators, genetic, biomechanical) are crucial in the development of OA (workshop OARSI 2014: "How to define OA; phenotyping OA from different perspective"). Differences in mediator profiles could be due to patient's (baseline) characteristics, e.g. BMI, age and sex, as they influence the joint environment and presence of inflammatory mediators (thesis L. Gierman) [221, 358]. Moreover, also the separate disease stages will each be characterised by their own synovial fluid mediator profiles. For example, in knee trauma patients, inflammatory cytokines such as IL-1β and IL-6 were highly elevated, but dropped to low values already after 1 week [241]. In the wrist, we could not discriminate between healthy and pre-OA (chapter 3). This can indicate the wrong mediator panel was chosen, or point to joint-specific characteristics, but also that trauma-induced upregulation of inflammation is only transient and that other factors play a role in the posttraumatic stage leading to early OA.
All in all, biomarker studies may help to identify different subgroups for OA, regardless of the joint affected or stage of the OA process. Patient selection, for example by the presence of specific inflammatory mediators in OA patients, may subsequently improve the success of treatment. Some patients showed high IL-6 and OSM production, whereas other synovial fluid donors were practically devoid of inflammatory markers (chapter 7 and 8). As wrist OA has a more inflammatory character than knee OA, indicated by the presence of IL-1α and high concentrations of IL-6 and other inflammatory mediators (chapter 4), one could speculate that this subgroup is more susceptible to anti-inflammatory therapy as it has a more pro-inflammatory character.
Also the potential of synovial fluid for biomarker analyses in the identification of early OA looks promising. The OARSI started the OA initiative to validate different biomarkers for the early detection of OA (www.OARSI.org). Today large cohort studies (e.g. CHECK, OA initiative) are identifying prognostic mediators which could predict early onset of OA and discriminate between early and end-stage stage OA (http://oai.epi-ucsf.org,/Wesseling et al. 2009) Finding a biomarker, or a set of biomarkers, which predict progression from early OA to end-stage OA, would be a big step forward in stopping OA development and progression.

Which inflammatory mediators are important in osteoarthritis and cartilage repair?

The name 'osteoarthritis' suggests that inflammation plays a crucial role in the pathogenesis of OA [242]. However, which role inflammatory mediators play in the pathogenesis is still a debate in the current OA research community (debate OARSI 2012). Is the production of inflammatory mediators in (early) OA the beginning of the OA process with a point of no return or is it a final attempt of the joint to stop the degenerative process? Moreover, OA is a heterogeneous disease and not all patients will have inflammation as the main feature for OA.
As already pointed out in the above, the postulated role of IL-1 and TNFα is highly unlikely, given their limited presence in synovial fluid and production by synovial tissue in culture. This is further supported by the complete lack of success of several clinical studies applying antibodies against TNFα or IL-1 receptor antagonist to stop cartilage degeneration in patients with osteoarthritis [24, 25, 27], in contrast to the breakthroughs in treatment of rheumatoid arthritis using these biologicals, with both clinical and radiological improvement [243], in parallel with the effects found of IL-6 inhibition [154]. However, in line with findings of increased inflammatory markers in acute knee trauma, promising results were obtained with the application of IL-1Ra in the treatment of pain [61].
Even the detection of increased levels of a particular cytokine does not necessarily imply its involvement in the degenerative process. Although IL-6 is involved in inflammation and joint pathology [110] and was increased in the synovial fluid of almost all OA patients (chapter 2-4), blocking IL-6 in osteoarthritic synovial fluid had no effect on cartilage repair or regeneration (chapter 7). In fact, when added to healthy chondrocytes during cartilage regeneration, IL-6 slightly increased GAG production and it decreased GAG release in osteoarthritic chondrocytes. As previously described, inflammation also plays an import role in joint development and regeneration [140], which may be one explanation why adding IL-6 is beneficial in cartilage regeneration.

One mediator did show to play a role in OA cartilage metabolism. OSM, upregulated in about 30% of the patients, was shown to inhibit cartilage repair in a culture model with OA synovial fluid and cartilage explants (chapter 8), although no effects on degradation were found. OSM, a pro-inflammatory mediator from the IL-6 family is a particularly potential catabolic mediator in combination with other inflammatory cytokines, in particular, IL-1 and TNFα [36, 244, 245]. Although OSM alone up-regulate ADAMTS4-5, combined with IL-1 or TNFα this increased exponentially (ten-fold). The relevance of these observations for cartilage destruction in OA is unclear. Generally, concentrations of 100-1000 fold higher than those observed in OA synovial fluid have been used *in vitro* for all three cytokines. [108]. Moreover, they may mask the actual process in the OA joint, where possibly their effects, if at all present, may all be limited to inhibition of regeneration, rather than destruction. In addition, their single addition to tissue and cell

monoculture *in vitro*, may mask the effects of interactions with other factors present occurring *in vivo*. Therefore, co-culture models or culturing in the presence of synovial fluid may be a better way to investigate the role of mediators present in the synovial fluid. Better understanding of the role of single inflammatory mediators and pro-inflammatory cytokines in the joint, as well as their interactions, will eventually aid in finding a treatment to slow down OA development.

What is the value of anti-inflammatory medication in osteoarthritis?

As long as no definitive role of a particular mediator has been shown, general inhibition of inflammation might be an option to treat OA. Different anti-inflammatory treatments are used for the treatment of OA such as NSAIDS and injection with corticosteroids [1]. Blocking COX-2 by the NSAID celecoxib is currently used in the treatment of OA and inhibits pain and decreases joint stiffness. Moreover, it was suggested to have a potential disease modifying effect, however, ambiguous results were obtained [29, 30, 148, 246]. Celecoxib has shown to stop OA degeneration in cartilage explant culture [22, 244]. Other studies failed to show a positive effect of COX-2 blocking on cartilage metabolism [124]. This raises the question if blocking COX-2 stops OA progression and is truly a DMOAD. We performed a study using 3 different culture models to elucidate the DMOAD potential of celecoxib (chapter 6). The production of inflammatory mediators by synovial tissue could be decreased by celecoxib in a co-culture with cartilage and synovial tissue; however, no effect was seen on cartilage metabolism (chapter 6). The severity of synovitis and degree of cartilage degeneration might explain these differences. The presence of inflammation in some OA subgroups could explain the ambiguous results reported in literature for celecoxib and for some patients celecoxib could truly be a DMOAD.

The corticosteroid triamcinolone decreased the production of inflammatory cytokines and decreased MMP production by cartilage and synovial tissue (chapter 5). The inhibition of cartilage repair in cartilage explant culture by triamcinolone was similar to the effects of corticosteroids found previously on chondrocytes and cartilage explants [139]. However, in co-culture, triamcinolone actually counteracted the inhibitory effect of synovial tissue on cartilage matrix production resulting in a protective effect on cartilage. This again stresses the importance of combining more joint tissues in OA *in vitro* models. However, due to the complete blocking of mediators by corticosteroids, also the long-term repair and normal cartilage turnover seems affected.

Many repair processes start with an acute inflammatory phase such as wound healing and fracture healing. When the inflammation phase is blocked by NSAIDS, fracture healing is impeded [247]. Recent findings evaluating inflammatory pathways in joint development and cartilage identified multiple genes up-regulated involved in inflammation [140]. Blocking complete inflammatory pathways or specific inflammatory mediators may likewise not be beneficial for cartilage repair. However, blocking COX-2 activity has been shown to inhibit hypertrophic differentiation, a

hallmark process in OA [125]. Hence NSAIDS may also prevent further deterioration of cartilage quality. Further elucidation of the role of specific inflammatory mediators in cartilage repair and regeneration and understanding of converging pathways in inflammation and regeneration will likely lead to drugs specifically targeting inflammation without affecting regeneration.

Concluding remarks

Osteoarthritis is a frequently occurring joint disease with multiple factors, e.g. inflammation, obesity, genetic and trauma, contributing to the development, progression and severity of the disease. With a better understanding of the pathogenesis of OA, more specific treatments to stop OA progression can be developed. Multiple inflammatory mediators are produced by osteoarthritic joints tissues and are differently increased in different stages of the OA process and subtypes of OA. Research focussing on specific inflammatory mediators in OA helps us to better understand OA. Inflammatory mediators produced by the synovial tissue and cartilage are capable of affecting OA progression, however, which factors are responsible for cartilage degeneration and how these factors influence cartilage repair in OA is still not clear. Treatments based on blocking these inflammatory factors are currently investigated. As OA progression and development is based on multiple processes (e.g. inflammation, chemotaxis, bone turnover) as indicated by cluster analysis targeting of specific processes maybe the key in stopping OA. However, targeting one mediator, or even one process, will probably not stop OA progression in *all* OA patients. Larger biomarker studies, which are currently being performed, will give insight in which mediators and processes are responsible for OA development and progression in specific subgroups of patients in specific phases of the disease. Finally, as in subgroups of OA patients inflammation is a key process and inflammation is capable of inducing cartilage degeneration, targeting inflammation to stop OA progression will be beneficial in these patients.

Addendum

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Nederlandse samenvatting

Ontstekingsmediatoren in artrose –identificatie en rol in kraakbeenherstel-

In een gezond gewricht bedekt een dunne laag kraakbeen het onderliggende bot. Kraakbeen is een avasculair weefsel dat dient als schokdemper voor het bot en zorgt voor het soepel bewegen van het gewricht. Het kraakbeen wordt in stand gehouden door een complex proces van afbraak en aanmaak van de matrix. In een gewricht dat is aangetast door artrose, breekt het kraakbeen langzaam af en treden de symptomen pijn, stijfheid en functiebeperking op. De behandeling van artrose richt zich momenteel voornamelijk op symptoombestrijding in de vorm van pijnmedicatie of operaties waarbij het gewricht wordt vervangen. Ondanks veel onderzoek is er nog veel onduidelijk over het ontstaan en beloop van artrose. Er bestaat nog geen geschikte behandeling die het ziekteproces van artrose kan remmen of genezen. Een aspect dat onderzoek naar artrose moeilijker maakt, is de heterogeniteit van de ziekte. Dit houdt in dat het ziekteverloop sterk verschilt bij verschillende personen.

Over het algemeen wordt aangenomen dat ontsteking een belangrijke rol speelt bij artrose. Het ontstekingsproces wordt aangestuurd door zogenoemde ontstekingsmediatoren. Deze ontstekingsmediatoren worden veelal geproduceerd door de gewrichtsweefsels, in het bijzonder door het synoviale weefsel dat het gewrichtskapsel van binnen bekleedt. De ontstekingsmediatoren geven specifieke signalen af aan de omliggende weefsels, wat kan leiden tot pijn en afbraak van het kraakbeen. Het is al bekend dat diverse mediatoren een cruciale rol spelen bij het ontstaan van kraakbeenschade in reumatoïde artritis. Deze mediatoren stimuleren de afbraak van het kraakbeen. Welke mediatoren een rol spelen bij artrose is echter nog onduidelijk. Als we hier meer inzicht in krijgen, kunnen we mogelijk een nieuwe behandeling vinden voor artrose.

In dit proefschrift doen we onderzoek naar de pathosfysiologie en behandeling van artrose. Ten eerste hebben we ontstekingsmediatoren geïdentificeerd die al dan niet verhoogd aanwezig zijn in het artrotische gewricht. Daarna hebben we gekeken welk weefsel in het gewricht verantwoordelijk is voor de productie van de geïdentificeerde mediatoren en wat het effect is van deze mediatoren op het kraakbeen. Als laatste hebben we onderzocht of door het gebruik van ontstekingsremmers en specifieke antilichamen het herstellende vermogen van kraakbeen verbeterd kan worden (interventie).

Identificatie van ontstekingsmediatoren

In hoofdstukken 2-4 bestudeerden wij de aanwezigheid van ontstekingsmediatoren in het artrotisch gewrichtsmilieu. Hierbij hebben we zowel de pols als de knie onderzocht. We hebben een breed scala aan mediatoren onderzocht, waaronder eiwitten die de ontsteking remmen en eiwitten die de ontsteking versterken. In hoofdstuk 2 presenteren we een overzicht van mediatoren (onder andere cytokines, chemokines en groeifactoren) die we hebben gemeten in de gewrichtsvloeistof van gezonde patiënten en van patiënten met knieartrose. In de gewrichtsvloeistof van artrosepatiënten zijn diverse ontstekingsmediatoren verhoogd in vergelijking met de gewrichtsvloeistof van gezonde mensen. Dit komt overeen met de gangbare hypothese dat in het ziekteproces van artrose ontsteking een cruciale rol speelt.

De knie is een gewricht dat regelmatig wordt bestudeerd voor onderzoek naar artrose, maar artrose kan in alle gewrichten voorkomen. Over artrose in de pols is nog weinig bekend. In hoofdstuk 3 hebben we gekeken naar de aanwezigheid van ontstekingsmediatoren in het polsgewricht. Hier zagen we een verhoogde concentratie van de ontstekingsmediatoren IL-6, IL-10 en interferon (IFN)-y in vergelijking met gezonde polsen, wat kan wijzen op een ontstekingsproces bij polsartrose. Als we de pols met de knie vergelijken (hoofdstuk 4), zien we dat in de pols meer van de gemeten mediatoren verhoogd zijn dan in de knie. Dit zou een verklaring kunnen zijn voor het meer agressieve natuurlijke beloop van polsartrose in vergelijking met knieartrose. Concluderend, als we de gewrichtsvloeistof van gezonde mensen en die van patiënten met artrose vergelijken, blijkt dat er duidelijk verschillen zitten in de aanwezigheid en concentratie van ontstekingsmediatoren. De aanwezigheid van deze ontstekingsmediatoren kan erop wijzen dat ontsteking een rol speelt bij het ontstaan en verergeren van symptomen van artrose, zoals kraakbeenschade.

Herkomst van ontstekingsmediatoren

In hoofdstuk 5 hebben we gekeken naar de productie van de verschillende ontstekingsmediatoren door het gewrichtsweefsel en wat het effect is van artrotisch synoviaal weefsel op het herstellende vermogen van artrotisch kraakbeen. Voor een goed functionerend gewricht zijn zowel het kraakbeen als het synoviale weefsel essentieel. Het kraakbeen wordt in stand gehouden door een complex proces van afbraak en aanmaak van de matrix. Deze afbraak en aanmaak wordt gestuurd door de mediatoren in de gewrichtsvloeistof. Het synoviale weefsel is de binnenbekleding van het gewricht en produceert moleculen die zorgen voor smering van het gewricht. Daarnaast heeft het synoviale weefsel een belangrijke functie in het voeden van het kraakbeen en zuiveren van de gewrichtsvloeistof. De interactie tussen het kraakbeen en het synoviale weefsel is nog niet

helemaal duidelijk. Gedacht wordt dat bij artrose het synoviale weefsel ontstoken is en door ontstekingsmediatoren te produceren een rol speelt bij het ontstaan en/of het verergeren van kraakbeenschade. Het is echter nog niet bewezen dat dit het geval is.

Om een zo goed mogelijk beeld te krijgen van wat er speelt bij artrose hebben we geprobeerd het gewricht na te bootsen door een kweekmodel te ontwikkelen met zowel artrotisch kraakbeen als artrotisch synoviaal weefsel. De mediatoren die werden geproduceerd door het kraakbeen en het synoviale weefsel tijdens het kweekproces, kwamen overeen met de mediatoren die we hebben gevonden in synoviale vloeistof van artrosepatiënten. Dit wijst erop dat het kweekmodel dat we gebruikten overeenkomt met het gewrichtsmilieu gevonden bij patiënten met artrose. Uit ons onderzoek met het kweekmodel blijkt dat de mediatoren die geproduceerd worden door het ontstoken synoviale weefsel niet de afbraak van kraakbeen versnellen maar wel het herstel van kraakbeenschade remmen. Daarnaast hebben we aangetoond dat er in het synoviale weefsel ontstekingscellen (macrophagen en lymfocyten) zitten. Mogelijk zijn deze ontstekingscellen in staat diverse ontstekingseiwitten, zoals IL-1, IL-6 en Oncostatin M (OSM), te produceren.

Interventie door modulatie van ontstekingsmediatoren

Om de remmende werking van het synoviale weefsel op kraakbeen te stoppen hebben we de ontsteking geremd door middel van verschillende medicamenten. Allereerst hebben we het corticosteroid triamcinolon gebruikt (hoofdstuk 5). Hierbij zagen we een vermindering van de productie van ontstekingseiwitten, met een positief effect op het herstel van het kraakbeen wanneer het samen met synoviaal weefsel werd gekweekt. Echter zagen we een negatief effect op het kraakbeen wanneer we het kraakbeen alleen kweekten met triamcinolon. Ook hebben we in diverse kweekmodellen gekeken of er een effect is op kraakbeenherstel bij het remmen van het ontstekingsproces door cyclo-oxygenase 2 (COX-2). Hiervoor gebruikten we de ontstekingsremmer celecoxib, een specifieke COX-2 remmer, die gebruikt wordt in de behandeling van artrose. In onze experimenten zagen we een daling van meerdere ontstekingsmediatoren. Dit wijst op een onderdrukking van de ontsteking door celecoxib. Echter, zagen we geen verbetering op kraakbeenherstel of vorming van nieuw kraakbeen (hoofdstuk 6).

Omdat we het effect van specifieke ontstekingsmediatoren in kraakbeenherstel wilden bestuderen hebben we in de laatste twee hoofdstukken gericht mediatoren geblokkeerd. Omdat zowel IL-6 als OSM verhoogd zijn in het gewricht van artrosepatiënten, en bekend is dat deze mediatoren een rol spelen in reuma, hebben we ons specifiek gericht op de rol van deze twee mediatoren in artrose.

Allereerst bestudeerden we IL-6 (hoofdstuk 7). Door een antilichaam te gebruiken tegen IL-6 of de receptor van IL-6 te blokkeren, hebben we onderzocht of het remmen van IL-6 positief was voor het kraakbeenherstel dan wel kraakbeenvorming. We vonden echter geen effect van het blokkeren van IL-6 op het kraakbeen. Daarnaast hebben we ook het effect onderzocht van een toevoeging van IL-6. Bij het toevoegen van IL-6 zagen we dat er juist een kleine toename was van kraakbeenvorming. Mogelijk is een (lichte) ontsteking nodig of bevorderlijk voor kraakbeenvorming, of is IL-6 -in deze *in vitro* modellen of concentraties- niet inflammatoir.

We hebben tevens gekeken naar de rol van het ontstekingseiwit OSM bij artrose (hoofdstuk 8). Zoals al eerder beschreven in hoofdstuk 2 is OSM aanwezig in de gewrichtsvloeistof van artrosepatiënten. Bij het bestuderen van het artrotische kraakbeen en synovium vonden we bovendien een toename van een tweetal receptoren waar OSM specifiek aan bindt. Beide worden geassocieerd met ontsteking en kunnen van invloed zijn op het ontstekingsproces. We hebben onderzocht of het blokkeren van OSM in gewrichtsvloeistof het kraakbeenherstel kon bevorderen. Dit bleek inderdaad het geval te zijn. Dit wijst erop dat het blokkeren van OSM een potentiële therapie kan zijn in de behandeling van artrose.

Concluderend hebben we meerdere mediatoren geïdentificeerd die geassocieerd worden met ontsteking in het gewricht van artrosepatiënten. Door kweekmodellen van zowel artrotisch kraakbeen als synoviaal weefsel te gebruiken, ontdekten we dat sommige van mediatoren bijna exclusief geproduceerd worden door het synoviale weefsel (IL-1RA, IL-6 en Il-8) en de meeste juist door kraakbeen, en vonden we specifieke ontstekingsmediatoren die invloed hebben op het herstellende vermogen van kraakbeen. Remming van de productie van enkele ontstekingsmediatoren uit het synoviale weefsel resulteerde in het stimuleren van kraakbeenherstel. Specifieke remming van de ontstekingsmediator OSM had een vergelijkbaar effect, maar remming van IL-6 had geen of zelfs een tegenovergesteld effect. Op deze manier hebben we aangetoond dat medicatie die ingrijpt in het ontstekingsproces een rol kan spelen in de behandeling van artrose. Het is echter niet zo dat een verhoogde productie van één bepaalde ontstekingsmediator in iedere patiënt in elk stadium van artrose altijd de belangrijkste mediator is voor kraakbeenafbraak of een verminderde aanmaak van kraakbeen. Uiteindelijk moet meer inzicht en begrip van de heterogeniteit van artrose bijdragen bij het vinden van de juiste behandeling voor patiënt met artrose.

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List of publications

An exploratory study of the differences between healthy and osteoarthritic synovial fluid. M. Beekhuizen, L.M. Gierman, W.E. van Spil, G.J.V.M. van Osch, T.W. Huizinga, D.B.F. Saris, L.B. Creemers, A-M Zuurmond. *Osteoarthritis Cartilage. 2013 Jul;21(7):918-22.*

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Comparison of soluble mediators in the osteoarthritic knee joint and wrist joint. T. Teunis, M. Beekhuizen, M. Kon, L.B. Creemers, A.H. Schuurman, L.P. van Minnen. *Submitted*

Osteoarthritic synovial tissue inhibits proteoglycan production in human osteoarthritic cartilage. M. Beekhuizen, Y.M. Bastiaansen-Jenniskens, W. Koevoet, D.B.F. Saris, W.J.A. Dhert, L.B. Creemers, G.J.V.M van Osch. *Arthritis Rheum. 2011 Jul;63(7):1918-27*

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Interleukin 6 is elevated in synovial fluid of patients with focal cartilage defects and stimulates cartilage matrix production during regeneration. A.I. Tsuchida, M. Beekhuizen, M. Rutgers, G.J.V.M. van Osch, J.E.J. Bekkers, A.G.J. Bot, B. Geurts, W.J.A. Dhert, D.B.F. Saris, L.B. Creemers. *Arthritis Res Ther. 2012 Dec 3;14(6):R262*

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Synovial fluid cytokine profiles depend on cartilage pathology, but are different between synovial fluid, cartilage tissue and cultured chondrocytes. A.I. Tsuchida, M. Beekhuizen, M.C. 't Hart, T.R.D.J Radstake, W.J.A. Dhert, D.B.F. Saris, G.J.V.M. van Osch, L.B. Creemers. *Submitted*

Pronounced biomaterial dependency in cartilage regeneration by non-expanded compared to expanded chondrocytes. A.I. Tsuchida, J.E.J. Bekkers, M. Beekhuizen, L. Vonk, W.J.A. Dhert, D.B.F. Saris, L.B. Creemers. *Regen Med. 2013 Sep 8(5):583-95*

CXCL8 (Interleukin-8) in synovial fluid and autocrine production by chondrocytes; association with cartilahe pathology, but no effect on regeneration or terminal differentiation. A.I. Tsuchida, D.B.F. Saris, M. Rutgers, M. Beekhuizen, J.E.J. Bekkers, B. Geurts, A. Kragten, W.J.A. Dhert, L.B. Creemers. *Submitted*

Curriculum Vitae

Michiel Beekhuizen was born on January 27th, 1983 in Arnhem. After graduating from athenaeum at the 'Pantarijn scholengemeenschap' in Wageningen he started his medical study at Utrecht University. During the final year of his medical study he participated in a research project on joint distraction at the department of Rheumatology (supervised by Dr. F. Intema, Dr. L.B. Creemers and Prof. Dr. F.P.J.G. Lafeber). In 2008 he started his PhD project entitled "Inflammatory mediators in osteoarthritis, identification and role in cartilage repair", supervised by Dr. L.B. Creemers, Prof. Dr. G.J.V.M. van Osch and Prof. Dr. W.J.A. Dhert. The research described in this thesis has resulted in publications in peer reviewed journals and (poster) presentations at (inter)national conferences. He continued his training for orthopaedic surgeon in the region Utrecht. The first 1.5 year he worked as a surgical resident at Meander Medical Center in Amersfoort (head: Dr. A.J. van Overbeeke). Currently he is working as an orthopaedic resident at the department of Orthopaedics at the Antonius Hospital in Nieuwegein (head: Dr. M.R. Veen). He will receive the rest of his orthopaedic training at Diakonessenhuis in Utrecht (head: Dr. A. de Gast) and the University Medical Center in Utrecht (head: Prof. Dr. D.B.F. Saris).

Michiel Beekhuizen lives in Utrecht with Suzanne Tiesema.