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DISSERTATION

Titel der Dissertation

„Inflammation and human chondrocytes: Glycobiological aspects and anti-inflammatory activity of *Caesalpinia sappan* isolates *in vitro*“

Verfasserin

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1. Introduction

1.1. Cartilage

Cartilage is an avascular, aneural and alymphatic connective tissue, covering the articulating ends of long bones. Cartilage is primarily (> 95%) composed of a highly organized extracellular matrix (ECM), comprising a collagen II network, proteoglycans and non-collagen proteins. Chondrocytes, the unique cell component of cartilage, are assumed to be responsible for the production and maintenance of the ECM by low-turnover replacement of certain matrix proteins [1].

ECM in cartilage consists of a fluid phase composed of high percentage (70-80% by wet weight) water and electrolytes (Fig.1). The solid phase of cartilage contains collagen fibrils, proteoglycans, and glycoproteins. Collagen is believed to contribute mostly to the tensile behaviour of the tissue. Proteoglycans are large molecules with a protein backbone named core protein and glycosaminoglycan (GAG) side chains. Beside the main proteoglycan-aggrecan, there are several other small proteoglycans, such as decorin, biglycan and fibromodulin. The most common types of GAGs are chondroitin sulphate and keratan sulphate. Trapped in the collagen network, proteoglycans are attached to a molecule of hyaluronan, forming a feathery structure called a proteoglycan aggregate. With numerous negatively charged sulphate groups, these aggregates attract positive ions in the surrounding fluid phase and lead to increased swelling pressures as compared to the outside of this tissue. The collagen fibrils in the matrix limit water imbibitions by limiting the degree to separation of

glycosaminoglycans. Thus, collagen fibrils and proteoglycans contribute mostly to the mechanical characteristics of cartilage [2-4].

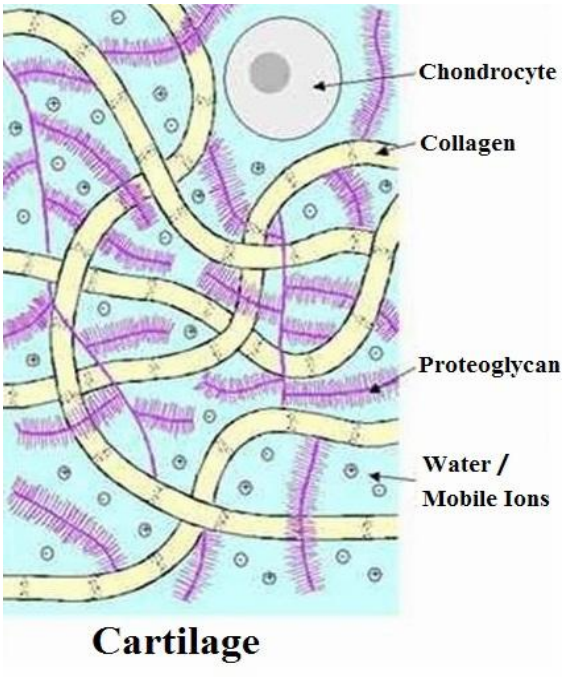


Figure.1 Structure of cartilage

http://www.bidmc.org/Research/Departments/Radiology/Laboratories/Cartilage.aspx

1.2. Osteoarthritis (OA)

OA is one of the most important causes of physical disability in adults and ranks among the most frequent and cost-intensive chronic diseases in advanced age. In the USA, Europe and Japan, the estimated total prevalence of OA ranges from 36% to 48% of the population. Due to the growing proportion of older age groups, the estimated number of total prevalent cases of OA is expected to grow by 1.1-1.2 % per year [5]. 27 million Americans have OA, and this arthritis patient number is projected to increase to 67 million in 2030. One out of four adults will be affected by arthritis and

OA will account for most cases. The annual cost of arthritis in the USA economy is total $128 billion and OA accounts for much of that. In 2004, over 11 million physician visits, 662,000 hospitalizations, and more than 632,000 total joint replacements were caused by OA in USA [6]. In Austria, OA resulted in 44,151 hospitalizations (15,601 men, 28,550 women) in 2000, and the average stay in hospital was 16.8 days (man 16 days, woman 17 days) [7].

As the most common joint disease in elderly, OA is characterized by progressive loss of articular cartilage. Generally, knees, hips, hands, and the spine are the most frequently affected sites. The symptoms are often associated with significant functional impairment, pain, stiffness, and disability. Traditionally, OA has been considered a disease of articular cartilage. Recently, evidence demonstrated that OA is not only a disorder of cartilage, but a disease affecting the whole joint, including subchondral bone sclerosis, osteophyte formation, and changes in the synovial tissue (Fig2.). Various components of the joint are involved in the pathological progression of OA, such as the synovial joint lining and peri-articular bone [8, 9]. In contrast to rheumatoid arthritis, OA is conventionally not considered a classical inflammatory arthropathy, but thought to develop from chronic overuse or injury of the joint. A variety of risk factors associated with OA include age, sex, overweight, trauma, inflammation diseases of bones or genetic predisposition [7, 10].

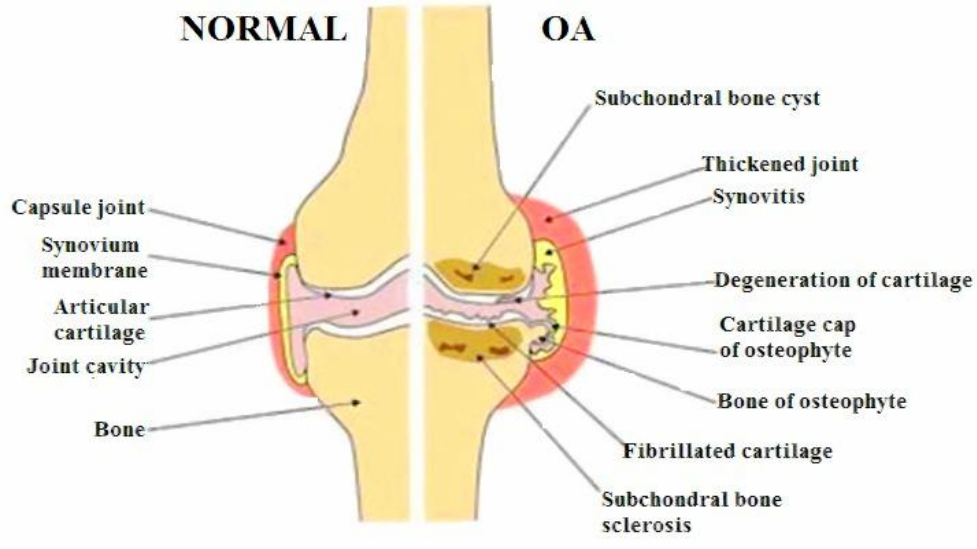


Figure.2 Healthy and osteoarthritic joint

http://www.mendmeshop.com/knee/knee_osteoarthritis_causes.php

The etiology of OA is multi-factorial involving both mechanical and biochemical factors (Fig.3). Chondrocytes embedded within the negatively charged ECM, serve as mechano-sensors and osmo-sensors, and change the metabolism in response to local physicochemical alteration of the microenvironment[11]. In OA, a shift in equilibrium between anabolic and catabolic activities occurs in cartilage and surrounding joint tissues. The metabolic imbalance results in increased cartilage degradation and insufficient reparative responses.

1.2.1. Cartilage metabolism in OA

Degeneration and loss of articular cartilage are characteristic features of OA. The loss of articular cartilage is a result of the action of unregulated and overproduced proteinases that degrade both proteoglycans and collagens. Matrix metalloproteinases

(MMPs) include collagenases (MMP-1, MMP-8, and MMP-13), stromelysin-1 (MMP-3), the gelatinaseses (MMP-2 and MMP-9) and membrane type 1 MMP (MMP14) [1, 12]. Of the collagensases, MMP-13 is assumed to play an important role in OA progression because of its preferential ability to degrade native type II collagen [11, 12]. MMP-3, MMP-8 and MMP14 are capable of degrading proteoglycans [1]. The aggrecanases belong to a family of extracellular proteases known as the ADAMTS (a distintergrin and metalloprotease with thrombospondin motifs). ADAMTS-4 and ADAMTS-5 are now regarded as the principal aggrecan degrading enzymes in cartilage [13].

In early OA, there is evidence of increased synthetic activity, which is considered as a compensatory response of chondrocyts to regenerate the matrix with cartilage specific components including types II, IX, and XI collagens, agggrecan, and type IV collagen [8]. The increased levels of cartilage anabolic factors such as bone morphogenetic proteins (BMPs) and insulin-like growth factor-α, TGF-ß, and fibroblast growth factors provide a possible explanation of the underlying mechanism [11,14,15]. However, once the cartilage is severely degraded the chondrocyte is not able to maintain or reconstruct the structure of cartilage. Along with the phenotypic modulation of the articular OA chondrocytes, the cells stop to expressing aggrecan and collagen type II, but start to synthesize collagen types I, III, and V [16].

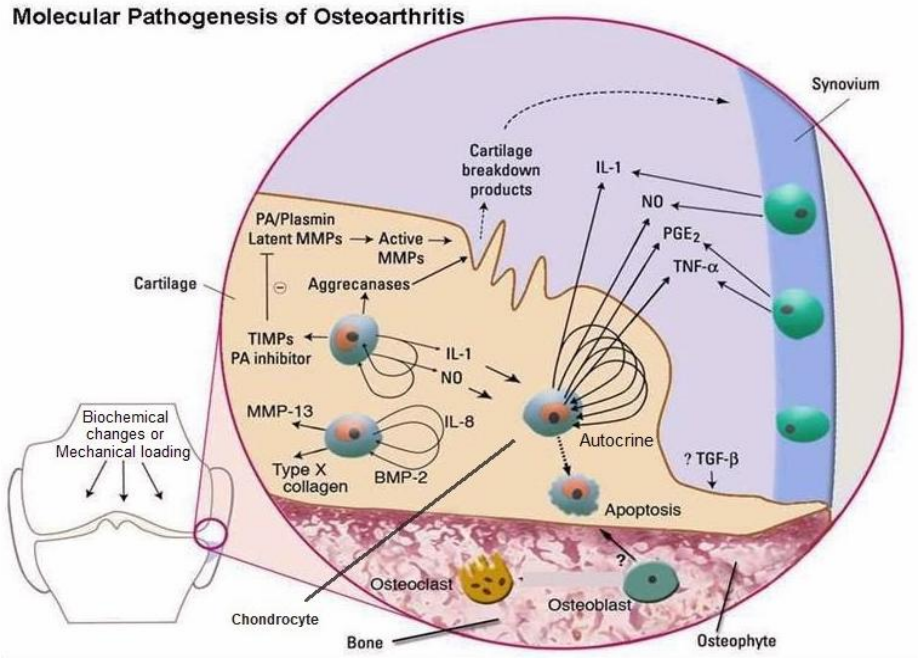


Figure 3. Molecular and cellular mechanisms of OA [11]

1.2.2. Inflammation and OA

Traditionally, OA has been classified as a non-inflammatory disease because of the absence of neutrophils in the synovium and a systemic manifestation of inflammation. However, in the last decade evidence indicated that inflammation contributes to the symptoms and the progression of OA. In response to biochemical changes or mechanical loading within cartilage, chondrocytes can produce a number of inflammatory mediators, which drive predominantly catabolic pathways and inhibit matrix synthesis in joint tissue [8].

Interleukin-1ß (IL-1ß) and tumor necrosis factor-α (TNF-α) are synthesized intracellularly as prescursors, converted to mature forms by the IL-1ß-converting enzyme (ICE, caspase-1) and the TNF-α-converting enzyme (TACE), respectively.

An increased production of both cytokines in joint tissues is a characteristic feature of OA [17]. IL-1ß and TNF-α stimulate chondrocytes to overexpress MMPs and suppress the synthesis of matrix collagen and proteoglycans. In addition, both cytokines in an autocrine/paracrine manner stimulate their own production and induce chondrocytes and synovial cells to produce other proinflammatory mediators, such as chemokines, prostanglandins and nitric oxide (NO). The action of both cytokines is in part mediated by activation of the transcription factor nuclear factor-κB(NF-κB). IL-1ß and TNF-α increase the synthesis of prostaglandin E_2 (PGE_2) via the inducing the expression or activities of cyclooxygenase(COX)-2, microsomal PGE synthase-I and soluble phospholipase A2, and they up-regulate the production of NO via inducible nitric oxide synthetase (iNOS, or NOS2). IL-1ß also has the capacity to induce IL-6, leukemia inhibitory factor (LIF), and IL-8 and suppress the expression of type II collagen (COL-2AI) [8, 18, 19, 20]. (Fig.3)

Although debate remains regarding the essential role of synovitis in OA, and regarding the mechanism underlying the initiation of inflammatory mediators, inflammation is a major factor contributing to the exacerbation of dysregulation of chondrocyte function and cartilage degradation in OA.

1.2.3. NF-κB signaling pathways in inflammation of cartilage

Various signaling pathways are involved in the progression of OA. NF-κB signaling pathways play a crucial role in inflammatory processes, cartilage degradation, cell

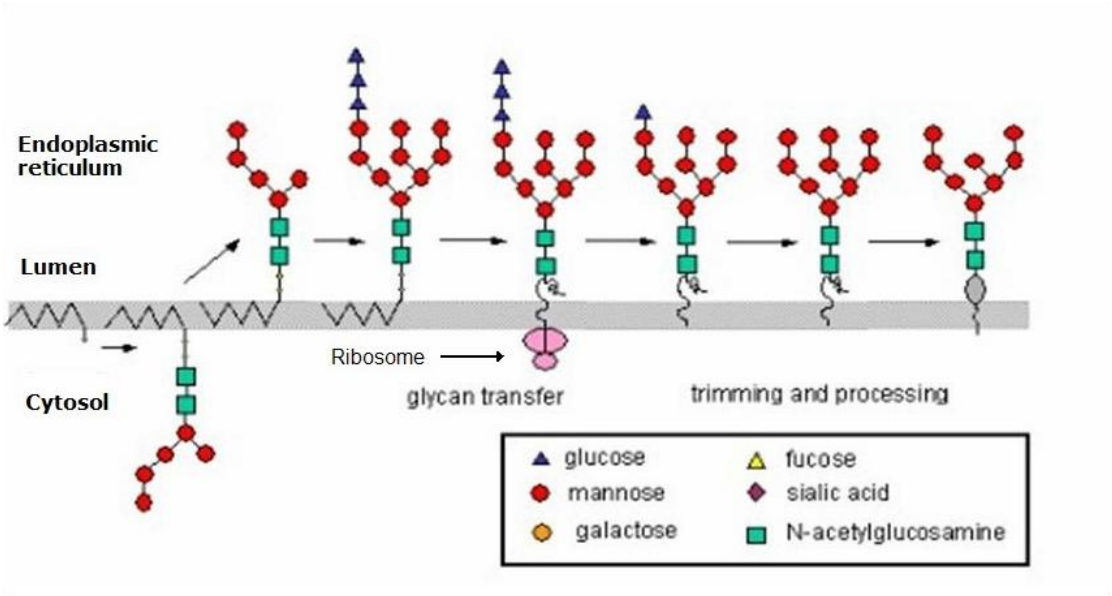
proliferation, and angiogenesis in cartilage.

In mammals, NF-κB dimers are homodimers or heterodimers of 5 different NF-κB proteins (RelA/p65, RelB, c-Rel, NFκB1/p105 and NFκB2/p100). NF-κB is present in the cytoplasm in an inactive form associated with the inhibitory NF-κB proteins (IκBα, IκBβ, IκBε) or the larger precursor proteins p105 (NFκB1) and p100 (NFκB2). Phosphorylation of the IκB kinase (IKK) results in nuclear translocation of NF-κB and induction of transcription of target genes. The IKK complex consists of two serine-threonine kinases, IKKα and IKKβ, and NEMO/IKKγ. The canonical NF-κB signaling module immediately reacts to most pro-inflammatory and stress-like responses. In this process, IKKβ is both necessary and sufficient to phosphorylate IκBα, or IκBβ in an IKKγ-dependent manner [21, 22, 23]. IKKα is specifically required for the activation of the so-called non-canonical or alternate NFκB pathway. The NF-κB pathway is essential for chondrocytes to express inflammation-related genes, including MMP-1, 3 and 13, iNOS/NOS-2, IL-6, IL-1, TNF-α, COX-2, and transcription factors such as ELF3/ESE-1 [23]. Evidence suggested that NF-κB and the MAP kinase ERK1/2 mediate inhibition of type II collagen and link protein expression [24], and mediate MMP-1, 3, and 13 RNA and protein expression [25] induced by in IL-1ß or TNF-α. Thus, the NF-κB pathways appear as potential therapeutic targets in OA.

1.3. Glycobiology of chondrocytes

The ECM of cartilage consists of protein fibers embedded in an amorphous mixture of huge glycosylated proteins (proteoglycans), which serve as structural components of ECM and play a role in maintaining matrix homeostasis [2]. The protein component of proteoglycans is synthesized by ribosomes and translocated into the rough endoplasmic reticulum (ER). Then, a core oligosaccharide is added to the protein chain, following trimming and processing of monosaccharides, until a core mannose structure remains added to the protein chain (Fig.4A). Additional glycosylation of the proteoglycan further occurs in the Golgi apparatus in multiple enzymatic steps and is a form of co-translational and post-translational modification (Fig.4B). The attachment of oligosachharide to asparagine (N-linked) or serine/threonine (O-linked) residues confers protection against proteolytic degradation or controls protein folding [26-28].

A)



B)

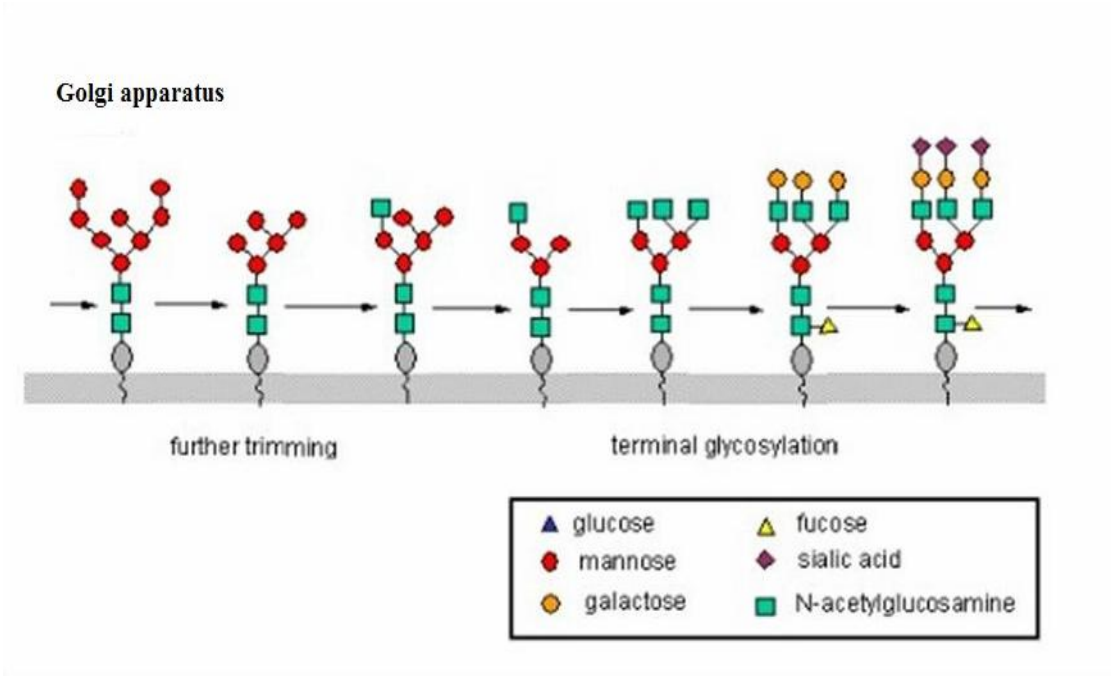


Figure 4. A) Glycosylation in ER. B) Glycosylation in Golgi apparatus. The newly synthesized protein is shown in the lumen side of the ER membrane. *http://employees.csbsju.edu/hjakubowski/classes/ch331/cho/glycoproteinshtm.htm*

Glycosyltransferases (GTs) are a class of enzymes, which are responsible for the catalyzation of the glycosylation reactions. In different tissues and cell types, the characteristic glycopheotype varies during development and oncogenesis. In addition, the glycosylation reaction and expression of GTs can also be regulated by various factors such as cytokines and differentiation factors under homeostasis or pathogenic conditions. It is well known that altered glycosylation various significantly in cancer cells as compared to normal cells [29-31]. Such changes in the cellular glycophenotype potentially affect cell functions, such as cellular adhesion, cell signaling, and apoptosis [32, 33].

Sialic acids are a diverse family of negatively charged sugar units that are typically found at the outermost end of glycan chains of all cell types, meditating or modulating a variety of normal and pathological processes [34, 35]. In mammals, 20 sialyltransferases (SiaTs) have been cloned and grouped into four families (*ST3GAL*, *ST6GAL*, *ST6Galnac*, *ST8Sia*) based on linkage (e.g., α-2,3 or α-2,6 linkage in case of N-linked glycans in glycoproteins) and acceptor substrate. The diverse enzymes of the SiaT family share the cytidine monophosphate-sialic acid as donor substrate, but are distinguished by the oligosaccharide acceptor [36]. Cell surface sialylation has been reported to affect osteoclastogenesis, endocytosis in dendritic cells, and adhesion properties of human colon cancer cells [37-39].

Recent studies have documented a relationship between the alteration of glycosylation and inflammatory conditions of cartilage. As such, OA was suggested to associate with a dysregulation in the hexosamine biosynthesis pathway that could lead to an

alteration of the level of o-linked n-acetylglucosamine (O-GlcNAc)-glycosylation in a variety of proteins [40]. Yang et al suggested that TNF-α and TGF-ß treatment cause specific glycosylation changes related to apoptosis and altered cell proliferation in human and bovine chondrocytes [32]. Thus, inflammatory conditions appear able to alter the synthesis and activities of glycosyltransferases that synthesize the glycan chains of glycoproteins, and these changes in turn can influence the functions of glycoproteins.

1.4. Nutraceuticals for OA management

In general, OA is a progressive disease, and not curable to date. Current treatment options are limited to non-pharmacological interventions range from physical exercise and weight loss to surgical intervention and symptomatic treatments using acetaminophen, oral and topical nonsteroidal anti-inflammatory drugs (NSAIDs) or intra-articular glucocorticoid and hyaluronic acid injection to the affected joint [5]. The mechanism of cartilage degradation in OA is known as a multifactorial process. However, standard pharmacological interventions, such as the use of NSAIDs often act in a monomodal way associated with significant adverse effects due to the requirement of extended duration of medication. Nutraceuticals and phytopharmaceuticals, that usually contain a range of active compounds targeting multiple pathways, could provide an alternative to conventional treatment of OA [41, 42]. Several studies suggest that glucosamine (GlcN), chondroitin sulphate, curcumin,

diacerin, s-adenosylmethionine, avocado/soybean unsaponisfiables, and numerous of nutraceuticals posses possible analgesic and structure-modifying effects.

Glucosamin (GlcN) is a natural aminomonosaccharide existing in healthy cartilage. D- Glucosamine, the biologically active form, serves as a metabolic precursor for the synthesis of proteoglycans, GAG and hyaluronate. It was reported that GlcN sulfate stimulates proteolycan production and inhibits IL-1ß- induced $NF_{\kappa}B$ activation in human OA chondrocytes and owns mild anti-inflammatory properties [43-45]. Over the last 20 years, numerous clinical studies on the use of GlcN as a medical therapy for OA were conducted worldwide, but there is conflicting outcome as to its effectiveness [46].

Curcumin (diferuloylmethane or 1,7-bis(40-hydroxy-30-methoxyphenyl)-1,6-heptadiene-3,5-dione) is an active phytochemical component from Indian curry spice tumeric (Curcuma longa) and has potent antioxidant, anti-inflammatory, anti-carcinogenic and anti-amyloid properties. It has been reported that curcumin is a potent inhibitor of NF-κB, c-jun N-terminal kinse (JNK), and signal transducers and activators of transcription (STAT) activation by oncostatin M (OSM) and IL-1ß. In chondrocytes, it down-regulates the expression of IL-1ß, TNF-α, cyclooxygenase- 2 (COX2), nitrous oxide systems (NOS), MMPs and chemokines. Further, curcumin is able to restore type II collagen and GAG synthesis in chondrocytes[47-50]. Therefore, data from recent studies suggested that curcumin may be a novel and safe nutraceutical alternative to the first-line used NSAIDs treatment for OA. Nevertheless, more information about solubility, application, bioavailability, efficiency and toxicity

of curcumin is required and is thus an interesting topic of ongoing research efforts.

A large number of other nutraceuticals and phytopharmaceuticals are currently widely studied. Avocado/soybean unsaponifiables, collage hydrolysates, vitamine D, resveratol, extracts from rose hip, ginger and bromelain have also been demonstrated to be effective for OA treatments [41, 42]. They could provide alternative for OA management, however, further evidence is needed for most of the compounds to draw final conclusions regarding their efficacy.

1.4.1. Isolates from *Caesalpinia sappan*

Sappan Lignum, the heartwood of *Caesalpinia sappan* L. (Leguminosae), is known as the source of a natural red dye (Fig.5A.). Besides, it has been widely used in oriental traditional medicine for improvement of blood circulation, sprains and emmeniopathy [51]. Recently, it has also been listed in the 15th edition of the Japanese Pharmacopoeia [52]. Different extracts and active compounds isolated from Sappan lignum have been reported to possess diverse biological activities including antioxidative [53], antiinflammtory [54, 55], antibacterial [56] and anticonvulsive [57] effects.

A **B**

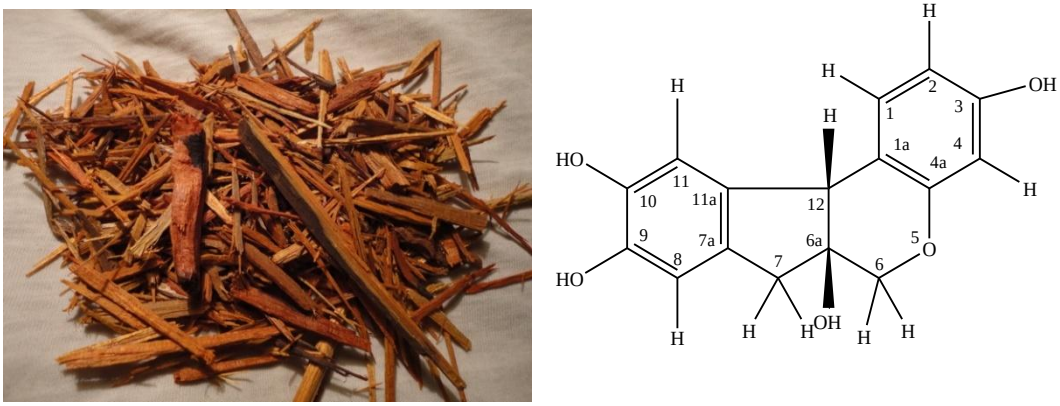


Figure 5. A) Sappan lignum used in traditional Chinese medicine B) the structure of Brazilin

Brazilin ((6a*S*,11b*R*)-7,11b-Dihydrobenz[b]indeno[1,2-d]pyran-3,6a,9,10(6H)-tetrol), the major compound of Sappan Lignum, was reported to possess anti-inflammatory [58], antibacterial [59] and antihepatotoxic effects [60]. In addition, numerous other compounds from Sappan Lignum such as prostosappanins A-E or sappanchalcone were shown to participate in the different biological activities [61].

Although brazilin possesses a defined chemical structure (Fig.5B), as well as its own IUPAC name and CAS-number, commercially available "brazilin" is mostly an extract of sappan wood consisting of several undefined compounds. Hu et al reported that brazilin possesses anti-inflammatory actions in macrophages and acts through a novel mechanism involving the action of HO-1. In this study, they used a commercially available "brazilin" (MP Biomedicals, USA) as pure substance to examine the effects of brazilin on LPS-stimulated NO and cytokine production and iNOS and PGE2 expression in RAW264.7 macrophages [62]. In preliminary experiments, however, we analyzed this product using analytical HPLC and verified

that it is actually a multi-component sappan wood extract (data not shown). A similar situation was observed in a study of neoflavonoids based on negative ion electrospray mass spectrometry [63]. The purchased “brazilin” used in that study was also proved as a multi-component sappan wood extract by the manufacturer when contacted. In order to obtain chemically pure brazilin, chemical synthesis might represent one possibility. Huang et al. reported a unique route to synthesis of (±)-Brazilin using IBX [64]**.** However, this chemical synthesis is highly complex and on inquiry the authors claimed that they are currently not able to provide a sample of the purified product. Thus, according our experience, the most straightforward way to obtain pure brazilin is to isolate it from sappan wood prior to careful identification of its structure.

1.5. Aim of the thesis

The present thesis aimed to investigate two separate aspects of inflammation in human chondrocytes.

First, we investigated the chondrocyte glycophenotype in different cell models and under inflammatory conditions *in vitro*.

The study *"**Lectin binding patterns reflect the phenotypic status of in vitro chondrocyte models***" aimed to evaluate the glycoprofile of primary human chondrocytes as well as the relationship between chondrocyte phenotype and glycophenotype by comparing primary chondrocytes and immortalized chondrocytes models.

The study *"**Phenotype-related differential α-2,6- or α-2,3-sialylation of glycoprotein N-glycans in human chondrocytes"*** aimed to compare the sialylation pattern of primary human chondrocytes with immortalized chondrocytes models and one chondrosarcoma cell model, and to investigate the effects of IL-1ß and TNF-α on primary chondrocyte sialylation.

The study *"**IL-1ß and TNF-α alter the glycophenotype of primary human chondrocytes in vitro"*** aimed to investigate the impact of IL-1ß and TNF-α on characteristics of chondrocyte glycophenotype focusing on (1) the ratio between oligomannosidic, hybrid and complex-type N-glycans, (2) the core fucosylation in N-glycans, and (3) the sialylation of N-and O-glycans with regard to linkage type and degree of sialylation.

As inflammatory events represent interesting targets for the treatment of OA, the second part of this thesis evaluated the activities of *Caesalpinia sappan* extracts (CSE) and the isolated compound brazilin in stimulated human chondrocytes and macrophages.

The study *"**Caesalpinia sappan extract inhibits IL1ß mediated over-expression of matrix metalloproteinases in human chondrocytes"*** aimed to investigate the effect of CSE on catabolic processes in human OA chondrocytes, particularly regarding the expression and activity of major MMPs.

The study *"**Anti-inflammatory activity of an ethanolic Caesalpinia sappan extract in human chondrocytes and macrophages"*** aimed to elucidate the anti-inflammatory activity of CSE in lipopolysaccharide (LPS)-stimulated human macrophages and IL-1ß-stimulated human chondrocytes, focusing on the inhibition of gene expression of proinflammatory factors such as IL-1ß and TNF-α, and on the interference with NO production and NF-κB-mediated COX-2 promoter activation.

The study *"**Brazilin from Caesalpina sappan inhibits pro-inflammatory mediators and metalloproteinase-3 expression in human chondrocytes"*** aimed to proof the hypothesis that brazilin is responsible for previous studies observing anti-inflammatory effects of CSE in chondrocytes by determining the effects of brazilin on chondrocytes in comparison with CSE.

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2. Specific topics

2.1. Author's contribution

I hereby declare to have significantly contributed to the realization of the studies included in the present thesis.

For the study"***Lectin binding patterns reflect the phenotypic status of in vitro chondrocyte models***", I carried out the cell culture, treatments and performed qPCR experiments.

For the study*"**Phenotype-related differential α-2,6- or α-2,3-sialylation of glycoprotein N-glycans in human chondrocytes"***, I carried out the cell culture, treatments, performed qPCR experiments, and was involved in data analysis.

For the study*"**IL-1ß and TNF-α alter the glycophenotype of primary human chondrocytes in vitro"*** I carried out the cell culture, treatments, performed qPCR experiments, and was involved in data analysis.

For the study *"**Caesalpinia sappan extract inhibits IL1ß mediated over-expression of matrix metalloproteinases in human chondrocytes"***, I participated in the study design, contributed to chondrocyte isolation and the maintenance of the cell culture, performed biochemical assays and qPCR analyses. Moreover, I was involved in data

analysis and interpretation.

For the study *"**Anti-inflammatory activity of an ethanolic Caesalpinia sappan extract in human chondrocytes and macrophages"***, I participated in the study design, contributed to the maintenance of the cell culture and chondrocyte isolation, performed biochemical assays and qPCR analyses. I was involved in data analysis and interpretation, and I drafted the manuscript.

For the study*"**Brazilin from Caesalpina sappan inhibits pro-inflammatory mediators and metalloproteinase-3 expression in human chondrocytes**"* , I participated in the study design, contributed to the maintenance of the cell culture and chondrocyte isolation, performed biochemical assays and qPCR analyses. I was involved in data analysis and interpretation, and I drafted the manuscript.

2.2. Glycophenotype of chondrocytes in inflammation (Studies 1-3)

Study 1. Lectin binding patterns reflect the phenotypic status of in vitro chondrocyte models.

S. Toegel, V.E. Plattner, **S.Q. Wu**, C. Chiari, F. Gabor, F.M. Unger, M.B. Goldring, S. Nehrer, H. Viernstein, M. Wirth. **Lectin binding patterns reflect the phenotypic status of in vitro chondrocyte models.** *In Vitro Cell. Dev. Biol. Anim.* 45 (2009) 351-360.

Study 2. Phenotype-related differential α-2,6- or α-2,3-sialylation of glycoprotein N-glycans in human chondrocytes.

S. Toegel, M. Pabst, **S.Q. Wu**, J. Grass, M.B. Goldring, C. Chiari, A. Kolb, F. Altmann, H. Viernstein, F.M. Unger. **Phenotype-related differential α-2,6- or α-2,3-sialylation of glycoprotein N-glycans in human chondrocytes.** *Osteoarthr. Cartilage* 18 (2010) 240-248.

Study 3. IL-1ß and TNF-α alter the glycophenotype of primary human chondrocytes in vitro.

M. Pabst, **S.Q. Wu**, J. Grass, A. Kolb, C. Chiari, H. Viernstein, F.M. Unger, F. Altmann, S. Toegel. **IL-1ß and TNF-α alter the glycophenotype of primary human chondrocytes in vitro.** *Carbohyd. Res.* 345 (2010) 1389-1393.

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Lectin binding patterns reflect the phenotypic status of in vitro chondrocyte models

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Abstract In vitro studies using chondrocyte cell cultures have increased our understanding of cartilage physiology and the altered chondrocytic cell phenotype in joint diseases. Beside the use of primary cells isolated from cartilage specimens of donors, immortalized chondrocyte cell lines such as C-28/I2 and T/C-28a2 have facilitated reproducible and standardized experiments. Although carbohydrate structures appear of significance for cartilage function, the contribution of the chondrocyte glycocalyx to matrix assembly and alterations of the chondrocyte phenotype is poorly understood. Therefore, the present study aimed to evaluate the glycoprofile of primary human chondrocytes as well as of C-28/I2 and T/C-28a2 cells in culture. First, the chondrocytic phenotype of primary and immortalized cells was assessed using real-time reverse transcriptase polymerase chain reaction, immunofluorescence, and glycosaminoglycans staining. Then, a panel of lectins was selected to probe for a range of oligosaccharide sequences determining specific products of the *O*-glycosylation and *N*-glycosylation pathways. We found that differences in the molecular phenotype between primary chondrocytes and the immortalized chondrocyte cell models C-28/I2 and T/C-28a2 are reflected in the glycoprofile of the cells. In this regard, the glycocalyx of immortalized chondrocytes was characterized by reduced levels of high-mannose type and sialic acid-capped *N*-glycans as well as increased fucosylated *O*-glycosylation products. In summary, the present report emphasizes the glycophenotype as an integral part of the chondrocyte phenotype and points at a significant role of the glycophenotype in chondrocyte differentiation.

Keywords Chondrocytes · Glycophenotype · Phenotype · Lectins · *O*- and *N*-glycans

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Introduction

Beside water, collagen fibers, and other components such as adhesion proteins and lipids, the extracellular matrix (ECM) of cartilage consists of numerous glycoproteins and proteoglycans which function in the maintenance of healthy cartilage (Knudson and Knudson 2001). The attachment of oligosaccharide chains to asparagine (*N*-linked) or serine/threonine (*O*-linked) residues represents a common post-translational modification of proteins potentially influencing biological processes such as apoptosis, inflammation, cell signaling, protection against proteolytic degradation, and protein folding (Knudson and Knudson 2001; Yang et al. 2007). Furthermore, the protein backbone of major cartilage proteoglycans is often associated with carbohydrate chains such as chondroitin sulfate, heparan sulfate, and keratan sulfate. Another key molecule required for matrix assembly is hyaluronan, a large linear glycosaminoglycan (GAG) composed of a repeating disaccharide (beta-D-glucuronyl-beta-D-*N*-acetylglucosamine) without core protein (Lee and Spicer 2000). It can either be secreted by chondrocytes into the ECM or stay associated with the plasma membrane to form a pericellular coat maintaining cell-substrate adhesion and reducing friction in cartilage. In general, carbohydrate structures are of fundamental importance for the functionality of cartilage since the high density of fixed charges from sulfated glycosaminoglycans (sGAG) is essential to maintain hydrostatic pressure and gives cartilage the resistance to bear loads.

In degenerative joint disorders such as osteoarthritis (OA), the breakdown of the cartilage matrix represents a major characteristic of the disease process. Typically, loosening of the tight collagen network as well as the loss of the interwoven proteoglycan aggregates determine the initiation and progression of cartilage degeneration (Aigner and Stove 2003). Being the only vital reactive elements within the tissue, chondrocytes play a pivotal role in the loss of cartilage function. It has been shown that increased matrix catabolisms as well as alterations of the matrix gene expression profile are important features of chondrocytes under osteoarthritic conditions (Martin et al. 2001). In vitro studies using chondrocyte cell cultures have increased our understanding of cartilage physiology and the altered chondrocytic cell phenotype in disease (Von der Mark et al. 1977; Martin et al. 2001; Stokes et al. 2002). In particular, immortalized chondrocyte cell lines such as the C-28/I2 and T/C-28a2 cell lines have facilitated reproducible and standardized experiments exploring chondrocyte-specific responses to stimuli such as cytokines or chondroprotective agents (Goldring et al. 1994; Oliver et al. 2005; Toegel et al. 2007b, 2008). Despite their high benefit, however, cell lines—as expected for cells in constant log-phase growth—were shown to differ from primary chondrocytes regarding mRNA profiles and other features of the chondrocyte phenotype (Finger et al. 2003).

Although carbohydrate structures appear of significance for cartilage function, the contribution of the chondrocyte glycocalyx to matrix assembly or alterations of the chondrocyte phenotype is poorly understood. Hence, the present study evaluates the glycoprofile of primary human chondrocytes in culture as well as the relation between chondrocytic phenotype and glycophenotype by comparing primary chondrocytes with the immortalized chondrocyte cell models C-28/I2 and T/C-28a2.

Materials and Methods

The fluorescein-labeled lectins from *Triticum vulgare* (wheat germ agglutinin (WGA); molar ratio fluorescein/protein (F/P)=3.1), *Solanum tuberosum* (STL; F/P=2.9), *Dolichos biflorus* (DBA; F/P=2.2), *Arachis hypogaea* (peanut agglutinin (PNA); F/P=5.2), *Ulex europaeus* (*Ulex europaeus* isoagglutinin I (UEA); F/P=2.7) and *Lens culinaris* (LCA; F/P=3.8) were purchased from Vector laboratories (Burlingame, USA) and contained >98% active conjugate and no free fluorescein. The Mx3000P QPCR instrument, the StrataScript First-Strand Synthesis System, and the Brilliant SYBR Green QPCR Master Mix were from Stratagene (La Jolla, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

Isolation and culture of primary human chondrocytes. Human OA cartilage was obtained during total knee replacement surgery performed in patients with osteoarthritis (six patients; age 60–68; blood group 0 RhD positive) with informed consent and in accordance with the terms of the ethics committee of the Medical University of Vienna (EK-Nr.: 081/2005). Only cartilage specimens from femoral condyles and the tibial plateaus showing no visible signs of pathology were used. Primary chondrocytes were isolated following standard protocols using 0.2% collagenase II (Gibco, Carlsbad, CA). Primary chondrocytes were seeded at high density (10^5 cells/cm^2) and grown to confluence in an atmosphere of humidified 5% CO_2/95% air at 37°C using Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (Biochrom AG, Berlin, Germany), 2 µl/ml gentamycin, and 50 µg/ml ascorbate as culture medium (full medium). For all assays, only freshly isolated and seeded cells without subculturing were used.

Culture of immortalized human chondrocytes. The T/C-28a2 chondrocyte cell line was derived from primary cultures isolated from costal cartilage from a 15-yr-old

female by retroviral-mediated transfection with the large T antigen of Simian virus 49 (Goldring et al. 1994). C-28/I2 represents a cell line obtained after clonal expansion of the T/C-28 cells. Both cell lines retain chondrocytic morphology and maintain continuous proliferation in monolayer culture (Finger et al. 2003). C-28/I2 and T/C-28a2 cells were verified to be mycoplasma-free using the Venor GeM Mycoplasma PCR Detection Kit (Minerva Biolabs, Berlin, Germany), seeded at 9.3×10^3 cells/cm^2 and maintained in full medium as described for primary cells.

Lectin binding assay. Immortalized chondrocytes were cultured in 96-well microplates (Iwaki, Tokyo, Japan). Confluent monolayers were washed three times with 50 µl phosphate buffered saline (PBS) using a Columbus washer (SLT, Salzburg, Austria) prior to incubation with 50 µl of a dilution series of the respective fluorescein-labeled lectin (3.125–100 pmol/well in PBS) for 60 min at 4°C. Selected lectins, associated binding specificities, and target structures are presented in Table 1. Monolayers were washed again and the mean cell-bound fluorescence intensity (MFI) was determined using a Spectra Fluor fluorescence microplate reader (SLT) equipped with Biolise software. Fluorescence emission of the lectin-loaded cell monolayers with 50 µl PBS as supernatant was measured at 535 nm after excitation at 485 nm. Each experiment was performed in quadruplicate and control samples consisting of unlabeled cells were included.

Lectin binding patterns of cultured primary human chondrocytes were assessed using 50 µl of fluorescein-labeled lectins (25 pmol/well) following the same procedure.

Flow cytometry. The glycocalyx of primary human chondrocytes was assessed directly after isolation from cartilage specimens and after trypsin treatment (described below) following recently published protocols (Toegel et al. 2007a). Briefly, 3×10^5 cells were incubated with 25 pmol fluorescein-labelled lectins (50 µl) for 5 min at 4°C. After washing, the cells were resuspended in particle-free PBS and analyzed using an Epics XL-MCL analytical flow cytometer (Coulter, FL). The MFI of the relevant cell population representing the binding of the conjugated lectins was used to quantify the presence of specific carbohydrate structures in the glycocalyx of chondrocytic cells. All MFI values were corrected for autofluorescence of cells.

Specificity of the lectin-chondrocyte interaction. Confluent monolayers of C-28/I2 and T/C-28a2 cells cultured in 96-well microplates were washed three times with 50 µl PBS. Then, 100 µl of a dilution series of the respective complementary carbohydrate (Table 2) and 50 µl of a solution containing 25 pmol fluorescein-labeled lectin were added followed by incubation for 60 min at 4°C. After washing, the cell-bound fluorescence intensity was determined by fluorimetry. Each experiment was performed in quadruplicate and reference values accounting for the fluorescence maximum (0% inhibition) were determined using 100 µl PBS instead of the carbohydrate solution. Specificity of lectin binding to primary OA human chondrocytes was controlled using the respective complementary sugars and 25 pmol fluorescein-labeled lectin as described above. Hyaluronan oligosaccharides used to characterize the binding specificity of WGA were generated by a 24-h digestion of 20 mg hyaluronan (MW ~ 1,500 kD) with 439 U of bovine testicular hyaluronidase in 1 ml digestion buffer (0.1 M phosphate puffer, pH 5.3, 0.15 M NaCl) at 37°C. It has been reported that the final reaction product of this digestion consists mainly of tetrasaccharides (Takagaki et al. 1994).

The binding sites of WGA and PNA were further evaluated after enzyme-catalyzed hydrolytic removal of sialic acid residues. Confluent T/C-28a2 monolayers were treated with 100 µl neuraminidase (Type V; Clostridium perfringens; 0.05 and 0.1 U/ml in 0.2 M sodium acetate, pH 5.5) for 60 min at 37°C. Subsequently, the monolayers were extensively washed with PBS prior to labeling with 50 µl fluorescein-labeled WGA or PNA (50 pmol; 60 min; 4°C). After washing, cell-bound fluorescence intensities were determined by fluorimetry. Each experiment was performed in triplicate and control samples consisting of

Table 1. Sources, specificities, and possible target structures at chondrocyte cultures of lectins used

Lectin	Source	Binding specificity	Target structures
WGA	*Triticum vulgaris*/wheat germ	*N*,*N*′,*N*″-triacetylchitotriose > GlcNAc > sialic acid	Hybrid-type *N*-glycans, keratin sulfate, hyaluronan, complex oligoantennary glycans
STL	*Solanum tuberosum*/potato	Poly-*n*-acetyllactosamine	Keratin sulfate
LCA	*Lens cultinaris*/lentil	D-Mannose	High-mannose type *N*-glycan
SNA	*Sambucus nigra*/elder	Sialic acido2-6Gal−	Sialic acid caps of complex-type *N*-glycan
DBA	*Dolichus biflorus*/horse gram	GalNAc−	First step of *O*-glycosylation pathway
PNA	*Arachis hypogaea*/peanut	Galβ1-3GalNAc−	Unmodified *O*-glycan core 1
UEA	*Ulex europaeus*/gorse	Fucα1-2Galβ1-4GlcNAc−	Fucosylated *O*-glycan

Table 2. Competitive inhibition of lectin binding (25 pmol/well) to confluent C-28/I2 or T/C-28a2 cell monolayers was performed by addition of increasing amounts of complementary carbohydrate

Lectin + inhibiting sugar	IC50	
	C-28/I2	T/C-28a2
WGA + GlcNAc	1.75	1.1
WGA + triacetylchitotriose	0.005	0.005
WGA + hyaluronan oligosaccharides	0.15	0.32
STL + GlcNAc	15	30
STL + triacetylchitotriose	0.02	0.01
LCA + mannose	2.5	15.3
SNA + sialyllactose	0.46	0.65
UEA + fucose	0.1	0.08
UEA + hyaluronan oligosaccharides	–	–
PNA + galactosamine	0.18	0.33

IC_{50} values represent the amount of complementary carbohydrate necessary for a 50% inhibition of the lectin binding to confluent cell monolayers. The concentration of hyaluronan oligosaccharides was calculated based on the molecular weight of hyaluronan of 1,500 kD prior to enzymatic digestion. Sialyllactose is commercially available as mixture of α-2,6- and α-2,3-isomers.

unlabelled cells were included. Additional controls were performed by substituting the neuraminidase with PBS.

Moreover, primary chondrocytes were treated with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) for 20 min at 37°C followed by washing with PBS. Then, flow cytometry using 25 pmol fluorescein-labeled WGA, *L. culinaris* agglutinin, and *Sambucus nigra* agglutinin was performed as described above.

Immunofluorescence and alcian-blue staining. Confluent monolayers of C-28/I2 cells, T/C-28a2 cells, and primary human OA chondrocytes on glass cover slides were washed with PBS and fixed with 400 µl methanol (10 min, −20°C) prior to incubation with mouse monoclonal primary antibodies against aggrecan, collagen type I, and collagen type II (Calbiochem, San Diego, CA) for 60 min at 37°C. Then, cells were washed using 1% bovine serum albumin (BSA) in PBS followed by incubation with a fluorescein isothiocyanate-conjugated antimouse-Ig secondary antibody (Dako, Hamburg, Germany) for 30 min at 37°C. Monolayers were rinsed again, embedded in FluorSave antifade medium (Calbiochem), and examined using a Nikon Eclipse 50i fluorescence microscope. Negative controls were prepared using 1% BSA/PBS instead of the primary antibodies.

Alcian blue 8GX was dissolved in 3% acetic acid or 0.1 M HCl to prepare 1% alcian blue solutions of pHs 2.5 and 1, respectively. C-28/I2 cells and primary human OA chondrocytes were seeded in triplicate into six-well plates and cultured as described above. Upon confluence, cells were fixed with 10% neutral buffered formalin for 10 min at room temperature. After washing twice with PBS, cells were incubated with 3% acetic acid for 10 min. The entire amount of GAG was stained with alcian blue solution at pH 2.5, whereas sGAG were stained with alcian blue solution at pH 1 (Kiernan 2006). After 30 min, cell monolayers were rinsed five times with PBS prior to extraction of alcian blue with dimethyl sulfoxide. Absorbance was measured in triplicate at 650 nm using a Tecan Infinite M200 microplate reader (SLT). Finally, absorbance values were normalized to equal DNA contents as evaluated using the CyQuant assay (Piana et al. 2007).

Quantitative. RT-PCR. C-28/I2 cells and primary human OA chondrocytes from three donors were seeded in duplicate into 12-well microplates. Upon confluence, total RNA was extracted using the NucleoSpin RNA II Kit (Macherey-Nagel, Dueren, Germany). Each sample was run on the Agilent 2100 Bioanalyzer Nano LabChip for quality control and quantification of total RNA prior to reverse transcription into cDNA using the StrataScript first-strand synthesis system. All quantitative reverse transcriptase polymerase chain reaction (qPCR) reactions for transcripts from collagen type I (COL1), collagen type II (COL2), and aggrecan (AGC) were performed in 25 µl reaction mixtures pursuing protocols published recently (Toegel et al. 2008). Expression levels were calculated by the equation $2^{-\text{deltaCt}}$ with respect to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) as reference gene.

Statistics. Hypothesis tests among two data sets were made using paired or unpaired Student's *t* test. Statistics cross-comparing several study groups were performed using one-way analysis of variance with post hoc Tukey test. Values of $p<0.05$ were considered significant.

Results

Lectin binding patterns of isolated human chondrocytes. Figure 1 shows the binding level of isolated human chondrocytes for WGA, *S. tuberosum* lectin, *L. culinaris* agglutinin, *S. nigra* agglutinin, and PNA as assessed by flow cytometry ($n=6$ donors). Mean ratios between WGA and *L. culinaris* agglutinin, *S. nigra* agglutinin, *S. tuberosum* lectin, and PNA were 0.7, 1.2, 4.1, and 31.1, respectively. At this, *S. nigra* agglutinin, *S. tuberosum* lectin, and PNA yielded significantly lower binding values than WGA ($p<0.05$, paired *t* test). When primary chondrocytes were treated with trypsin-EDTA, a significant drop in binding of *L. culinaris* agglutinin and *S. nigra* agglutinin

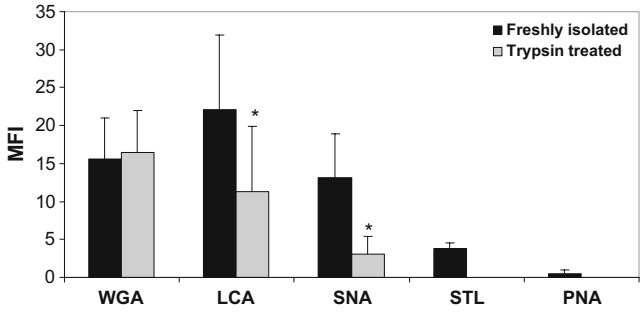


Figure 1. Flow cytometric analyses of lectin binding by primary chondrocytes (mean ± standard deviation, n=6) using fluorescein-labeled WGA, LCA, SNA, STL, and PNA. Chondrocytes isolated by collagenase II digestion of cartilage specimens and chondrocytes treated subsequently with 0.25% trypsin-EDTA (20 min, 37°C) are compared. Significant differences were determined using paired Student's t test and marked with *asterisks* (p<0.05).

was observed (Fig. 1; p<0.05, paired t test). In contrast, WGA binding sites were not affected by trypsinization.

Lectin binding patterns of human chondrocyte monolayers. The glycoprofile of primary chondrocyte monolayers was evaluated using seven fluorescence-labeled plant lectins (Table 1; Fig. 2). The ranking of bound lectins remained stable among the donors (PC1-3), with either WGA or *L. culinaris* agglutinin exhibiting the highest binding followed by *S. nigra* agglutinin, *S. tuberosum* lectin, PNA, *D. biflorus* agglutinin, and *U. europaeus* isoagglutinin I. The mean fluorescence intensities among the three individual donors were 589.3±88.9 for WGA, 393.7±109.9 for *S. tuberosum* lectin, 608.3±254.5 for *L. culinaris* agglutinin, 429.0±167.3 for *S. nigra* agglutinin, 10.7±4.6 for *U. europaeus* isoagglutinin I, 23.7±1.1 for *D. biflorus* agglutinin, and 119.7±54.2 for PNA. Mean ratios between WGA and *L. culinaris* agglutinin, *S. nigra* agglutinin, *S. tuberosum* lectin, PNA, *U. europaeus* isoagglutinin I, and *D. biflorus* agglutinin were 0.9, 1.5, 1.6, 5.7, 64.5, and 24.9, respectively. This indicates a comparable distribution pattern of lectin binding sites between freshly isolated cells and confluent chondrocyte monolayers.

The glycoprofile of immortalized C-28/I2 and T/C-28a2 cells was evaluated in more detail using rising concentrations of fluorescence-labeled plant lectins. Resulting binding curves increased as a function of increasing lectin concentrations and leveled off between 50 and 100 pmol/well lectin indicating saturable binding processes for both cell lines (Figs. 3 and 4). The figures illustrate that the fluorescence intensity of bound WGA was significantly higher than that of any other lectin (12.5–100 pmol WGA/well). At 50 pmol/well, WGA binding was 1.9, 2.5, 2.9, 3.5, 3.9, and 14.3-fold higher than that of *S. tuberosum* lectin, *L. culinaris* agglutinin, PNA, *U. europaeus* isoagglutinin I, *S. nigra* agglutinin, and *D. biflorus* agglutinin in the case of C-28/I2 cells (Fig. 3). In the case of T/C-28a2 cells, ratios of 2.0, 2.8, 7.9, 7.3, 2.8, and 43.6 were obtained. Together with a direct comparison of the respective fluorescence intensities, this indicates that binding levels of PNA, *U. europaeus* isoagglutinin I, and *D. biflorus* agglutinin were significantly lower in T/C-28a2 cells.

In comparison to primary cells, immortalized C-28/I2 chondrocytes bound significantly lower amounts of WGA and *S. tuberosum* lectin (both 1.7-fold), whereas no significant differences were found in case of *D. biflorus* agglutinin and PNA. Of note, remarkable differences were observed for binding levels of *L. culinaris* agglutinin, *S. nigra* agglutinin, and *U. europaeus* isoagglutinin I, which were 3.5-fold lower, 5.7-fold lower, and 11.7-fold higher in case of C-28/I2 chondrocytes, respectively (p<0.05).

Specificity of the lectin–chondrocyte interaction. Competitive assays revealed that lectins were inhibited in their ability to bind to the cell monolayers by increasing amounts of complementary sugars. From these data, IC_{50} values were calculated (Table 2). Due to the low binding of *D. biflorus* agglutinin at the chondrocyte glycocalyx, its binding specificity was only ascertained using excess amounts of *N*-acetylgalactosamine. Using free sialic acid as inhibitory sugar, the preparation of inhibition curves for *S. nigra* agglutinin failed (not shown), whereas sialyllactose as inhibitor markedly inhibited *S. nigra* agglutinin binding to chondrocytes, indicating the necessity of 2,6-linked galactose (Gal) residues for specific *S. nigra* agglutinin binding.

Regarding the specificity of WGA, we found that both *N*-acetylglucosamine (GlcNAc) and triacetylchitotriose induced a high degree of inhibition of the interaction between WGA and the chondrocyte glycocalyx. IC_{50} values resulting from triacetylchitotriose addition, however, were 350 times lower in case of C-28/I2 cells and 220 times lower in

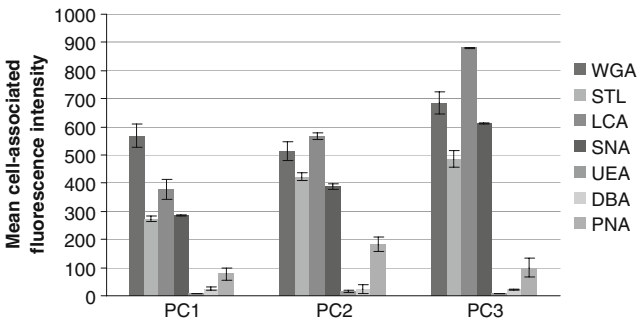


Figure 2. Lectin binding patterns of cultured primary human chondrocytes isolated from three different patients (*PC1–3*). Confluent cell monolayers were incubated with 25 pmol/well fluorescein-labeled WGA, STL, LCA, SNA, UEA, DBA, and PNA for 60 min at 4°C. Results were related to an apparent fluorescein/protein ratio of 1 (mean ± standard deviation, n=4).

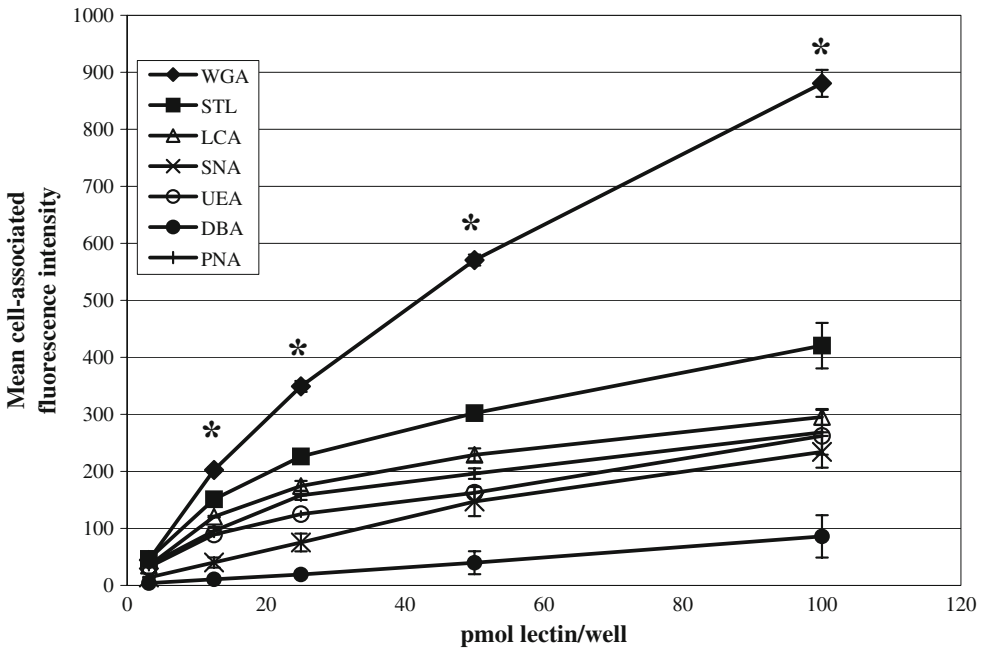


Figure 3. Saturation analysis of lectin binding sites on C-28/I2 chondrocyte monolayers with fluorescein-labeled WGA, STL, LCA, SNA, UEA, DBA, and PNA related to an apparent fluorescein/protein ratio of 1 (mean ± standard deviation, n= 4). Confluent cell monolayers were incubated with 3.125–100 pmol/well fluorescein-labeled lectin in PBS for 60 min at 4°C. The significant differences (p<0.05) between WGA and other lectins at different concentrations are marked with *asterisks*.

case of T/C-28a2 cells as compared to GlcNAc, indicating the preferred binding of WGA to oligosaccharide structures (Table 2). Hence, hyaluronidase-digested hyaluronan consisting mainly of hyaluronan tetrasaccharides was prepared and used as competitive sugar. IC_{50} values observed with these oligosaccharides were 11.6-fold lower in case of C-28/I2 cells and 3.4-fold lower in case of T/C-28a2 cells compared to the monosaccharide GlcNAc. When hyaluronan oligosaccharides were added to *U. europaeus* isoagglutinin I, the low and concentration-independent binding inhibition indicated that hyaluronan oligosaccharides did not exert any unspecific interactions.

For further characterization of WGA and PNA binding specificities, sialic acid residues were enzymatically removed from the chondrocyte glycocalyx. Following neuraminidase treatment, binding of PNA significantly

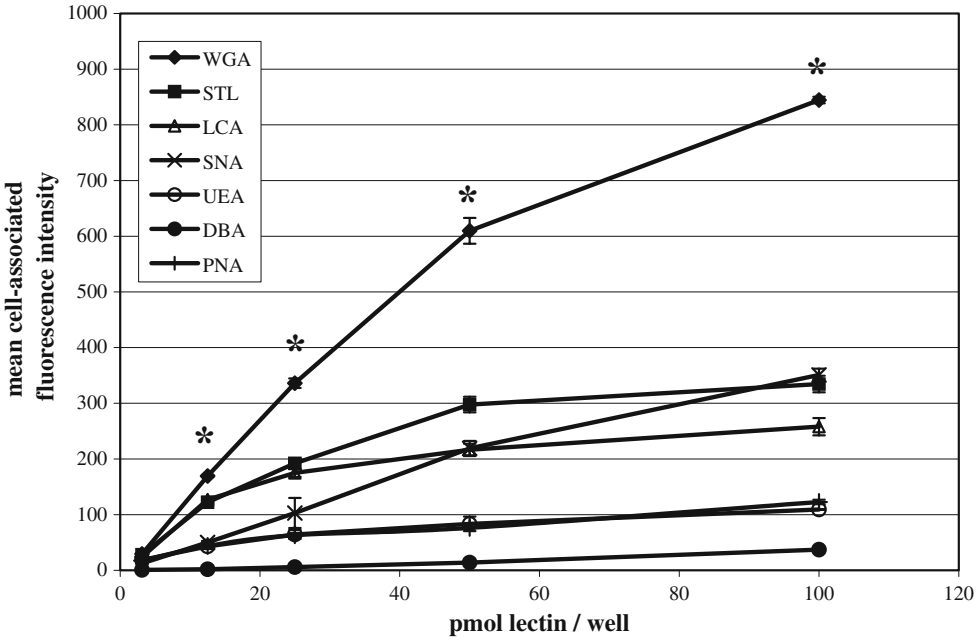


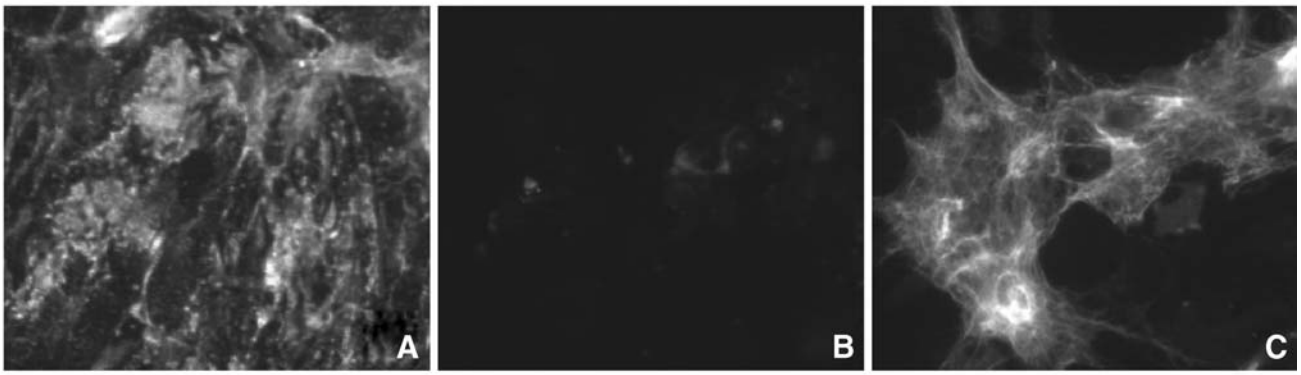
Figure 4. Saturation analysis of lectin binding sites on T/C-28a2 chondrocyte monolayers with fluorescein-labeled WGA, STL, LCA, SNA, UEA, DBA, and PNA related to an apparent fluorescein/protein ratio of 1 (mean ± standard deviation, n=4). Confluent cell monolayers were incubated with 3.125–100 pmol/well fluorescein-labeled lectin in PBS for 60 min at 4°C. The significant differences (p< 0.05) between WGA and other lectins at different concentrations are marked with *asterisks*.

increased (5.3±0.7-fold at 0.05 U/ml neuraminidase) and remained constant upon elevation of neuraminidase concentration. PNA has been shown to specifically bind terminal residues of keratan sulfate which are known to be masked with sialic acid groups (Hoedt-Schmidt et al. 1989). The exposure of these PNA binding sites indicated the successful removal of sialic acids from the chondrocyte glycocalyx and suggested the presence of keratan sulfate. Of note, WGA binding was not significantly affected by neuraminidase treatment (p=0.14 at 0.1 U/ml neuraminidase).

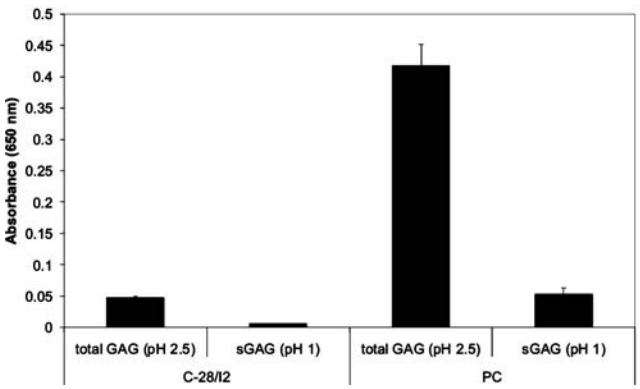
For primary human chondrocytes, competitive assays were performed similarly to the cell lines. Specificity of lectin binding was verified by a continuous decline of fluorescence intensities after addition of increasing amounts of the complementary sugars (data not shown).

Phenotypic characterization of primary and immortalized chondrocytes. In order to associate the evaluated glycophenotype with the general chondrocyte phenotype, the presence of specific chondrocyte markers at C-28/I2 cells and primary OA chondrocytes was assessed using qPCR, immunofluorescence, and GAG analysis. Examination of genes that encode ECM proteins revealed minimal expression of AGC and COL2 in C-28/I2 cells (Fig. 5C), whereas these genes were expressed to a markedly higher extent in primary chondrocytes (13,996 and 32,951-fold, respectively). Regarding COL1, comparable mRNA levels were found in both populations. Of note, COL2 to COL1 ratios, previously defined as a chondrocyte differentiation index (Martin et al. 2001), were markedly higher in the primary cells (53.6 vs. 0.002) and indicated the difference in the molecular phenotype between immortalized and primary chondrocytes. In addition, both cell populations were characterized at the ECM protein level (Fig. 5A). Primary chondrocytes formed a dense ECM including the chondrocyte-specific marker proteins aggrecan and collagen type II, whereas collagen

A)



B)



C)

	AGC	COL2	COL1
C-28/I2	0.00004	0.0009	0.46
PC	0.57	31.1	0.58
Ratio	13996	32951	1.27

Figure 5. (*A*) Matrix formation of primary human chondrocytes was evaluated by immunofluorescence staining with primary antibodies for aggrecan (*A*), collagen type I (*B*), and collagen type II (*C*). (*B*) Quantification of alcian-blue stainable proteoglycans present in cultures of immortalized human C-28/I2 chondrocytes and primary human chondrocytes (*PC*). Total glycosaminoglycans (*GAG*) were stained at pH 2.5, whereas sulfated glycosaminoglycans (*sGAG*) were stained at pH 1. Absorbance values were normalized to equal DNA contents as evaluated using the CyQuant assay. (*C*) Quantitative analysis of the mRNA expression of aggrecan (*AGC*), collagen type II (*COL2*), and collagen type I (*COL1*) in immortalized C-28/I2 cells and in primary chondrocytes (*PC*). Expression levels of the PC represent the mean values of three different patients. mRNA levels of each target gene were normalized to GAPDH and calculated as $2^{-\text{deltaCt}}$. For comparison, target gene ratios between PC and C-28/I2 are presented.

type I was not present in the ECM of primary chondrocytes. Consistent with the lack of synthesis and secretion of ECM by cells in log-phase growth, immortalized C-28/I2 cells did not stain with either of the antibodies, indicating negligible amounts of ECM accumulated during culture (not shown).

To evaluate the presence of GAG structures in the pericellular coat, C-28/I2 cells and primary OA chondrocytes were stained with alcian blue. At pH 2.5, alcian blue stains most polyanions including polycarboxylic acids such as hyaluronan, whereas at pH 1, only those polyanions that owe their acidity to sulfate groups (e.g., keratin sulfate) are stained. The present experiments revealed alcian blue stainable proteoglycans at the surface of C-28/I2 monolayers both at pHs 2.5 and 1 (Fig. 5B). In comparison to C-28/I2 cells, cultured primary OA chondrocytes revealed significantly higher amounts of alcian blue stainable structures at both pHs 2.5 and 1.

Discussion

In this study, we compared the glycophenotype of primary human chondrocytes and the human chondrocyte cell lines C-28/I2 and T/C-28a2 in relation to their chondrocytic molecular characteristics.

The explicit chondrocytic phenotype of the isolated primary chondrocytes was demonstrated by high expression of ECM genes, ECM proteins, as well as GAG and sGAG structures. The basically chondrocytic phenotype of the C-28/I2 and T/C-28a2 cell lines has been recently demonstrated by detecting SOX9 mRNA levels and marker genes of catabolic activity (Finger et al. 2003). Here, we confirmed the expression of the anabolic marker genes COL2 and AGC in C-28/I2 cells using qPCR assays. As expected for cells in constant log-phase growth, however, the expression of both matrix genes as well as COL2/COL1 ratios were less than in primary chondrocytes in our study. Moreover, alcian blue staining and immunofluorescence confirmed the reduced chondrocytic phenotype of immortalized cell lines as compared to primary cultures.

The present study highlights that differences in the molecular phenotype between immortalized and primary human OA chondrocytes are also reflected in the glycoprofile of the cells. A panel of lectins was selected to probe for a range of oligosaccharide sequences determining specific products of the *O*-glycosylation and *N*-glycosylation pathways (Liener et al. 1986; Baldus et al. 1996; Yang et al. 2004, 2007). In the *O*-glycosylation pathways of most cell types, the primary GalNAc− epitope is converted to the core 1 structure Galβ1-3GalNAc−. These carbohydrate structures may provide binding sites for *D. biflorus* agglutinin and PNA, respectively (Yang et al. 2007). Subsequently, the core 1 structure can be further extended in different ways, e.g., by fucosylation reactions forming the Fucα1-2Galβ1-4GlcNAc− residue which can be specifically detected by *U. europaeus* isoagglutinin I (Baldus et al. 1996). The present experiments show that the C-28/I2 and the T/C-28a2 cell lines differ in their expression of *O*-linked carbohydrates. The binding levels of *D. biflorus* agglutinin, PNA, and *U. europaeus* isoagglutinin I were significantly lower for T/C-28a2 cells, indicating minor biosynthesis of GalNAc− epitopes, core 1 structures, and fucosylation products. The altered glycoprofile of T/C-28a2 cells might be related to their higher proliferation rate as compared to C-28/I2 cells (Goldring et al. 1994; Finger et al. 2003). It has been suggested that the matrix-producing chondrocytic phenotype is negatively correlated with proliferative activity (Finger et al. 2003; Toegel et al. 2008), a fact that might also be reflected in *O*-glycan expression patterns. The average biosynthesis of *O*-glycans in primary human chondrocytes did not significantly differ from that of C-28/I2 cells with respect to the amount of expressed GalNAc− epitopes and core 1 structures. It is remarkable, however, that the expression of Fucα1-2Galβ1-4GlcNAc− residues appeared to be markedly reduced in primary OA chondrocytes.

The biosynthesis of *N*-linked oligosaccharide chains is an intricate process involving the transformation of a high-mannose precursor oligosaccharide to the complex-type oligosaccharide structure by trimming and modification (Helenius and Aebi 2001). To account for *N*-linked oligosaccharide chains, the binding of *L. culinaris* agglutinin and *S. nigra* agglutinin was assessed identifying the expression of high-mannose type oligosaccharides and sialylα2-6Gal termini capping some complex-type *N*-glycans, respectively (Akama et al. 2006; Yang et al 2007). No differences in the expression of these *N*-glycan structures were found between C-28/I2 and T/C-28a2 cells. At primary chondrocytes, the observed susceptibility of *S. nigra* agglutinin and *L. culinaris* agglutinin binding sites to trypsin treatment (Fig. 1) supports the presence of glycoproteins with high-mannose and sialyl structures. Of note, primary OA chondrocytes were characterized by significantly elevated amounts of high-mannose *N*-glycans and sialic acid-capped *N*-glycans in comparison to the cell lines. This observation is in line with previous findings (Bernard et al. 1984) showing that chondrocytes produce mature glycoproteins such as fibronectin containing oligosaccharides of the high mannose or hybrid type in contrast to fibroblast fibronectin, which contains only the complex-type structure. Therefore, we consider the possibility that the reduced levels of high-mannose *N*-glycans in C-28/I2 and T/C-28a2 cells indicate the altered chondrocytic phenotype of immortalized cell lines.

Due to the ubiquitous presence of GlcNAc moieties in the glycocalyx of cells, the target structures of WGA are difficult to specify. First, possible binding sites could be

provided by *N*-linked glycans of the hybrid type. It has been reported that the binding of WGA to the hybrid-type *N*-glycans was mediated by the *N*,*N*′-diacetylchitobiose core portion as well as a GlcNAc residue at the reducing end (Yamamoto et al. 1981). Also, an interaction of WGA with keratan sulfate in cultured human chondrocytes appears conceivable, considering the components of this sGAG. In contrast to *S. tuberosum* lectin, however, WGA has been shown to possess lesser binding affinity for keratan sulfate in its highly sulfated form (Toda et al. 1981). Based on the observation that the binding of WGA to the chondrocyte glycocalyx was higher than that of *S. tuberosum* lectin throughout the study, keratan sulfate might not contribute markedly to the interaction between WGA and cultured chondrocytes. Similarly, the binding of WGA to sialic acid residues in the glycocalyx of cultured chondrocytes appears negligible in view of stable binding values of WGA following enzymatic removal of sialic acids. It has to be underlined that the binding level of WGA was among the highest of all tested lectins with both immortalized and primary chondrocytes. Moreover, the low interindividual variability of WGA binding among different patients might indicate that the WGA binding sites at the glycocalyx are part of the basic repertoire of chondrocytes. Recently, it has been depicted that chondrocytes are surrounded by a several micrometer-thick hyaluronan coat with gel-like properties (Cohen et al. 2003). In our study, alcian blue assays at pH 2.5 suggested high levels of polycarboxylic acids such as hyaluronan covering cultured human chondrocytes. Given the components of hyaluronan, we hypothesize that hyaluronan might represent a target structure for WGA in the pericellular matrix of cultured chondrocytes. The resistance of WGA binding sites against trypsin treatment (Fig. 1) further supports their localization at a nonprotein target such as hyaluronan. In spite of a previous study reporting that WGA does not interact with hyaluronan (Carlsson et al. 1976), our assumption was corroborated by a concentration-dependent inhibition of WGA binding using hyaluronan oligosaccharides. IC_{50} values observed with hyaluronan oligosaccharides were several times higher than those observed with the monosaccharide GlcNAc. Thus, these data suggest the partial binding of WGA to oligosaccharide structures of the chondrocyte hyaluronan coat.

In summary, the present report emphasizes the glycophenotype as an integral part of the chondrocyte phenotype in vitro. In this regard, lectin binding patterns of primary and immortalized chondrocytes indicate that alterations of the chondrocytic molecular phenotype are reflected in the glycophenotype of the cells. In addition to the decreased production of GAGs, the pattern of *O*-linked and *N*-linked oligosaccharides appears shifted toward reduced levels of high-mannose type and sialic acid-capped *N*-glycans as well as increased fucosylated *O*-glycosylation products in immortalized chondrocyte cell lines. A limitation of the current study is that conclusions regarding the glycophenotype of healthy or OA chondrocytes in vivo cannot be definitely drawn from any in vitro model. Based on the presented results, however, future studies might aim to specify the glycoprofile of chondrocytes that is related to OA. This could further clarify the relevance of the glycophenotype for chondrocyte function or its significance, if any, as a marker for the development of new chondroprotective agents.

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Osteoarthritis and Cartilage

Phenotype-related differential α-2,6- or α-2,3-sialylation of glycoprotein N-glycans in human chondrocytes

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Summary

Objective: Sialic acids frequently occur at the terminal positions of glycoprotein N-glycans present at chondrocyte surfaces or in the cartilage matrix. Sialic acids are transferred to glycoproteins in either α-2,3 or α-2,6 linkage by specific sialyltransferases (SiaTs) and can potentially affect cell functions and cell–matrix interactions. The present study aimed to assess the relationship between the expression of the human chondrocyte phenotype and the sialylation of chondrocyte glycoprotein N-glycans.

Methods: The transcription of 5 SiaT was quantified using real-time Reverse transcription polymerase chain reaction (RT-PCR) assays. N-glycan analysis was performed using LC–ESI-MS. Primary human chondrocytes were cultured in monolayer or alginate beads and compared to the chondrocyte cell lines C-28/I2 and SW1353. In addition, effects of interleukin-1β (IL-1β) or tumour necrosis factor-α (TNF-α) on primary cells were assessed.

Results: Primary human chondrocytes predominantly express α-2,6-specific SiaTs and accordingly, α-2,6-linked sialic acid residues in glycoprotein N-glycans. In contrast, the preponderance of α-2,3-linked sialyl residues and, correspondingly, reduced levels of α-2,6-specific SiaTs are associated with the altered chondrocyte phenotype of C-28/I2 and SW1353 cells. Importantly, a considerable shift towards α-2,3-linked sialic acids and α-2,3-specific SiaT mRNA levels occurred in primary chondrocytes treated with IL-1β or tumour necrosis factor-alpha (TNF-α).

Conclusion: The expression of the differentiated chondrocyte phenotype is linked to the ratio of α-2,6- to α-2,3-linked sialic acids in chondrocyte glycoprotein N-glycans. A shift towards altered sialylation might contribute to impaired cell–matrix interactions in disease conditions.

Key words: Chondrocytes, Extracellular matrix, Cell–matrix interaction, Glycoproteins, Sialic acids, Sialyltransferases, Differentiation, Phenotype.

Introduction

The extracellular matrix (ECM) of cartilage consists of numerous glycosylated proteins, which contribute to the maintenance of its specific functions[1]. Beside structural proteoglycans such as aggrecan transcript (mRNA), and adhesive proteins such as chondroadherin, glycosylated cell surface receptors, including CD44 and integrins, play critical roles in the mediation of chondrocyte–matrix interactions[1–3]. Protein glycosylation, i.e., the attachment of oligosaccharide chains to asparagine or serine/threonine residues, represents a common co-translational and post-translational modification of proteins, conferring protection against proteolytic degradation or controlling protein folding. It is known that carbohydrate chains vary according to tissue and cell type and are subject to change during development and oncogenesis[4–6]. In addition, various factors such as cytokines or differentiation factors can regulate cellular glycosylation and the expression of specific glycosyltransferases[7–11]. Thus, changes in the cellular glycophenotype can potentially affect cell functions such as cell adhesion, cell surface receptor activity, and the onset of apoptosis[12].

Sialic acids (Neu5Ac in humans) are negatively charged sugars typically found at the terminal positions of N- and O-linked oligosaccharides attached to cell surfaces or secreted glycoproteins. As a result of their non-reducing terminal position, sialic acids are involved in highly specific recognition phenomena, as well as in invasive properties of cancer cells[13–16]. The transfer of sialic acids to penultimate galactose or *N*-acetylgalactosamine groups of glycoproteins is mediated by sialyltransferases (SiaTs). The diverse enzymes of the SiaT family share the cytidine monophosphate-sialic acid as donor substrate, but are

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distinguished by the oligosaccharide acceptor on which they act and by the linkage type they generate (e.g., α-2,3 or α-2,6 linkage in case of N-linked glycans in glycoproteins)[16]. The expression patterns of SiaTs show remarkable tissue specificity and appear to be regulated at the transcriptional level. In particular, the altered expression of α-2,3- or α-2,6-SiaTs and subsequent effects on cell surface sialylation have been demonstrated to influence osteoclastogenesis, endocytosis in dendritic cells, and adhesion properties of human colon cancer cells[17–19].

Regarding inflammatory and degenerative joint diseases, recent studies have suggested a relationship between altered glycosylation and disease conditions. It is known that altered N-glycans of serum immunoglobulin G molecules contribute to the pathophysiology of rheumatoid arthritis[20]. In addition, initial evidence for altered N-glycosylation of cartilage tissue has been presented using an animal model of osteoarthritis[21]. Despite the significance of glycoproteins for ECM assembly in cartilage tissue, however, little is known about the regulation of the chondrocyte glycophenotype. Recently, we found that differences in the molecular phenotype between primary human chondrocytes and chondrocyte cell lines are reflected by specific cellular lectin-binding patterns[22,23]. In a further study, Yang and coworkers have shown that cytokine treatment of human and bovine chondrocytes results in specific glycosylation changes related to apoptosis and altered cell proliferation[12].

Owing to the potential implications of the chondrocyte glycophenotype in the pathophysiology of joint diseases such as osteoarthritis, we set out to examine the sialylation processes in human chondrocytes using a combination of gene expression and N-glycan structural analyses. We compared the sialylation pattern of primary human chondrocytes with those of immortalized chondrocytes C-28/I2 and chondrosarcoma cells SW1353, and investigated the effects of interleukin-1β (IL-1β) and tumour necrosis factor-alpha (TNF-α) on primary chondrocyte (PC) sialylation. Our findings provide new information on N-glycan sialylation in human chondrocytes and demonstrate that the status of the chondrocyte phenotype is critically linked to the ratio of α-2,6- to α-2,3-linked sialic acids of chondrocyte glycoprotein N-glycans.

Methods

HUMAN CHONDROCYTE CULTURES

Human articular cartilage was obtained during total knee replacement surgeries in patients with osteoarthritis with informed consent and in accordance with the terms of the ethics committee of the Medical University Vienna (EK-Nr.: 081/2005). PCs were isolated according to protocols published previously[22], seeded as high density monolayers (MLs) (10^5 cells/cm^2), and grown in an atmosphere of humidified 5% CO_2/95% air at 37°C using Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum (Biochrom AG, Germany), 2 μl/ml gentamycin, and 50 μg/ml ascorbate as culture medium (complete medium). Only freshly isolated cells were plated for all assays throughout the study. To induce inflammatory conditions, PCs were treated with 10 ng/ml IL-1β or 40 ng/ml TNF-α for the time periods indicated. Three-dimensional (3D) chondrocyte cultures were established in alginate beads according to standard procedures and cultured for 3 weeks as described[24]. Immortalized chondrocytes C-28/I2, and chondrosarcoma cells SW1353, were seeded at 9.3×10^3 cells/cm^2 and maintained in complete medium.

QUANTITATIVE REAL-TIME PCR

Chondrocytes were grown in duplicate wells in 12-well tissue culture plates (Iwaki, Japan) to 90% confluence prior to treatment with cytokines for 24 h. Total RNA was extracted using the NucleoSpin RNA II Kit (Macherey-Nagel, Germany). Each sample was run on the Agilent 2100 Bioanalyzer Nano LabChip for quality control and quantification of total RNA prior to reverse transcription into cDNA using the high capacity cDNA reverse transcription kit (Applied Biosystems, Austria).

SYBR-green based qPCR assays for the sialyltransferase transcripts ST6Gal1 (NM_003032), ST6Gal2 (NM_032528), ST3Gal3 (NM_174963), ST3Gal4 (NM_006278), and ST3Gal6 (NM_006100) were established. Amplification efficiencies of all primers were between 90 and 105% as evaluated using dilution series of cDNA prepared from chondrocyte mRNA. Primers and results of melting curve analyses are shown in Supplementary Table 1 and Supplementary Fig. 1, respectively. qPCR reactions for transcripts from collagen type I (COL1), collagen type II (COL2) and aggrecan AGC were performed according to protocols published recently[25]. mRNA expression levels were calculated as relative copy numbers considering actual amplification efficiencies and with respect to that of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) set at 1000. The regulation of a target gene by cytokines was calculated as quantities relative to the untreated control group using the MxPro real-time QPCR software, considering both amplification efficiencies and normalization to GAPDH.

PROTEIN ISOLATION AND N-GLYCAN PREPARATION

Chondrocytes were seeded in 25 cm^2 flasks (Greiner bio-one, Austria) and allowed to settle for 48 h. Then, the culture medium was changed and cells were maintained in the presence of 10 ng/ml IL-1β or 40 ng/ml TNF-α for 5 days. Cells were carefully washed five times with PBS buffer and scraped in the presence of 200 μl lysis buffer (containing protease inhibitor). After centrifugation for 5 min at 12,000 rpm, the pellet was washed once with PBS buffer and centrifuged again. The combined supernatants were mixed with three times the volume of acetone (−20°C for 30 min) to precipitate proteins. Further protein purification and glycan isolation was performed using the 'in-gel release method' as described by Rendic *et al.*[26] Briefly, the precipitate was dissolved in SDS-PAGE sample buffer and subjected to SDS-PAGE, but only to the point when the unseparated protein zone had just entered the separation gel. Free N-glycans were isolated from this band by trypsin and PNGase F digestion. The released asparagine-linked glycans were reduced with 50 μl of 1% $NaBH_4$ at room temperature for 4 h. To remove excess salt, the sample was further purified using a 10 mg HyperSep Hypercarb SPE cartridge (Thermo Scientific, USA), according to previous protocols[27].

QUANTIFICATION OF OLIGOSACCHARIDES USING LC–ESI-MS

Recent work on fibrin N-glycans and immunoglobulin glycosylation revealed the ability of porous graphitic carbon (PGC) phases to separate isomeric glycans with a subsequent sensitive detection of both neutral and sialylated glycans by using ESI-MS[28,29]. The recently established retention time library of mammalian, diantennary glycans varying in the linkage of sialic acid was applied for the structural assignment of the peaks. LC–ESI-MS, using graphitized carbon as the separation phase (PGC–ESI-MS), outperforms other glycan analysis methods including MALDI-TOF or NMR spectroscopy in terms of minimal sample consumption, minimal sample preparation, and the reliable qualitative and quantitative recording of neutral, mono- and di-sialylated structures with concomitant identification of isomeric structures[26–28].

Analysis of borohydride-reduced oligosaccharides (from an aliquot equivalent to 2.5×10^5 cells) by positive-ion LC–ESI-MS was performed with a 100 × 0.32 mm PGC column (Thermo) and a flow rate of 5 μl/min maintained with a Dionex Ultimate 3000 cap flow system, as described recently[28,29]. Mass analysis was done with a Waters Q-TOF Ultima Global mass spectrometer with standard ESI-source and MassLynx V4.0 SP4 software for evaluation and quantification of obtained peak areas. Individual analyses were averaged, with a mean deviation for the relative glycan quantities of approximately 0.05 units.

CELL MORPHOLOGY

PCs were seeded on glass cover slips and cultured for 48 h in complete medium. Then, cells were exposed to 10 ng/ml IL-1β or 40 ng/ml TNF-α for up to 5 days. Using conventional light microscopy, cell morphological alterations were observed with regard to the formation of a spindle-shaped cell configuration, which is associated with a dedifferentiated fibroblast-like phenotype.

CELL PROLIFERATION

PCs of two donors were seeded into 96-well microplates (Iwaki, Japan) and cultured for 48 h. Then, cells were treated in quadruplicate with 10 ng/ml IL-1β or 40 ng/ml TNF-α for 24 h. The total number of cells was determined using the CyQuant assay kit (Molecular Probes, USA) pursuing a modified protocol[30]. Moreover, the rate of proliferation of cells was assessed by incorporation of

2-bromodeoxyuridine into the DNA of dividing cells using the colorimetric BrdU assay (Roche, Germany), according to the manufacturer's instructions.

SULFATED GLYCOSAMINOGLYCAN (sGAG) ASSAY

PCs were seeded in triplicate into six-well tissue culture plates (Iwaki, Japan) and cultured for 48 h prior to treatment with 40 ng/ml TNF-α for 5 days. An improved 1,9-dimethyl-methylene blue (DMMB) colorimetric assay was performed according to Barbosa *et al.*[31] to assess both cell-associated sGAG and sGAG released into the culture medium. Absorbance of the samples was measured at 656 nm and related to equivalent cell numbers, which were determined using the CyQuant assay kit. The experiment was repeated twice using cells from different donors.

STATISTICS

Data were exported to the GraphPad Prism statistics software package (GraphPad Prism Software, USA). The Gaussian distribution of the data was verified using the Kolmogorov–Smirnov test. Statistics were performed using one-way analysis of variance (ANOVA) with post-hoc Tukey tests, cross-comparing all study groups (95% confidence interval) consisting of independent mean values. *P*-values <0.05 were considered significant.

Results

EXPRESSION PROFILES OF SiaT GENES IN HUMAN CHONDROCYTE MODELS

The mRNA levels of five SiaTs were determined with respect to GAPDH in human chondrocytes using qPCR. Table I shows the SiaT expression levels in PCs cultured as MLs or in alginate beads (3D), as well as in SW1353 and C-28/I2 cell lines. In general, SiaTs were expressed at low levels in all cell models. In MLs of PCs ($n=10$ donors), ST6Gal1 showed the highest abundance of all evaluated SiaTs. In comparison to ST6Gal1, ST6Gal2 was expressed at more than 400-fold lower levels. In addition, the mRNA levels of the α-2,3-SiaTs (ST3Gal3, ST3Gal4, and ST3Gal6) were all low compared to ST6Gal1. Among the α-2,3-SiaTs, ST3Gal4 was the gene with the highest expression level. All in all, the SiaT expression profile of PCs predicts a sialylation pattern in which α-2,6-linked sialylation predominates over α-2,3-sialylation. When cultured in alginate beads, PCs expressed comparable levels of α-2,6-SiaTs and slightly reduced levels of ST3Gal3 and ST3Gal4 (1.6-fold and 1.9-fold, respectively). When comparing the chondrosarcoma cell line SW1353 with PCs, we observed remarkably diminished (2.5-fold) ST6Gal1 expression in SW1353 cells, whereas mRNA levels of ST3Gal4 were slightly increased. The SiaT profile of C-28/I2 chondrocytes was characterized by almost completely suppressed ST6Gal1 mRNA levels, whereas the α-2,3-SiaT profile only showed minor alterations in comparison to PCs. Taken together, primary human chondrocytes are characterized by a predominant expression of α-2,6-SiaT, predicting the preponderant presence of α-2,6-linked terminal sialic acid residues on PC glycoprotein N-glycans. In contrast, immortalized and chondrosarcoma cell models feature a SiaT expression profile shifted towards α-2,3-SiaTs.

Table I

mRNA expression of N-glycan related sialyltransferases (SiaTs). mRNA levels were quantified using qPCR and expressed as relative copy numbers with respect to GAPDH set at 1000. PCs cultured in ML or alginate beads (3D) were compared to SW1353 chondrosarcoma cells and immortalized C-28/I2 chondrocytes. cDNA of PCs from 10 donors was pooled and analyzed in triplicate. Mean values of the samples are presented

Cells	ST6Gal1	ST6Gal2	ST3Gal3	ST3Gal4	ST3Gal6
PC (ML)	17.5	<0.1	3.8	4.7	1.1
PC (3D)	15.8	<0.1	2.3	2.5	1.1
SW1353	6.8	<0.1	1.3	5.8	0.4
C-28/I2	<0.01	0.2	2.2	5.3	1.1

SIALYLATION OF CHONDROCYTE GLYCOPROTEIN N-GLYCANS IN HUMAN CHONDROCYTE MODELS

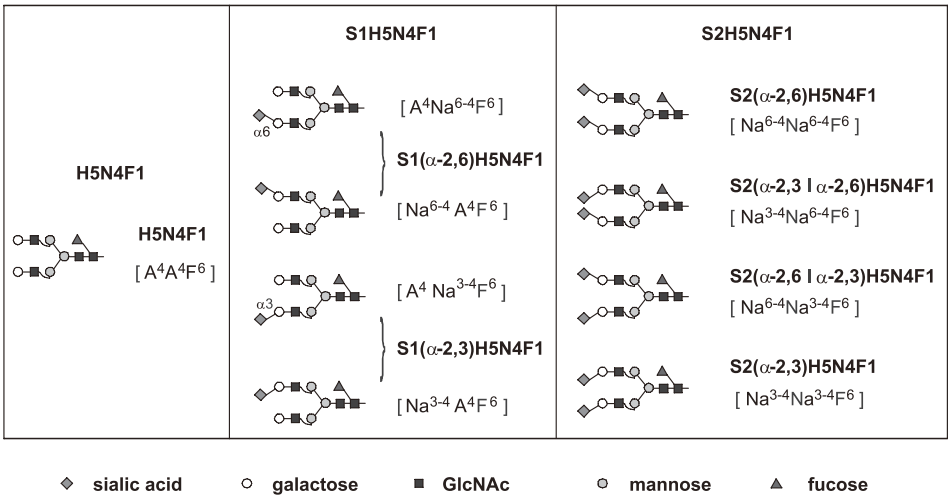
Sialylation patterns and linkage types of chondrocyte glycoprotein N-glycans were determined using LC–ESI-MS. Figure 1(A) shows the structure of the diantennary H5N4F glycan and its possible sialylated isomers analyzed in this study. Both the abbreviated composition and the full structural description according to the proglycan system are presented[29,32]. These glycans were selected for quantification since they represent a common modification of cellular proteins and are among the most abundant glycan structures in the human chondrocyte glycome (own unpublished result). Moreover, they are practically absent in fetal calf serum-containing cell culture medium minimizing the risk of medium-dependent glycan structure contamination.

Using LC–ESI-MS online analysis *via* PGC column, we quantified free H5N4F1 structures as well as H5N4F1 glycans carrying one or two sialic acid residues in α-2,6- or/and α-2,3-linkage and compared them across various PC models. As shown in Fig. 1(B), the proportional distribution of the different H5N4F1 glycans was strongly dependent on cell type and culture conditions. In PCs cultured as MLs, the majority of diantennary glycans carried one α-2,6-linked sialic acid (45.4%), whereas only 11.3% were decorated by one α-2,3-linked sialic acid residue. Among the S2H5N4F1 glycans, most structures (21.2%) carried both one α-2,6-linked and one α-2,3-linked sialic acid. In comparison to this mixed type, S2(α-2,6)H5N4F1 and S2(α-2,3)H5N4F1 structures were generated at 3-fold lower levels. In 3D-culture systems, PCs expressed a sialylation pattern characterized by increased levels of H5N4F1 glycans carrying one or two α-2,6-linked sialic acids. Concomitantly, those structures decorated with one or two α-2,3-linked sialic acids were produced at reduced levels. Of note, the sialylation pattern of N-glycans of SW1353 chondrosarcoma cells showed an inverse trend towards significantly reduced S1(α-2,6)H5N4F1 and S2(α-2,6)H5N4F1 structures and increased S1(α-2,3)H5N4F1 and S2(α-2,3)H5N4F1 glycans. This trend was even intensified in the C-28/I2 cell line, where α-2,6-linked sialylation was practically absent, while the majority of H5N4F1 glycans (87.8%) carried terminal sialic acids in α-2,3-linkage. In summary, sialylation patterns of chondrocyte N-glycans showed alteration towards increased α-2,6-/α-2,3-sialylation ratios in 3D-culture and decreased α-2,6-/α-2,3-sialylation ratios in SW1353 and C-28/I2 cells.

INFLAMMATORY CYTOKINES INDUCE CHONDROCYTE DEDIFFERENTIATION AND A SHIFT OF α-2,6-/α-2,3-SIALYLATION

Figure 2 confirms that PCs undergo dedifferentiation under treatment with pro-inflammatory cytokines IL-1β and TNF-α[33]. Morphological changes of chondrocytes towards a spindle-shaped fibroblast-like phenotype were observed after exposure to cytokines [Fig. 2(A)]. Moreover, both IL-1β and TNF-α reduced chondrocyte proliferation [Fig. 2(B)] and expression of chondrocyte marker genes AGC and COL2 [Fig. 2(D)], resulting in decreased COL2/COL1 ratios[34]. In addition, TNF-α decreased the production of both cell-associated and secreted sGAG, further supporting cellular dedifferentiation [Fig. 2(C)].

A



B

Cells	H5N4F1	S1(α-2,6) H5N4F1	S1(α-2,3) H5N4F1	S2(α-2,6) H5N4F1	S2(α-2,6/α-2,3) H5N4F1	S2(α-2,3) H5N4F1
PC (ML)	7.9	45.4	11.3	7.0	21.2	7.2
PC (3D)	10.3	50.8	7.7	10.8	17.3	3.1
SW1353	11.6	28.6	18.3	4.8	23.4	13.3
C-28/I2	11.7	0.4	24.0	0.0	0.0	63.8

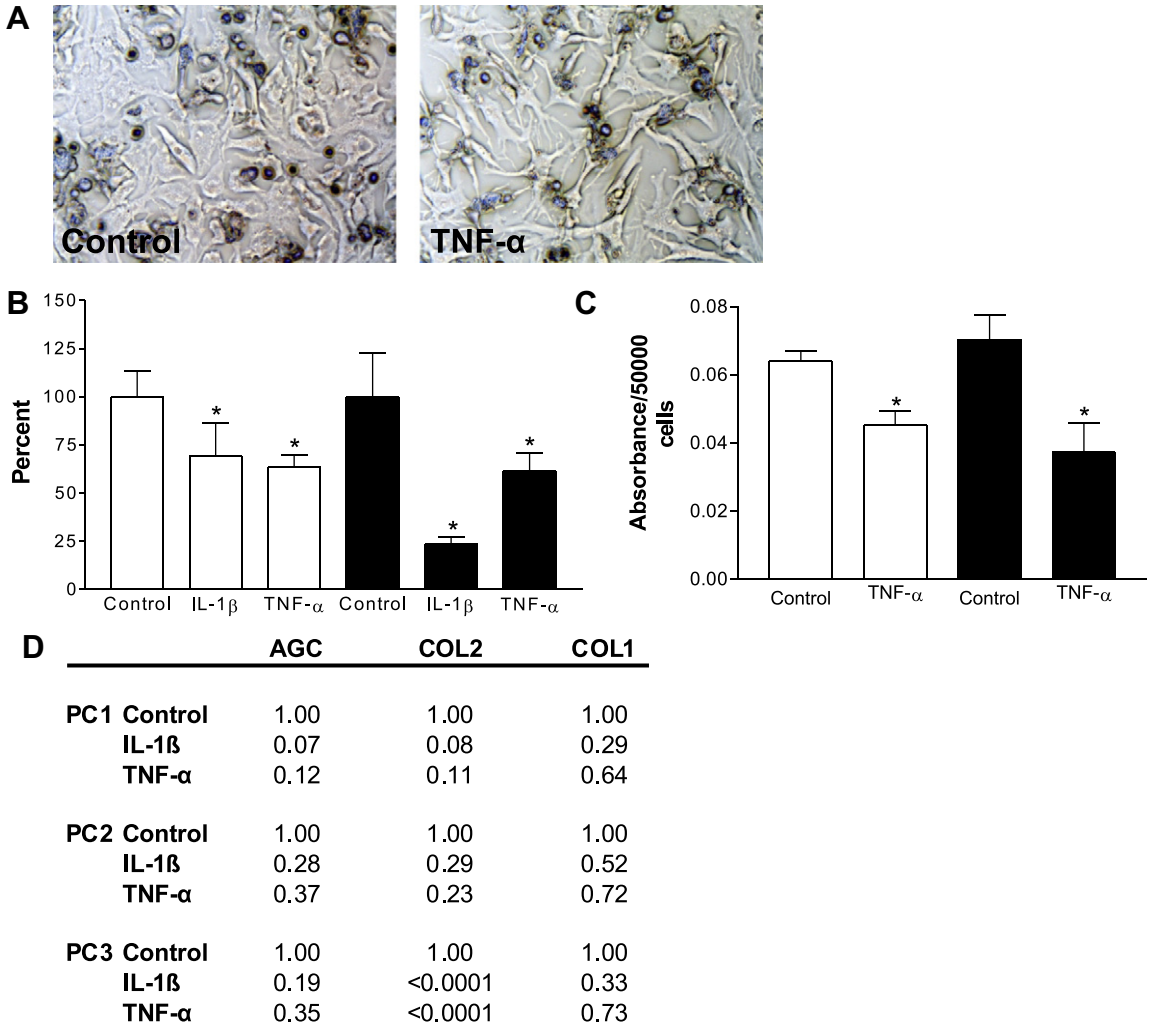
Fig. 1. Analysis of sialylated and free H5N4F1 glycans in human chondrocytes. (A) Scheme showing all the diantennary N-glycans considered in this study with the abbreviated composition and the full structural description according to the proglycan system[29,32]. S stands for sialic acid, H for hexose, *N* for N-acetylglucosamine (GlcNAc) and F for fucose. (B) Proportional distribution of free and sialylated H5N4F1 glycans in human chondrocytes. PCs cultured in ML or alginate beads (3D) are compared to SW1353 chondrosarcoma cells and immortalized C-28/I2 chondrocytes. PCs from three donors were analyzed in triplicate and respective mean values are presented. The standard deviations were between 1 and 10% of the mean values for all measurements.

To test whether the linkage type of chondrocyte N-glycans was altered under inflammatory conditions, SiaT mRNA expression and N-glycan sialylation were determined in PCs ($n = 6$ donors) after treatment with pro-inflammatory cytokines. Figure 3(A) and (B) shows the log-fold change of SiaT mRNA levels following IL-1β and TNF-α treatment, respectively. ST6Gal1 mRNA levels were markedly reduced under the influence of the cytokines (4.7-fold in case of IL-1β and 3.3-fold in case of TNF-α), whereas the expression of ST3Gal4 was enhanced under the same conditions (2.8-fold and 3.2-fold, respectively). ST6Gal2, ST3Gal3, and ST3Gal6 were also subject to reduction in the presence of IL-1β or TNF-α. Given the different steady-state expression levels of the single SiaTs (Table I), the contribution of each gene to the entire SiaT pool is displayed graphically in Fig. 3C. It is obvious that the α-2,3-SiaTs predominate after exposure of chondrocytes to IL-1β or TNF-α. In general, diminished ST6Gal1 and increased ST3Gal4 levels appear to be responsible for this shift towards reduced α-2,6-/α-2,3-SiaT mRNA ratios under inflammatory conditions.

Analysis of N-glycan sialylation using LC–ESI-MS confirmed the predicted differential expression of α-2,6- and α-2,3-sialic acid residues after cytokine treatment. Interestingly, the described shift in SiaT mRNA levels was not immediately followed by a preponderance of α-2,3-linked sialyl residues in the total of glycoprotein glycans, since no significant alteration was detected by LC–ESI-MS after 24 h of cytokine treatment (data not shown). However, following a 5-day exposure of the cells to TNF-α or IL-1β, a corresponding shift of α-2,6- towards α-2,3-linked sialylation was observed. Figure 4 presents detailed information on the alteration of sialylated H5N4F1 structures in chondrocytes from three donors, indicating that both S2(α-2,6)H5N4F1 and S1(α-2,6)H5N4F1 were significantly decreased, whereas those structures containing α-2,3-linked sialic acids (S1(α-2,3)H5N4F1, S2(α-2,6/α-2,3)H5N4F1, S2(α-2,3)H5N4F1) were enhanced by both cytokines. The most striking differences were observed in shift of S1(α-2,6)H5N4F1 and S2(α-2,3)H5N4F1 glycans under TNF-α exposure, resulting in a 0.6-fold decrease and 2.5-fold increase, respectively. A representative LC–ESI-MS chromatogram of S2H5N4F1 glycans under the influence of TNF-α or IL-1β is provided as Supplementary Fig. 2 and illustrates the underlying analytic principle, used to compare untreated PCs with cytokine-treated cells. Taken together, alterations of the chondrocyte phenotype induced by TNF-α or IL-1β were accompanied by decreasing α-2,6-/α-2,3-SiaT mRNA ratios and corresponding shifts of sialylated N-glycans towards α-2,3-sialylated H5N4F1 structures.

Discussion

Sialylation of cell surface glycoproteins plays a key role in many biological processes, including cell–matrix interactions. Although disturbed cell–matrix interactions are of fundamental importance in the onset of degenerative joint



D

		AGC	COL2	COL1
PC1	Control	1.00	1.00	1.00
	IL-1ß	0.07	0.08	0.29
	TNF-α	0.12	0.11	0.64
PC2	Control	1.00	1.00	1.00
	IL-1ß	0.28	0.29	0.52
	TNF-α	0.37	0.23	0.72
PC3	Control	1.00	1.00	1.00
	IL-1ß	0.19	<0.0001	0.33
	TNF-α	0.35	<0.0001	0.73

Fig. 2. Phenotypic changes of primary human chondrocytes under exposure to inflammatory cytokines. (A) Morphological change of chondrocytes under exposure to TNF-α for 24 h. (B) Altered cell number (white bars) and proliferation rate (black bars) of chondrocytes treated with IL-1β and TNF-α. Data are presented as mean values ± SD. (C) Reduced sGAG content in chondrocytes treated with TNF-α measured in cells (white bars) and culture medium (black bars). Data are presented as mean values ± SD. (D) Fold-changes of AGC, COL2 and COL1 transcription in human chondrocytes isolated from three donors (PC1, PC2, PC3) and exposed to IL-1β and TNF-α with respect to untreated cells (control).

diseases including osteoarthritis[1,35,36], little is known about the sialylation patterns in human chondrocytes and their involvement, if any, in cartilage breakdown processes. This gap in our knowledge may result, at least in part, from the methodological challenges due to low SiaT transcription levels and linkage-type specific glycoprotein N-glycan analysis in human chondrocytes. Up to the present, evidence for the sialylation of chondrocytes has been generated using lectin histochemistry[37], lectin-binding studies[22], enzyme-linked lectin assays[12,38], and enzyme activity assays[12]. In this study, we have employed an advanced approach to characterize the chondrocyte glycophenotype. Linking together qPCR and LC–ESI-MS techniques, we correlated quantitatively the gene expression of α-2,6- and α-2,3-specific SiaTs with the presence of α-2,6- and α-2,3-linked sialyl groups in chondrocyte glycoprotein N-glycans.

We here present two lines of evidence indicating that the chondrocyte phenotype is linked to specific sialylation patterns of chondrocyte glycoprotein N-glycans. Firstly, the sialylation of various *in vitro* chondrocyte models with well known phenotypic differences was investigated. Secondly, the sialylation of primary human chondrocytes was evaluated after treatment with IL-1β or TNF-α, two cytokines with pathophysiological significance in inflammatory joint diseases.

Despite the value of chondrocytic cell lines, such as the C-28/I2 and SW1353 cell lines used here, that are used frequently as surrogates for primary human chondrocytes *in vitro*, they differ markedly from PCs in their molecular phenotype[22,39,40]. For instance, the reduced expression of chondrocyte marker genes such as AGC or COL2, as well as altered reactivity to IL-1β, may be related to their high state of proliferation and continuous synthesis of proteins associated with log-phase growth. The present study clearly demonstrates that the presence of α-2,6-linked sialyl residues constitutes a typical feature of the differentiated phenotype of primary human chondrocyte cultures, whereas the abundance of α-2,3-linked sialyl residues is associated

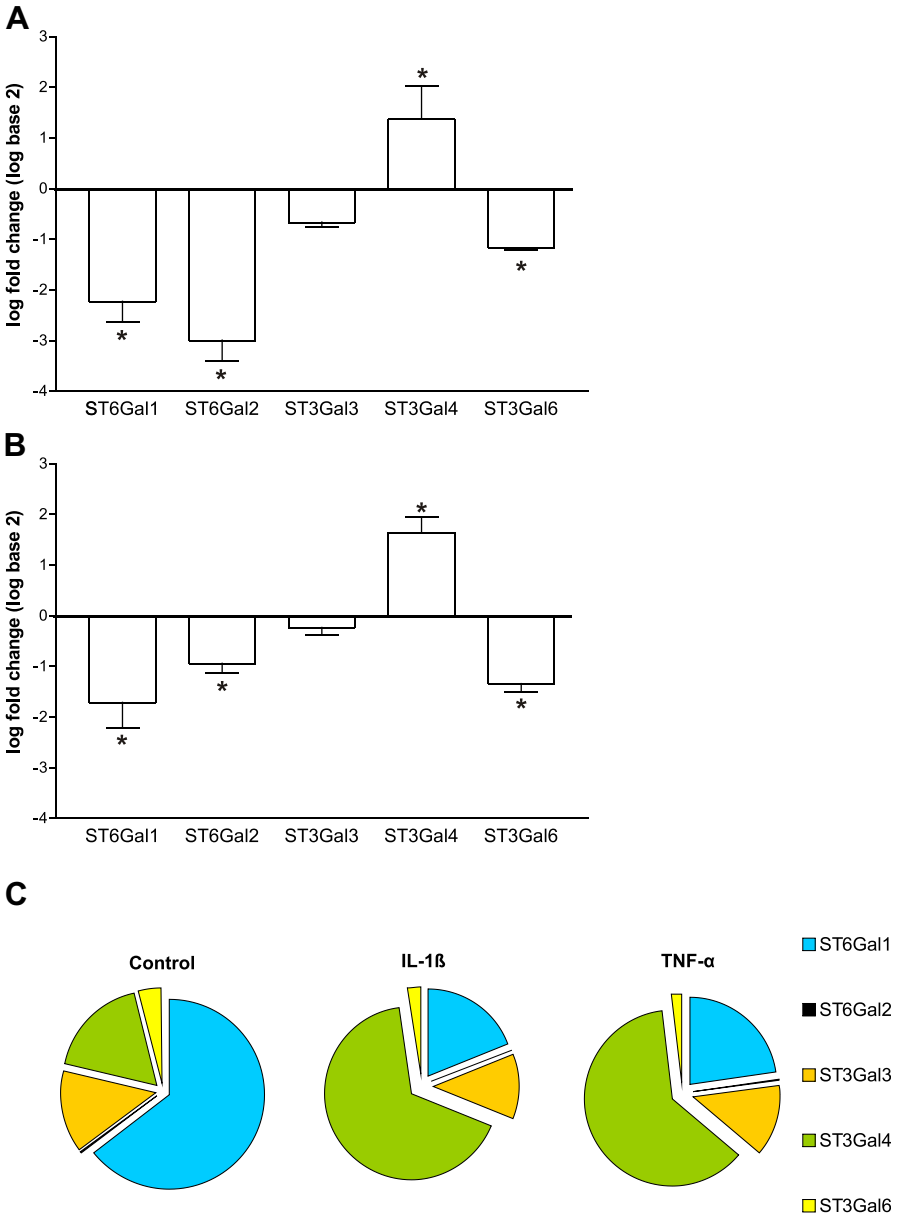


Fig. 3. mRNA levels of N-glycan related sialyltransferases in human chondrocytes exposed to pro-inflammatory cytokines. PCs were treated in duplicate with 10 ng/ml IL-1β (A) or 40 ng/ml TNF-α (B) for 24 h and SiaT expression was analyzed in duplicate using qPCR. Expression levels were log transformed (log base of 2) and alterations with respect to the control group were displayed as log-fold changes. Mean values and SD of six donors are presented. Asterisks indicate significant differences with respect to untreated controls ($P < 0.05$). (C) Proportional distribution of SiaT mRNA levels in untreated control cells and under exposure to IL-1β or TNF-α.

with the altered phenotype of chondrocytic cell lines. This result further supports our recent observation that immortalized cell lines are characterized by a reduced binding of *Sambucus nigra* agglutinin (SNA)[22]. Importantly, differences in N-glycan sialylation among the chondrocyte cell models were in correlation with differences in SiaT expression levels, indicating a crucial role of ST6Gal1 transcription for the phenotype-specific sialylation of chondrocyte glycoproteins. Our finding on the preponderant α-2,6-sialylation of human chondrocytes is in agreement with a previous lectin histochemical study of human knee joints reporting that chondrocytes in zones I–IV of normal hyaline cartilage were stained to a higher degree with SNA lectin than with Maackia amurensis agglutinin[37]. Interestingly, this feature distinguishes articular chondrocytes from their progenitor cells, the mesenchymal stem cells (MSC), which were previously shown to carry sialic acids predominantly in α-2,3-linkage[41], corresponding to the phenotype of the proliferating chondrocyte cell lines studied here.

It is tempting to speculate about the physiological reasons underlying the predominant α-2,6-sialylation of chondrocyte glycoprotein N-glycans. Homeostasis in cartilage tissue is maintained by chondrocyte–matrix interactions and the signals that chondrocytes receive in contact with

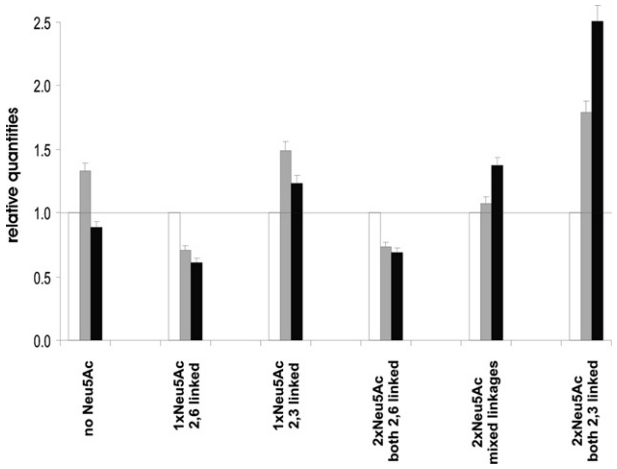


Fig. 4. Regulation of H5N4F1 glycan sialylation under IL-1β and TNF-α exposure. PCs of three donors were cultured separately for 5 days in the presence of 10 ng/ml IL-1β (grey bars) or 40 ng/ml TNF-α (black bars). N-glycan sialylation was analyzed in triplicate using LC–ESI-MS. Values are means ± SD relative to that of untreated cells (grey bars) set at 1.0.

matrix components. The glycoprotein CD44 is the major receptor for the glycosaminoglycan hyaluronan (HA), anchoring the proteoglycan network of cartilage to the surface of chondrocytes. Detailed studies have shown that at least two of the five N-glycosylation sites within the HA binding domain of CD44 can modify binding activity and that both carry terminal α-2,3-linked sialic acids[42,43]. Furthermore, modification of CD44 glycosylation by inhibition of N-glycan biosynthesis or enzymatic cleavage of N-glycans or terminal sialic acids can trigger the activation of CD44-mediated HA binding in various cells[43–46]. Therefore, the presence of terminal α-2,3-linked sialic acid on N-glycans of the CD44 receptor inhibits the binding to HA[46]. Given the observation that α-2,6-SiaTs and α-2,3-SiaTs compete for the same limited pool of N-glycan substrates[47,48], it is conceivable that the predominant expression of α-2,6-SiaTs in human chondrocytes facilitates the activation of CD44 receptors, which in fact is required for a functional matrix assembly of cartilage. In contrast, the dominant α-2,3-sialylation of MSC[41] may be a physiological requirement for migration and homing of MSC to sites of condensation and cartilage formation in the embryo. A similar physiological mechanism has been proposed recently for hematopoietic stem and progenitor cells (HSPC)[48]. In that study, an increased relative abundance of α-2,3-sialylation was found in HSPC whereas α-2,6-sialylation was prevalent in peripheral blood-derived $CD34^+$ and $CD34^-$ cells. Thus, the authors suggested that the enriched α-2,3-sialylation of HSPC might constitute a mechanism to facilitate migration of HSPC from bone marrow into the circulation.

In recent years, the inflammatory component of osteoarthritis has been recognized as a decisive factor for the progression of the disease. The release of cytokines such as IL-1β or TNF-α from both the synovium and the chondrocytes themselves may be involved in the induction of inflammatory factors, including inducible nitric oxide synthetase, cyclooxygenase 2, and phospholipase A2[49–51], as well as proteinases that promote the destruction of cartilage matrix. The present work shows that IL-1β and TNF-α induce alterations of the differentiated chondrocyte phenotype and that these alterations are associated with a remarkable reorganization of chondrocyte N-glycan sialylation. Strongly enhanced ST3Gal4 mRNA levels and the parallel drop of ST6Gal1 expression result in reduced α-2,6- to α-2,3-sialylation ratios in chondrocyte glycoprotein N-glycans. We found that this linkage-type remodeling occurred at both S1H5N4F1 and S2H5N4F glycans, i.e., those H5N4F structures carrying one or two sialic acid residues. Of note, this finding is in line with recent *in vivo* experiments studying N-glycans in rabbit cartilage after ligament transection[20]. There, it was shown that the amount of S1(α-2,6)H5N4F1 structures significantly decreased in cartilage tissues excised from animals with induced osteoarthritis as compared to those of healthy animals. It appears noteworthy that the shift towards reduced α-2,6- to α-2,3-sialylation ratios in human chondrocytes under exposure to IL-1β and TNF-α, as observed in our study, is not a general cellular phenomenon. As reported recently, human endothelial cells respond to IL-1β, TNF-α, and lipopolysaccharide (LPS) with increased α-2,6-SiaT mRNA levels, increased α-2,6-SiaT enzyme activity, and enhanced cellular binding of SNA lectin[52]. In contrast, stimulation of human monocyte maturation with TNF-α, IL-1β, LPS, or IFN-γ leads to downregulation of both ST6Gal1 and ST3Gal4 transcription[19]. Thus, it can be concluded that the effect induced by pro-inflammatory molecules on cellular sialylation is strongly dependent on cell and tissue type. For human chondrocytes we propose a novel mechanism involving α-2,6-linked sialic acids that represent a typical structure of the differentiated chondrocyte phenotype, whereas the enhanced expression of α-2,3-linked sialic acids accompanies cellular dedifferentiation induced by IL-1β and TNF-α. In light of the significance of sialic acids in CD44-matrix interactions, a shift towards α-2,3-sialylation under inflammatory conditions may modify the binding ability of CD44 to the HA substrate, influencing cellular anchoring of matrix components and affecting signal transduction and gene expression[53,54]. Similarly to CD44, the β1 subfamily of integrins is substrate of several glycosyltransferases including ST6Gal1[55–58]. Although controversial and highly dependent on the cell type, those studies have demonstrated the relationship between sialylation of β1 integrins and adhesion to ECM components. Since chondrocytes express a number of different integrins including α1β1, α2β1, α3β1, α5β1, and α6β1 integrins[59], the altered N-glycan sialylation in the presence of TNF-α or IL-1β may potentially influence the binding affinity of these receptors for their matrix substrates. This hypothesis, however, still remains to be experimentally proven.

In conclusion, the present report has revealed that the expression ratio of α-2,6- to α-2,3-linked terminal sialic acids constitutes a signature of the differentiation status of human chondrocytes. Whereas α-2,6-linked sialic acids are the typical structure in primary human chondrocytes, the altered phenotype observed in chondrocytic cell lines or induced by IL-1β and TNF-α is associated with abundant α-2,3-linked sialic acids. This finding may shed light on the significance of sialylated N-glycan structures for cartilage homeostasis and enhance our understanding of cartilage pathophysiology. Future studies clarifying the molecular basis, as well as the functional consequences of shifting sialylation patterns in human chondrocyte N-glycans, are therefore anticipated.

Conflict of interest

No conflicts of interest.

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Supplementary material

Supplementary material associated with this paper can be found, in the online version, at doi:10.1016/j.joca.2009.09.004.

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IL-1β and TNF-α alter the glycophenotype of primary human chondrocytes in vitro

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ABSTRACT

Despite the significance of glycoproteins for extracellular matrix assembly in cartilage tissue, little is known about the regulation of the chondrocyte glycophenotype under inflammatory conditions. The present study aimed to assess the effect of IL-1β and TNF-α on specific features of the glycophenotype of primary human chondrocytes in vitro. Using LC–MS, we found that both cytokines increased overall sialylation of N- and O-glycans and induced a shift towards α-(2→3)-linked sialic acid residues in chondrocyte glycoproteins. These results were supported by quantitative PCR showing increased expression of α-(2→3) sialyltransferases in treated cells. Moreover, we found that both IL-1β and TNF-α induced a considerable shift from oligomannosidic glycans towards complex-type N-glycans. In contrast, core α-(1→6)-fucosylation of chondrocyte N-glycans was found to be reduced particularly by TNF-α. In summary, inflammatory conditions induce specific alterations of the chondrocyte glycophenotype which might affect cell–matrix interactions or the function of endogenous lectins.

1. Introduction

In recent years, inflammatory processes have been recognized to contribute to the progression of degenerative joint diseases such as osteoarthritis.[1] The release of interleukin-1β (IL-1β) or tumor necrosis factor-alpha (TNF-α) from joint tissues may be involved in the induction of pro-inflammatory mediators and proteinases that promote the destruction of cartilage matrix.[2] Interestingly, recent studies provide evidence for a relationship between osteoarthritic conditions and altered glycosylation of cartilage tissue or chondrocytes. In an animal model of osteoarthritis, altered N-glycosylation of cartilage has been observed.[3] This finding is in line with a report showing that cytokine treatment of human and bovine chondrocytes results in specific glycosylation changes related to apoptosis and altered cell proliferation.[4] Recently, we found that differences in the molecular chondrocyte phenotype are reflected by specific cellular lectin-binding patterns.[5,6] In a further study employing quantitative PCR and liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS), we demonstrated that primary human chondrocytes predominantly express α-(2→6)-sialyltransferases (SiaT) and accordingly, α-(2→6)-linked sialic acid residues in glycoprotein N-glycans. Upon treatment with IL-1β or TNF-α, however, an overall shift towards α-(2→3)-sialylation of diantennary fucosylated N-glycans and towards α-(2→3)-SiaT mRNA levels was observed in primary chondrocytes.[7]

To expand our previous work, the present study aimed to investigate the impact of IL-1β and TNF-α on three characteristics of the chondrocyte glycophenotype: (1) the ratio between oligomannosidic, hybrid and complex-type N-glycans, (2) the core fucosylation in N-glycans, and (3) the sialylation of N- and O-glycans with regard to linkage type and degree of sialylation.

2. Results and discussion

In osteoarthritis, articular chondrocytes undergo phenotypic modulation in response to mechanical injury and inflammation.[2] In addition to changes of cell morphology and marker gene expression, inflammatory conditions induce alterations of the chondrocyte glycophenotype. As shown in Figure 1, a series of glycosyltransferases is inducible by pro-inflammatory cytokines in primary human chondrocytes. IL-1β significantly down-regulated MAN1C1 (14.3-fold), MGAT3 (7.1-fold) and ST6GalNAc3 (11.1-fold) mRNA expression in all three donors (Fig. 1A). Consistently, TNF-α reduced these genes to a similar extent (12.5-, 4.8-, and 25-fold, respectively) (Fig. 1B). In contrast, ST3Gal1 was up-regulated 4.1- and 2.7-fold

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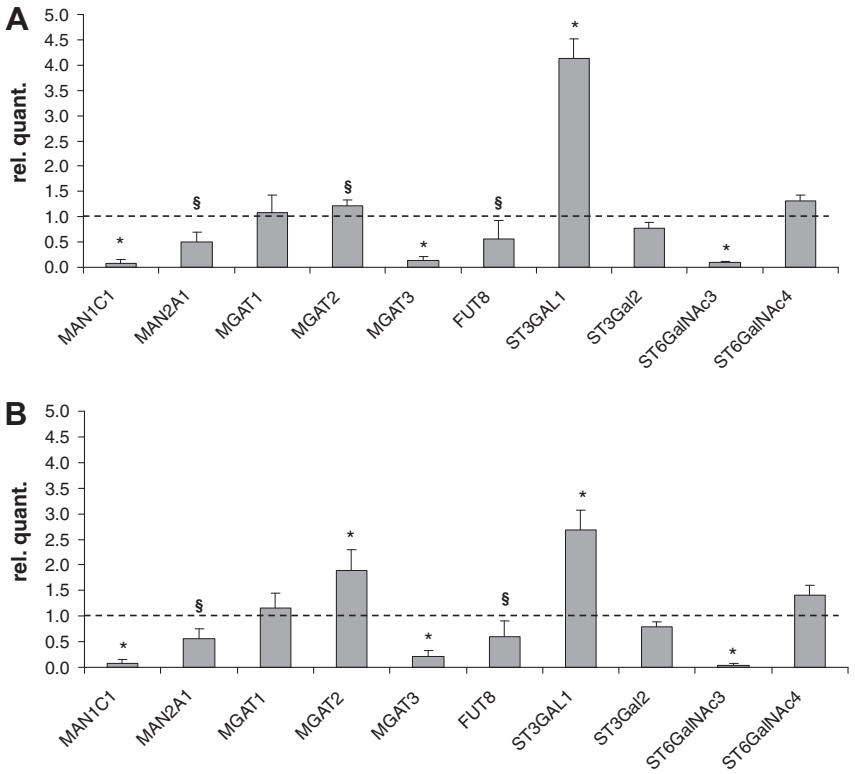

Figure 1. mRNA levels of glycosyltransferases in human chondrocytes exposed to pro-inflammatory cytokines. Primary chondrocytes were treated with 10 ng/mL IL-1β (A) or 40 ng/mL TNF-α (B) for 24 h and mRNA expression was analyzed using RT-qPCR. Cytokine-induced alterations of mRNA levels are displayed as relative quantities (mean ± SD; $n = 3$ donors) with respect to untreated controls set at 1. $^{*}p < 0.05$ in three cell populations. $^{§}p < 0.05$ in two cell populations.

by IL-1β and TNF-α, respectively. MGAT2 mRNA was expressed at 1.9-fold higher levels in the presence of TNF-α.

To estimate changes due to cytokine treatment we identified the most representative major N-glycan structures for five different criteria (oligomannosidic, hybrid-type, complex-type, degree of fucosylation and sialylation) by LC-ESI-MS with a graphitized carbon column (Fig. 2). For oligomannosidic glycans we quantified the amounts of Man4, Man5, Man6, Man7, Man8 and Man9 glycans, whereas hybrid-type glycans were represented by the series of Man3Na, Man4Na and Man5Na glycans, core α-1,6 fucosylated as well as unsubstituted. Major complex type structures were identified as $A^4A^4F^6$ glycans (see Fig. 2), either non-, mono- or disialylated. A more detailed analysis concerning sialylation linkage distribution of the $A^4A^4F^6$ glycan in human chondrocytes can be

#	Oligomannosidic	Mass	H	N	F	S
	Man4 - Man9	x	x	2		
	Complex-type					
1	$A^4A^4F^6$	1788.67	5	4	1	
2	$A^4Na^{6\text{-}4}F6$	2079.76	5	4	1	1
3	$A^4Na^{3\text{-}4}F6$	2079.76	5	4	1	1
4	$Na^{6\text{-}4}Na^{6\text{-}4}F6$	2370.86	5	4	1	2
5	$Na^{3\text{-}4}Na^{6\text{-}4}F6$	2370.86	5	4	1	2
6	$Na^{3\text{-}4}Na^{3\text{-}4}F6$	2370.86	5	4	1	2
	Hybrid-type ($Na^{3\text{-}4}$ isomers not listed)					
7	$Man3Na^{6\text{-}4}$	1568.57	4	3		1
8	$Man4Na^{6\text{-}4}$	1730.62	5	3		1
9	$Man5Na^{6\text{-}4}$	1892.68	6	3		1
10	$Man3Na^{6\text{-}4}F^6$	1714.63	4	3	1	1
11	$Man4Na^{6\text{-}4}F^6$	1876.68	5	3	1	1
12	$Man5Na^{6\text{-}4}F^6$	2038.73	6	3	1	1
	O-glycans					
13	A^4A-O2	750.29	2	2		
14	$Na^{3\text{-}4}A$-O2	1041.39	2	2		1
15	A^4Na^3-O2	1041.39	2	2		1
16	$Na^{3\text{-}4}Na^3$-O2	1332.48	2	2		2

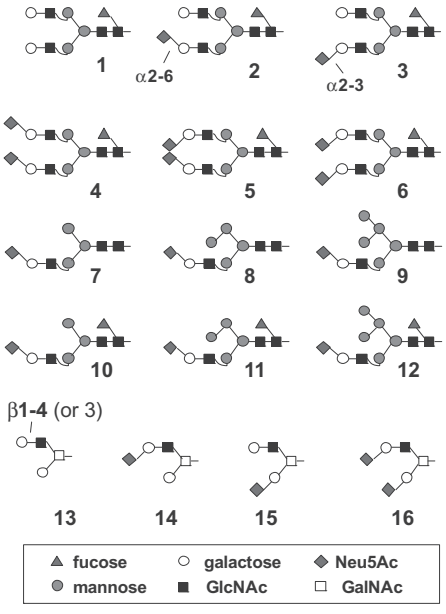

Figure 2. Chondrocyte glycan structures quantified by LC–ESI-MS. In addition to the structures shown here, hybrid-type N-glycans with α-(2→3)-linked sialic acid were considered for quantification. Regarding the fine structure of O-glycans, we assume the presence of β-(1→4)-linked galactose on the upper arm from the coelution of these substances with O-glycans from bovine fetuin.[9] Glycans are named according to the 'proglycan' nomenclature (http://www.proglycan.com).

found in our recent report.[7] (N-glycans are named according to the Proglycan nomenclature (http://www.proglycan.com)).

A considerable shift from oligomannosidic glycans towards complex-type N-glycans occurred in primary human chondrocytes under the influence of IL-1β and TNF-α. While untreated control cells showed an average of 46% complex type glycans, these structures increased to 52.7% and 50% after treatment with TNF-α or IL-1β, respectively (Table 2). This finding is in line with RT-qPCR results of MGAT2 expression levels (Fig. 1). Concomitantly, the proportion of oligomannosidic glycans decreased from 41.0% in control cells to 34.4% after TNF-α and to 38.0% after IL-1β treatment (Table 2). Interestingly, this result appears to be inconsistent with RT-qPCR results on MAN1C1 and MAN2A1, which both were markedly decreased in the presence of cytokines (Fig. 1). However, in both cases only one of the three genes encoding Golgi α-mannosidase I and Golgi α-mannosidase II was considered.[8] Hybrid-type structures were detected to represent about 13% in control samples and no significant changes were detected after cytokine treatment (Table 2). In addition, Figure 3 displays the percent differences of oligomannosidic, hybrid-type and complex-type structures with respect to untreated cells and clearly shows that the results were reproducible, albeit to a different extent, in cells isolated from three donors.

The degree of fucosylation was analyzed monitoring hybrid-type glycans. For these structures a contamination by non-fucosylated glycans from the FCS in the cell culture medium can be excluded. Core α-(1→6)-fucosylation was found to be 58.4% on average. A significant decrease of 6.5% was found for TNF-α treated cells of two donors (Table 2). For IL-1β on the other hand, fucosylation appeared to be almost unaltered. Similarly, FUT8 mRNA levels were slightly decreased by the cytokines, however, significance levels (p <0.05) were reached in just two of three donors (Fig. 1).

Primary human chondrocytes strongly express the α-(2→6)-SiaT ST6Gal1 and accordingly, mainly α-(2→6)-linked sialic acid residues are found on $A^4A^4F^6$ N-glycans. However, when primary chondrocytes were treated with IL-1β or TNF-α, a considerable shift towards the α-(2→3)-SiaT ST3Gal4 occurred.[7] LC–ESI-MS analysis of non-, mono-, and disialylated $A^4A^4F^6$ N-glycans confirmed the increase of α-(2→3) sialylation on glycoprotein N-glycans of TNF-α treated chondrocytes (Fig. 4). The degree of sialylation of diantennary (core fucosylated) N-glycans was found to be 68.4% in the control samples on average and was increased to 71.8% with TNF-α and 70.3% with IL-1β (Table 2). In case of TNF-α treated cells, these small changes were significant for two of three donors (Table 2). In summary, the degree of sialylation remained almost unchanged, or just slightly enhanced by treatment with TNF-α.

Regarding O-glycan sialylation, we observed that the two α-(2→3) SiaTs ST3Gal1 and ST3Gal2 were expressed at 5 ± 1 and 3 ± 2 molecules/1000 molecules GAPDH, respectively. In comparison, the α-(2→6) SiaTs ST6GalNAc1 and ST6GalNAc2 were not detectable with RT-qPCR and ST6GalNAc3 and ST6GalNAc4 were expressed at 0.2 ± 0.1 and 3 ± 1 molecules/1000 molecules GAPDH. Accordingly, α-(2→6) sialylation was practically absent at chondrocyte glycoprotein O-glycans as revealed by LC–MS (data not shown). Upon treatment with IL-1β or TNF-α, the SiaT expression profile of primary chondrocytes predicted an O-glycan sialylation pattern characterized by increased α-(2→3)-linked sialylation (Fig. 1). In fact, ST3Gal1 mRNA levels were markedly up-regulated under the influence of the cytokines (4.1-fold in case of IL-1β and 2.7-fold in case of TNF-α), whereas the expression of ST6GalNAc3 was decreased under the same conditions (11.1-fold and 25-fold, respectively).

LC–MS analysis of O-glycans from chondrocytes revealed changes in the sialylation of the core 2 O-glycan number 13 (A^4A-O, Fig. 2). In untreated chondrocytes, we found 7.4% of the nonsialylated as well as 44.1% and 49.4% mono- and disialylated structures, respectively (Table 3). Upon TNF-α treatment, the amount of disialylated glycans (number 16, Fig. 2) increased to 67% at the expense of non- and monosialo O-glycans. A comparable increase of sialylation of O-glycans was observed in presence of IL-1β (Table 3).

Taken together, this study shows that primary human chondrocytes display an altered glycophenotype in response to the pro-inflammatory cytokines IL-1β and TNF-α at the mRNA and glycan level. Regarding the functional consequences of altered glycophenotypes, it is noteworthy that the glycosylation of structural proteins and cell surface receptors contributes to the maintenance of articular cartilage functionality.[10] In particular, glycosylated receptors including CD44 and integrins play critical roles in the mediation of chondrocyte-matrix interactions which trigger signal transduction and gene expression.[11,12] Increased overall sialylation as well as shifting sialic acid linkage types could modify chondrocyte–matrix interactions or signaling by endogenous lectins. In this regard, galectins were shown to tolerate α-(2→3) sialylation[13], whereas galectin-1 does not bind sialylated N-glycans with terminal α-(2→6)-linked sialic acids of the type elaborated by

Table 2
Glycoprotein N-glycan characteristics in primary human chondrocytes treated with IL-1β or TNF-α

	Oligo-mann.	Hybrid	Complex	Fucosylation	Sialylation
CTRL	41.0	13.0	46.0	58.4	68.4
TNF-α	34.4*	12.9	52.7*	51.9§	71.8§
IL-1β	38.0§	12.0	50.1§	56.8	70.3

Data show mean values of cells isolated from 3 donors in percent (2 repeats per measurement). TNF-α and IL-1β treated cells are compared to untreated controls. On average, standard deviations were 2.5% of the respective values. (Oligo.mann. = oligomannosidic structures, hybrid = hybrid-type glycans, complex = complex-type glycans).
* p <0.05 in three cell populations.
§ p <0.05 in two cell populations.

Table 1
Primer sequences for qPCR assays

Gene	Accession number	Forward primer	Reverse primer	Amplicon size
ST3GAL1	NM_003033	CATCCTCTCGGTCATCTTCTC	CAGTAGTGGTGCCAGTTCC	93 bp
ST3GAL2	NM_006927	GGACGGGCACAACTTCATC	CAGGTTCTTGGCACTCTCAG	115 bp
ST6GalNAc1	NM_018414	TCTGGCTGTCCTGGTCTTC	TAATGTTCTCTGTGCGTTGATG	95 bp
ST6GalNAc2	NM_006456	CCAAGTCATCGCCTCCAC	TCAGAATGCCTCCGTTGC	125 bp
ST6GalNAc3	NM_152996	GATACCATTCTCCTACACATACAG	AAGGTCACAGTCCAGTTGC	97 bp
ST6GalNAc4	NM_175039	GGCTTTGAGGCGGATGTG	CTGGAAGTAGTGTGAATAGTTGC	96 bp
MGAT1	NM_002406	GGTGGAGTTGGTGGGTCATC	GAGGAGAGGTCTTGCTTGCC	142 bp
MGAT2	NM_002408	CTGCTGCTGGACTCACTTC	AATGCTGAAAGGAAAGAACACC	147 bp
MGAT3	NM_002409	ATGGAGGAGAAGAGAATGGATATG	GAGGGAAACAGGAGCAAGC	132 bp
MAN1C1	NM_020379	CCCGCTCAGACACCAAAC	TCCGACAGTATTTCTCCAAGG	204 bp
MAN2A1	NM_002372	GACTCTTACTCTCCTGATACCTTC	TACCTTGGCTCCGCACTC	104 bp
FUT8	NM_178155	GGTTCAGCGGAGAATAACATATC	GACAGCCATAGCCACAGC	98 bp

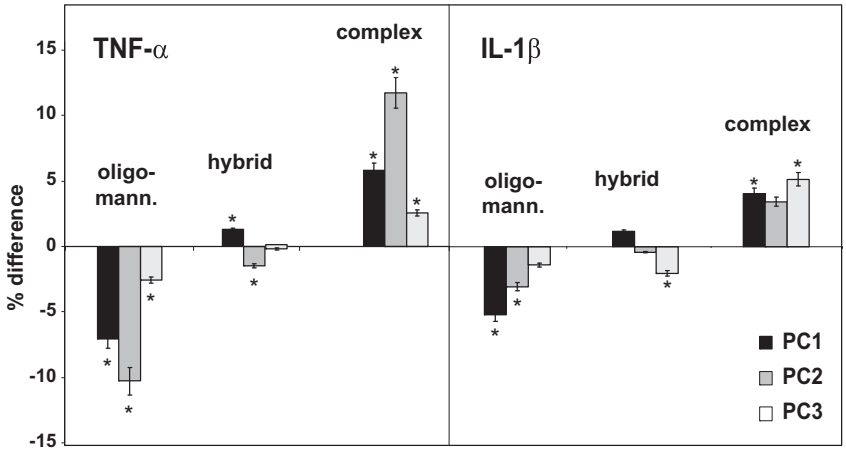

Figure 3. Changes of oligomannosidic, hybrid and complex N-glycans in primary human chondrocytes exposed to TNF-α (A) or IL-1β (B). N-Glycans of cells isolated from three donors (PC1, PC2, and PC3) were analyzed separately by porous graphitized carbon chromatography with ESI-MS detection. Alterations of the N-glycans in comparison with untreated cells are presented as percent difference (*p <0.05). (Oligo-mann. = oligomannosidic structures, hybrid = hybrid-type glycans, complex = complex-type glycans).

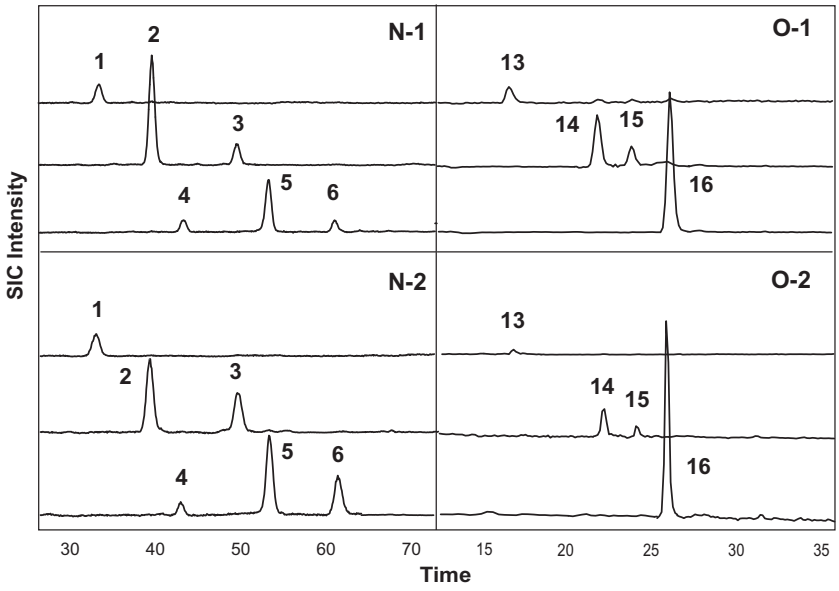

Figure 4. Carbon–LC–ESI-MS analysis of N- and O-glycans from chondrocytes. Enzymatically released N-glycans were reduced with borohydride and O-glycans were obtained by reductive β-elimination. Panels N-1 and N-2 show the selected ion chromatograms (SIC) of non-, mono-, and disialylated diantennary N-glycans from a control sample and of cells after TNF-α treatment, respectively. Similarly, panels O-1 and O-2 show the O-glycans form control and TNF-α treated samples. Numbers above peaks refer to the structures shown in Figure 2.

Table 3
O-Glycan sialylation in primary human chondrocytes treated with IL-1β and TNF-α

	#13	#14 & #15	#16
CTRL	7.4	44.1	49.4
TNF-α	3.3*	29.7*	67.0*
IL-1β	3.8§	30.8*	66.1*

TNF-α and IL-1β treated cells are compared to untreated controls. Data show mean values of cells isolated from three donors in percent (two repeats per measurement). In average, standard deviations were 3.5% of the respective values. Numbers (#13–#16) refer to the O-glycan structures shown in Figure 2.
* p <0.05 in three cell populations.
§ p <0.05 in two cell populations.

ST6Gal1.[14] This might support speculations on altered galectin function under inflammatory conditions. Similarly, changing ratios between oligomannosidic and complex-type structures of glycoproteins might influence protein folding and stabilization of functional conformations.[15] Based on the present report, future studies are expected to clarify the functional role of modulated glycosylation in physiological cartilage homoeostasis and joint disease.

3. Experimental

3.1. Cell culture

Primary human chondrocytes were isolated from cartilage specimens as described in detail previously.[5,7] The study was performed in accordance with the terms of the ethics committee of the Medical University Vienna (EK-Nr.: 081/2005). Isolated cells were grown to confluence in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum (Biochrom AG, Germany) and 2 μL/mL gentamycin. To induce inflammatory conditions, cells

were treated with 10 ng/mL IL-1β or 40 ng/mL TNF-α for the time periods indicated.

3.2. Quantitative real-time RT-PCR (RT-qPCR)

Chondrocytes were plated on 12-well tissue culture plates (Iwaki, Japan) and grown as described above prior to treatment with cytokines for 24 h. Total RNA was extracted using the NucleoSpin RNA II Kit (Macherey-Nagel, Germany) and processed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Austria).

SYBR-green based RT-qPCR assays for glycosyltransferases were established following published protocols.[16,17] Details on primer sequences are given in Table 1. All RT-qPCR reactions were performed in 25 μL reaction mixtures containing 1 μL cDNA, 12.5 μL Brilliant SYBR Green QPCR Master Mix, 100 nM primers, and nuclease-free water to 25 μL, and run in duplicate on a Mx3000P QPCR system (Stratagene). Melting curves were generated to confirm a single gene-specific peak and no-template-controls were included in each run to control for contaminations. mRNA expression levels were calculated as relative copy numbers with respect to the expression of glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) set at 1000. The regulation of genes by cytokines was calculated as quantities relative to the untreated control group using the MxPro real-time QPCR software.

3.3. Protein isolation, glycan preparation, and oligosaccharide analysis

Chondrocytes were seeded in 25 cm^2 flasks (Greiner bio-one, Austria) and allowed to settle for 48 h. Then, the culture medium was changed and cells were maintained in the presence of 10 ng/mL IL-1β or 40 ng/mL TNF-α for 5 days. Protein isolation and N-glycan preparation was done after cell lysis by acetone precipitation and a 'mini-prep SDS page purification'[18] of the proteins as described in our recent work.[7] O-glycans were released by reducing β-elimination[19] and were further analyzed in the same way as borohydride reduced N-glycans.[7,20,21] Porous Graphitized Carbon was selected as stationary phase for separation of borohydride reduced glycans as described recently.[20,21] Structural identification and differentiation of isobaric glycans was performed by elution times. For statistical evaluation, representative major glycan structures were identified and evaluated.

3.4. Quantification of LC–MS peaks

Glycans were detected using an ESI Q-TOF MS from Waters (Micromass, Q-TOF Ultima Global). A first data analysis was performed using a MassLynx 4.0 SP4 Software, where further automatic peak detection and quantification was done employing MassMap (Thermo-Fisher, http://www.massmap.de).

3.5. Statistics

RT-qPCR data were exported to the GraphPad Prism statistics software package (GraphPad Prism Software, USA). Statistics were performed using one-way analysis of variance (ANOVA) with post hoc Tukey tests, cross-comparing all study groups (95% confidence interval). p-values < 0.05 were considered significant.

Descriptive statistics and statistical significances of LC–MS data were calculated using SPSS (Statistical Package for the Social Sciences, IBM). One-way ANOVA was performed to evaluate statistical significance. Changes were considered significant if their corresponding p values were below p <0.05 (n = 3, 2 repeats).

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2.3. Effects of *Caesalpinia sappan* extracts and the isolated compound brazilin in human chondrocytes (Studies 4-6)

Study 4. *Caesalpinia sappan* extract inhibits IL1ß mediated over-expression of matrix metalloproteinases in human chondrocytes

S. Toegel, **S.Q. Wu,** M. Otero , M.B. Goldring , P. Leelapornpisid , C. Chiari , A. Kolb , F.M. Unger , R. Windhager , H. Viernstein. ***Caesalpinia sappan* extract inhibits IL1β-mediated overexpression of matrix metalloproteinases in human chondrocytes.** *Genes Nutr.* (2011), in press.

Study 5. Anti-inflammatory activity of an ethanolic *Caesalpinia sappan* extract in human chondrocytes and macrophages.

S.Q. Wu, M. Otero, F.M. Unger, M.B. Goldring, A. Phrutivorapongkul, C. Chiari, A. Kolb, H. Viernstein , S. Toegel. **Anti-inflammatory activity of an ethanolic *Caesalpinia sappan* extract inhuman chondrocytes and macrophages.** *J Ethnopharmacol.* (2011), *in press.*

Study 6. Brazilin from *Caesalpina sappan* inhibits pro-inflammatory mediators and metalloproteinase-3 expression in human chondrocytes.

S.Q. Wu, F.M. Unger, H. Viernstein, G. Reznicek, S. Toegel. **Brazilin from *Caesalpina sappan* inhibits pro-inflammatory mediators and metalloproteinase-3 expression in human chondrocytes.** To be submitted.

Caesalpinia sappan *extract inhibits IL1#-mediated overexpression of matrix metalloproteinases in human chondrocytes*

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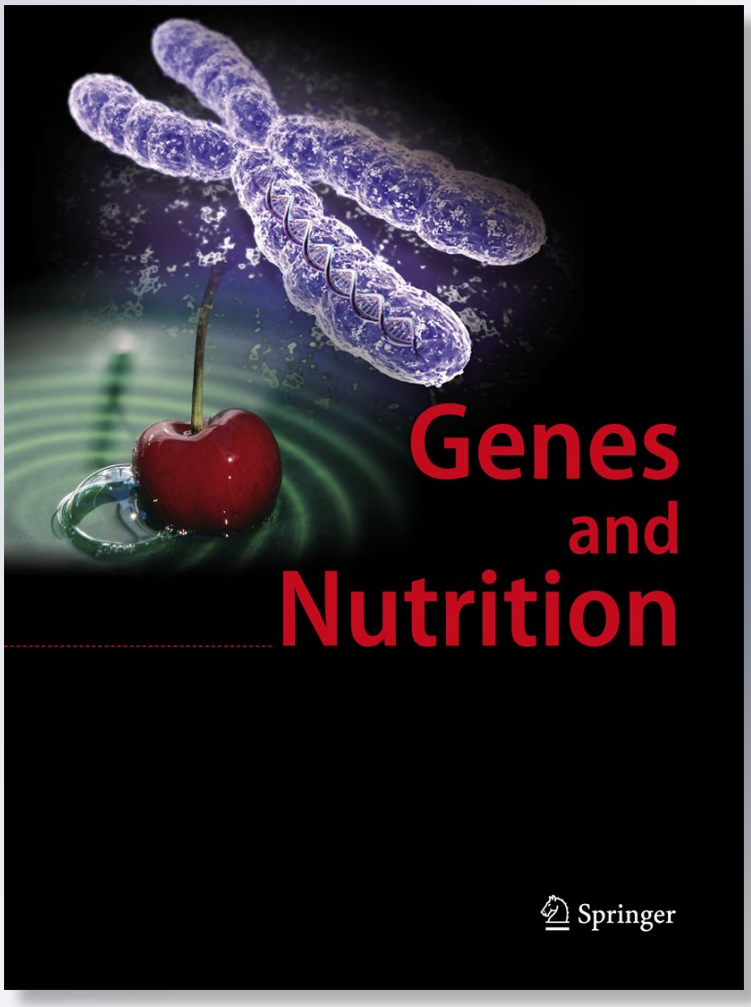

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RESEARCH PAPER

Caesalpinia sappan extract inhibits IL1β-mediated overexpression of matrix metalloproteinases in human chondrocytes

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Abstract Exacerbated production of matrix metalloproteinases (MMPs) is a key event in the progression of osteoarthritis (OA) and represents a promising target for the management of OA with nutraceuticals. In this study, we sought to determine the MMP-inhibitory activity of an ethanolic *Caesalpinia sappan* extract (CSE) in human OA chondrocytes. Thus, human articular chondrocytes isolated from OA cartilage and SW1353 chondrocytes were stimulated with Interleukin-1beta (IL1β), without or with pretreatment with CSE. Following viability assays, the production of MMP-2 and MMP-13 was assessed using ELISA, whereas mRNA levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13 and TIMP-1, TIMP-2, TIMP-3 were quantified using RT-qPCR assays. Chondrocytes were co-transfected with a MMP-13 luciferase reporter construct and NF-kB p50 and p65 expression vectors in the presence or absence of CSE. In addition, the direct effect of CSE on the proteolytic activities of MMP-2 was evaluated using gelatin zymography. We found that CSE significantly suppressed IL1β-mediated upregulation of MMP-13 mRNA and protein levels via abrogation of the NF-kB(p65/p50)-driven MMP-13 promoter activation. We further observed that the levels of IL1β-induced MMP-1, MMP-3, MMP-7, and MMP-9 mRNA, but not TIMP mRNA levels, were down-regulated in chondrocytes in response to CSE. Zymographic results suggested that CSE did not directly interfere with the proteolytic activity of MMP-2. In summary, this study provides evidence for the MMP-inhibitory potential of CSE or CSE-derived compounds in human OA chondrocytes. The data indicate that the mechanism of this inhibition might, at least in part, involve targeting of NF-kB-mediated promoter activation.

Keywords Chondrocytes · Osteoarthritis · *Caesalpinia sappan* · Matrix metalloproteinase · Tissue inhibitors of MMP · NFkB

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Background

Osteoarthritis (OA), a chronic and degenerative joint disease, has the highest prevalence and economic impact among arthritic maladies. Besides mechanical and genetic factors, pro-inflammatory cytokines such as interleukin-1β (IL1β) or tumor necrosis factor alpha (TNFα) contribute to the impaired chondrocyte function and extracellular matrix (ECM) integrity in OA. Histological examination has demonstrated the localization of IL1β in the zones of

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degenerated OA cartilage (Saha et al. 1999) as well as in the synovial membranes and synovial fluids of OA patients (Smith et al. 1997; Kubota et al. 1997). At the cellular level, chondrocytes respond to this cytokine via a cascade of events leading to loss of balance between cartilage anabolism and catabolism, associated with phenotypic modulation and activation of the normally quiescent chondrocytes (Goldring and Marcu 2009; Goldring et al. 2008). In OA cartilage, IL1β and TNFα co-localize with increased levels of matrix metalloproteinases (MMPs) (Tetlow et al. 2001), and IL1β was shown to upregulate MMP expression levels in normal human chondrocytes, OA chondrocytes, and chondrosarcoma cells (Tetlow et al. 2001; Fan et al. 2005; Mix et al. 2001).

According to their main substrate specificity, proteins of the MMP family can be divided into collagenases (MMP-1, MMP-8, MMP-13), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, MMP-11), and membrane type I MMPs (MMP-14-17). Restriction of the proteolytic activities of MMPs is mediated by endogenous tissue inhibitors of MMPs (TIMPs) (Nagase and Woessner 1999). MMPs play important roles in physiological processes such as tissue repair, but also become part of destructive disease processes due both to its overexpression and the excessive activation of pro-MMPs into their active forms. Interestingly, a recent study by Little et al. demonstrated that MMP-13-knockout mice were less prone than wild-type mice to develop tibial cartilage structural damage following medial meniscal destabilization surgery in the knee (Little et al. 2009). Taken together, MMPs might represent promising targets for therapeutic intervention in OA.

To date, classic therapeutic approaches of OA are still limited to exercise, palliative drugs or surgical intervention. There is growing awareness, however, that nutritional factors can positively influence the maintenance of bone and joint health. Articular cartilage is critically dependent upon the regular provision of nutrients, vitamins, and essential trace elements (Goggs et al. 2005). Thus, dietary supplementation programs and nutraceuticals might provide long-term benefits to patients with a chronic disease such as OA. In general, the benefits offered by nutraceuticals are based on the lack of adverse effects and the targeting of multiple signaling pathways addressed by nutritional compound mixtures such as plant extracts (Bjorkman 1999). A comprehensive review on current nutraceuticals for the management of OA has been published elsewhere (Ameye and Chee 2006).

Caesalpinia sappan L. (Leguminosae) is distributed in Southeast Asia, and its dried heartwood (also known as Sappan wood) has been a traditional ingredient of food or beverages such as Bir Pletok, a Batavian spice drink. The extract was shown to lack toxic effects and to possess anti-atherogenic potential in rats based on its constituent hematein (Sireeratawong et al. 2010; Oh et al. 2001). In traditional Chinese medicine, *Caesalpinia sappan* is known to promote blood circulation and to possess analgesic or anti-inflammatory properties (China Pharmacopoeia 2010). In Japan, Sappan wood was listed in the 15th edition of the Japanese Pharmacopoeia (2006). A range of constituents including brazilin, hematoxylin, brazilein, photosappanin derivatives, flavones, and other homoisoflavonoids has been isolated from *Caesalpinia sappan* (Chen et al. 2008; Washiyama et al. 2009; Xie et al. 2000). Extracts of *Caesalpinia sappan* as well as some of their defined constituents have been investigated as functional food, revealing potential anti-inflammatory (Jeong et al. 2008), vasorelaxing (Xie et al. 2000), immunosuppressive (Ye et al. 2006), and hepatoprotective effects (Srilakshmi et al. 2010). At the time of finalizing this manuscript, Sun et al. published a study demonstrating the efficacy of a *Caesalpinia sappan* extract in an animal model of rheumatoid arthritis (Sun et al. 2011). Rats treated daily with 1.2, 2.4, or 3.6 g/kg extract showed reduced signs of paw swelling and arthritis severity as well as lower levels of proinflammatory mediators in the plasma as compared to untreated animals. The mechanisms underlying these effects, however, are still not fully understood.

The present study was driven by the hypothesis that the extract of *Caesalpinia sappan* (CSE) interferes with catabolic processes in human OA chondrocytes. We cultured human chondrocytes in the presence of IL1β and examined the biological effects of CSE co-treatment on the expression and activity of major MMPs. The results presented here demonstrate that CSE strongly inhibits chondrocyte catabolism by suppressing IL1β-induced MMPs mRNA and protein levels. We further present evidence that CSE interferes with MMP-13 promoter activation mediated by NF-kB subunits p50 and p65.

Materials and methods

Preparation and characterization of CSE

The heartwoods of *Caesalpinia sappan* were collected in June 2006 in San-Pa-Thong district, Chiang Mai province, Thailand, and identified by comparison with the voucher specimen (No. 87-1631) at the Herbarium Section, Northern Research Center for Medicinal Plants, Faculty of Pharmacy, Chiang Mai University, Thailand. Ethanolic extraction was performed by continuously extracting 30 g powdered heartwood in 350 ml 96% EtOH for 24 h using a Soxhlet apparatus. Then, the liquid extract was evaporated under reduced pressure to yield solid CSE. The composition of CSE has been described in detail previously (Chen et al. 2008). For further characterization, 4 batches of CSE

were analyzed qualitatively using HPLC. Briefly, CSE samples were dissolved in MeOH (1 mg/ml), filtrated through a 0.2 µm sterile filter, and analyzed by HPLC (ICS-3000, Dionex, USA) using a Nucleodur C-18 column (Macherey–Nagel, D), a PDA-100 detector (Dionex) and a solvent gradient of MeOH and bidistilled H_2O (both containing 2.5% acetic acid). At 280 nm, all 4 batches yielded congruent chromatograms which were also comparable to that of the standardized reference Natural Red 24 (MP Biomedicals, USA, LOT #: R25179), a commercially available Sappan wood extract (Supplementary Figure 1). For cell treatment, CSE was dissolved in 96% EtOH (10 mg/ml) and used in appropriate dilutions for the in vitro assays. The activity of specific components was not addressed in the current study.

Cell culture

Human articular cartilage was obtained from intact regions of femoral condyles and tibial plateaus at the time of total knee replacement surgery in OA patients with informed consent and in accordance with the terms of the ethics committee of the Medical University Vienna (EKNr.: 081/2005). Primary chondrocytes (PCs) were isolated according to standard protocols (Toegel et al. 2009), seeded at 10^5 cells/cm^2, and grown in an atmosphere of humidified 5% CO_2/95% air at 37°C using Dulbecco's Modified Eagle Medium (DMEM; Gibco, A) supplemented with 10% fetal calf serum (FCS; Biochrom AG, D) and gentamycin (complete medium). Only freshly isolated PCs were used for all assays throughout the study. Human SW1353 chondrosarcoma cells were seeded at 10^4 cells/cm^2 and maintained in complete medium, whereas T/C28a2 immortalized chondrocytes were seeded at 10^4 cells/cm^2 and cultured using DMEM/F12 containing 10% FCS. Prior to cell treatment, chondrocytes were starved for 6 h in serum-free DMEM containing 2% insulin-transferrin-selenium solution (Gibco, A) (ITS medium). Then, cells were pretreated for 1 h with CSE or vehicle in ITS medium followed by the addition of 10 ng/ml IL1β (Strathmann Biotec, D) and further incubation for the indicated times.

Viability assay

Primary chondrocytes were seeded at 3×10^3 cells/well into 96-well microplates (Iwaki, J), serum-starved, and treated in quadruplicate with serial dilutions of CSE for 72 h. Cell proliferation was evaluated using the MTT-based EZ4U cell proliferation assay (Biomedica, A) following the manufacturer's instructions. The experiment was repeated twice with cells from different donors.

Gelatin zymography

Serum-starved cells were cultured in the presence of 5 µg/ml CSE for 48 h. Ten microliter of cell culture supernatant was mixed with 10 µl Sample buffer (0.5 M Tris–HCl, 20% Glycerol, 10% SDS, 0.1% Bromophenol Blue) and incubated for 10 min at room temperature. The samples were loaded on gelatin (1 mg/ml)—containing SDS–polyacrylamide gels for electrophoresis with constant voltage (125 V). After running, the gel was incubated in renaturation buffer (2.5% Triton X-100) with gentle agitation for 30 min at room temperature. Afterward, the gel was equilibrated for 30 min in developing buffer (50 mM Tris-Base, 0.2 M NaCl, 5 mM CaCl2, 0.02% Brij35) and then incubated in fresh developing buffer overnight at 37°C. The zymographic activities were revealed by staining the gel with 0.2% Coomassie Blue. Gels were destained with a destaining solution (7.5% acetic acid in 20% ethanol). Areas of protease activity appeared as clear bands against a dark blue background, where the substrate digestion by the protease occurred. Molecular weights were estimated by comparison with prestained SDS–PAGE markers and a human pro-MMP-2 standard (AnaSpec, USA).

In a different approach, 0.1 µg/ml human pro-MMP-2 (AnaSpec, USA) were mixed with sample buffer and subjected to gelatine zymography as described above. Following electrophoresis, the gel was divided into equal parts, and the individual strips were developed in different developing buffers containing 5 µg/ml CSE, 10 µg/ml CSE, or 1 mM EDTA as positive control, respectively (Albini et al. 2001; Mazzoni et al. 2007).

MMP ELISA

Primary human chondrocytes ($n = 3$ donors) and SW1353 cells were cultured as described in 12-well plates and pretreated with 5 µg/ml CSE followed by the addition of 10 ng/ml IL1β. After 48 h, the culture supernatants were collected, centrifuged, and stored at −80°C. ELISA assays for MMP-2 and MMP-13 were carried out following the protocols provided by the manufacturer (RayBiotech, USA).

Quantitative real-time RT-PCR

Primary chondrocytes and SW1353 cells were cultured as described in 12-well plates and pretreated for 1 h with 5 µg/ml CSE followed by the addition of 10 ng/ml IL1β. After 6 h, cell monolayers were washed with PBS and total RNA was extracted using the NucleoSpin RNA II Kit (Macherey–Nagel, D) according to the manufacturer's instructions. On-column DNase digestion was performed

for all samples during the isolation process. To quantify and control for the integrity of the isolated RNA preparations, each sample was run on the Agilent 2100 Bioanalyzer Nano LabChip prior to reverse transcription of equal RNA quantities into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, A). RNA integrity numbers (RINs) were found to range from 7.8 to 10 for all samples.

SYBR green-based RT-qPCR assays for MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, and MMP-13 as well as TIMP-1, TIMP-2, and TIMP-3 were established according to previously published protocols (Toegel et al. 2007, 2008). Details on primer sequences are given in Table 1. Amplification efficiencies of all primers were evaluated using dilution series of cDNA prepared from chondrocyte mRNA. All RT-qPCR reactions were performed in 25-µl reaction mixtures containing 1 µl cDNA, 12.5 µl SensiMix SYBR Green Master Mix (GenXpress, A), 100 nM primers (Metabion, D), and nuclease-free water to 25 µl and run in duplicate on an Mx3000P QPCR system (Stratagene, USA). Melting curves were generated to confirm a single gene-specific peak and no-template-controls were included in each run to control for contaminations.

The regulation of target genes was calculated as quantities relative to the untreated control group using the MxPro real-time QPCR software, considering both amplification efficiencies and normalization to GAPDH as reference gene. In preceding experiments, the expression stability of 5 candidate reference genes (GAPDH, ACTB, HPRT1, SDHA, B2M) was determined in human chondrocytes and SW1353 cells treated with 5, 10, and 20 µg/ml CSE, as described above. Then, the expression stabilities were evaluated using the geNorm software (Vandesompele et al. 2002), and GAPDH was selected as stable reference gene under the experimental conditions of this study.

We declare that the current study has adhered to the minimal guidelines for the design and documentation of qPCR experiments as recently outlined by Bustin et al. (2010). A qPCR checklist listing all relevant technical information has been provided for the reviewers to assess the technical adequacy of the used qPCR protocols (Supplementary Table 1).

Transfection and reporter assays

The MMP-13 promoter sequence spanning −1007/+27 bp of the human MMP13 promoter was prepared by PCR and cloned into the pGL2-Basic luciferase reporter vector (Promega; USA) as previously described (Ijiri et al. 2005). The expression vectors encoding the NF-kB p50 and p65 subunits were previously described (Grall et al. 2005). The sequences of all constructs were confirmed by DNA sequencing.

Transient transfection experiments were carried out in T/C-28a2 cells using LipofectAMINE PLUS™ Reagents (Invitrogen; USA). Cells were seeded in 24-well tissue culture plates and cultured to 80% confluence. Transfections were carried out in serum-free antibiotic-free medium using no more than 325 ng of plasmid DNA, including 300 ng of reporter constructs and 25 ng of expression vectors or pCI empty vector (control). After incubation for 1 h at 37°C with the transfection mixture, cells were treated with 1 ml of serum-free medium containing CSE or caffeic acid phenethyl ester (CAPE; Calbiochem, D) and incubation was continued for 23 h. Cell lysates were prepared using the Reporter Lysis Buffer (Promega), and luciferase activities were determined by chemiluminescence assay using the LMaxII384 luminometer (Molecular Devices, USA) and the Luciferase Assay Substrate (Promega). Data are representative of two independent experiments performed in triplicate and expressed as fold change versus control.

Table 1 Details on primers for RT-qPCR

Gene	Accession number	Forward primer	Reverse primer	Amplicon size (bp)	Efficiency (%)
MMP1	NM_002421	CTGGAATTGGCCACAAAGTT	TCCTGCAGTTGAACCAGCTA	144	93.9
MMP2	NM_004530	TGACGGTAAGGACGGACTC	ATACTTCACACGGACCACTTG	124	91.3
MMP3	NM_002422	GGTGTGGAGTTCCTGATGTTG	AGCCTGGAGAATGTGAGTGG	184	99.9
MMP7	NM_002423	GAGTGCCAGATGTTGCAGAA	TCTCTTTGCCCCACATGTTT	156	106.9
MMP8	NM_002424	TCTGCAAGGTTATCCCAAGG	ACCTGGCTCCATGAATTGTC	154	95.3
MMP9	NM_004994	CTGGGCAGATTCCAAACCT	TACACGCGAGTGAAGGTGAG	170	91.9
MMP13	NM_002427	TCAGGAAACCAGGTCTGGAG	TGACGCGAACAATACGGTTA	196	102.3
TIMP1	NM_003254	AATTCCGACCTCGTCATCAG	GTTGTGGGACCTGTGGAAGT	195	100.3
TIMP2	NM_003255	GTAGTGATCAGGGCCAAAGC	GGGGGCCGTGTAGATAAACT	153	101.9
TIMP3	NM_000362	GTACCGAGGCTTCACCAAGA	ACCTCTCCACGAAGTTGCAC	164	95.9

Statistics

Data were exported to the GraphPad Prism statistics software package (GraphPad Prism Software, USA). The Gaussian distribution of the data was verified using the Kolmogorov–Smirnov test. Statistics were performed using one-way analysis of variance (ANOVA) with post hoc Tukey tests, cross-comparing all study groups (95% confidence interval). *P*-values <0.05 were considered significant.

Results

Cytotoxicity

Aiming to test the potential use of CSE as MMP inhibitor in chondrocytes, we first evaluated the cytotoxic effects of the extract on primary human chondrocytes using an MTT-based viability assay. As shown in Fig. 1, CSE concentrations up to 20 µg/ml did not induce cytotoxicity in chondrocytes, whereas 40 µg/ml CSE significantly reduced viability (47.0 ± 2.2%). In the subsequent assays, therefore, we used the concentrations of CSE below 20 µg/ml.

CSE does not directly modify gelatinolytic activities

Previous work has suggested that MMP-2 is involved in both the physiological collagen turnover in articular cartilage and the matrix degradation in OA cartilage (Duerr et al. 2004). Using gelatin zymography, the present study confirms that chondrocytes isolated from OA cartilage, as well as SW1353 cells (data not shown), express functional pro-MMP-2 (Fig. 2a). In contrast, bands corresponding to MMP-9 activity were hardly detectable (data not shown), reflecting the differences in MMP-2 and MMP-9 mRNA levels (Table 2). The images also indicate that the activity of MMP2 was not affected in primary chondrocytes treated with 5 µg/ml CSE (Fig. 2a). Similarly, when added to the buffer during zymography development, CSE did not substantially affect the enzymatic activity of recombinant MMP-2, whereas the addition of EDTA completely abrogated the gelatinolytic activity (Fig. 2b).

CSE inhibits IL1β -stimulated production of MMP-13

In order to investigate the effect of CSE on MMP-13 and MMP-2 protein levels produced by human chondrocytes, supernatants of treated cell cultures were collected and subjected to ELISA. We observed that neither IL1β nor CSE significantly modified the expression of MMP-2 in primary or SW1353 cells (data not shown). In contrast, MMP-13 levels were significantly up-regulated in both SW1353 and primary cells after treatment with IL1β (Fig. 3). Pretreatment of SW1353 cells with 5 µg/ml CSE significantly reduced IL1β-stimulated MMP-13 production from 6.7 ± 1.6 to 2.5 ± 1.4 pg/ml, which was comparable to the levels found in untreated cells (1.2 ± 0.5 pg/ml; $P > 0.05$). In primary chondrocytes, CSE significantly reduced IL1β-stimulated MMP-13 levels from 16.3 ± 1.4 to 13.1 ± 2.7 pg/ml ($P < 0.05$; $n = 3$ donors).

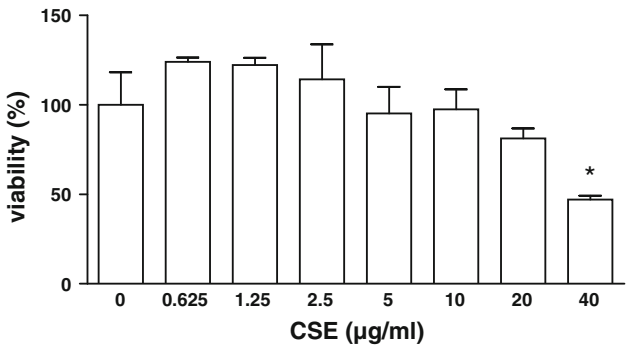

Fig. 1 Cell viability of primary chondrocytes treated with CSE. Serum-starved primary chondrocytes were treated for 72 h with varying concentrations of CSE (up to 40 µg/ml). Cell-viability was evaluated using a MTT-based proliferation assay. The experiment was repeated twice with cells from different donors, and each experimental condition was set up in quadruplicate. Mean values and standard deviations are given with respect to untreated cells (100% viability). *indicates significant differences with respect to untreated controls ($P < 0.05$)

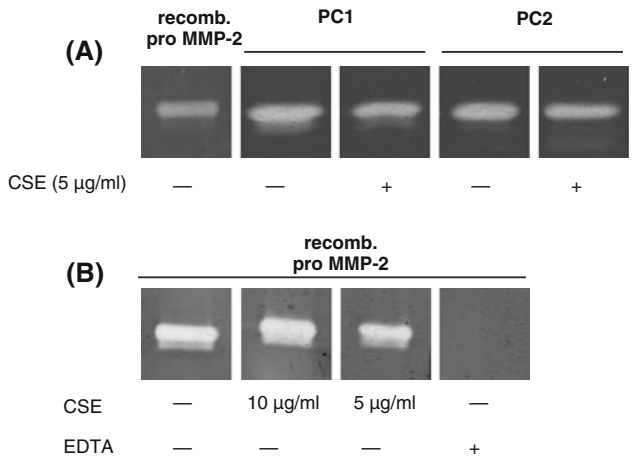

Fig. 2 CSE does not directly interfere with the gelatinolytic activity of MMP-2. **a** Cell culture supernatants of primary chondrocytes isolated from 2 patients (PC1 and PC2) were treated and subjected to gelatine zymography as described in Materials and Methods. *Lane 1* shows the activity of a recombinant human pro-MMP-2. *Lanes 2* and *4* show the supernatants of untreated chondrocytes, whereas *lanes 3* and *5* present the supernatants of the chondrocytes treated with 5 µg/ml CSE, respectively. **b** Recombinant human pro-MMP-2 was subjected to gelatine zymography and developed separately in different developing buffers containing 5 µg/ml CSE, 10 µg/ml CSE or 1 mM EDTA

Table 2 mRNA expression levels of MMPs and TIMPs in SW1353 cells and primary chondrocytes

	MMP1	*MMP2*	*MMP3*	*MMP7*	*MMP8*	*MMP9*	*MMP13*	*TIMP1*	*TIMP2*	*TIMP3*
SW1353	**0.001**	**0.22**	**0.004**	**0.0003**	**0.000004**	**0.0001**	**0.00003**	**0.06**	**0.13**	**0.01**
PC1	0.21	0.01	26.0	0.0000003	0.00003	0.000837	0.031	0.51	0.16	0.05
PC2	0.29	0.09	3.4	0.0000367	0.00006	0.000015	0.023	0.67	2.38	0.36
PC3	0.25	0.01	7.0	0.0000437	0.00008	0.000005	0.018	0.73	0.04	0.01
Mean PC (n=3)	**0.25**	**0.04**	**12.1**	**0.00003**	**0.00006**	**0.0003**	**0.024**	**0.64**	**0.86**	**0.14**
SD	**0.04**	**0.05**	**12.2**	**0.00002**	**0.00002**	**0.0005**	**0.007**	**0.11**	**1.32**	**0.19**

mRNA levels were quantified using RT-qPCR and expressed as number of molecules per molecules of GAPDH. Articular chondrocytes were isolated from cartilage specimens of 3 donors (PC1, PC2, PC3) and cultured and analyzed separately. Mean values and standard deviations (SD) of the 3 primary cell populations are also presented

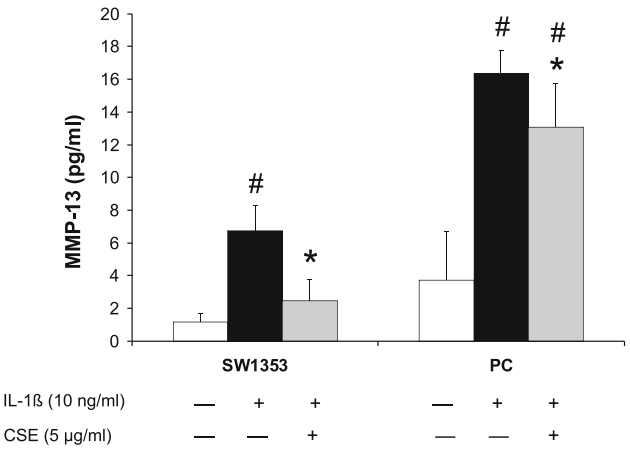

Fig. 3 CSE inhibits IL1β-stimulated MMP-13 production in human chondrocytes. Primary chondrocytes (PC) were isolated from 3 donors and cultured and analyzed separately. Serum-starved SW1353 cells and PC were pretreated with 5 µg/ml CSE followed by the coincubation with 10 ng/ml IL1β for 48 h. MMP-13 concentration was determined in the cell culture supernatants using ELISA. Mean values and standard deviations from 2 independent experiments (SW1353) and from 3 donors (PC) are given. *indicates significant differences with respect to cells treated with IL1β only ($P < 0.05$). #indicates significant differences with respect to untreated controls ($P < 0.05$)

CSE inhibits gene expression of MMPs, but not of TIMPs

To evaluate the effects of CSE on MMP and TIMP expression in human chondrocytes, mRNA levels of 7 MMPs (MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13) and 3 TIMPs (TIMP-1, TIMP-2, TIMP-3) were quantified using RT-qPCR (Table 2). In agreement with previous reports (Fan et al. 2005), MMP-3 was the most abundant (12.1 ± 12.2 molecules/molecules GAPDH) of the MMPs analyzed in human OA primary chondrocytes, with MMP-7 and MMP-8 showing the lower expression levels. The expression levels of TIMP-1, TIMP-2, and TIMP-3 were relatively higher compared with most MMPs, with lower expression of TIMP-3 (Table 2). Interestingly, the SW1353 cell line showed a different MMP and TIMP expression profile as compared to primary cells (Table 2), with MMP-2 being the most abundant (0.22 molecules/molecule GAPDH) and very low levels of MMP-1, MMP-3, and MMP-13 mRNA.

IL1β up-regulated the expression of a number of MMPs in SW1353 cells, including MMP-1 (Fig. 4a, 64.0 ± 3.1 fold), MMP-3 (Fig. 4c, 27.4 ± 0.8 fold), MMP-7 (Fig. 4d, 3.2 ± 0.1 fold), MMP-8 (Fig. 4e, 6.3 ± 1.1 fold), MMP-9 (Fig. 4f, 1.8 ± 0.1 fold), and MMP-13 (Fig. 4g, 71.4 ± 1.1 fold). Preincubation of SW1353 cells with CSE suppressed the IL1β-induced mRNA levels of these MMPs in a dose-dependent manner. Of note, 5 µg/ml CSE strongly inhibited the expression of MMP-1 (Fig. 4a), MMP-3 (Fig. 4c), and MMP-13 (Fig. 4g) mRNA as compared to IL1β-treated cells ($P < 0.05$), and 10 µg/ml CSE further down-regulated the expression of these genes to levels comparable to control cells ($P > 0.05$). In addition, 5 µg/ml of CSE reduced the IL-1β-induced MMP-7 (Fig. 4d), MMP-8 (Fig. 4e), and MMP-9 (Fig. 4f) expression to basal levels. Regarding MMP-2 gene expression, no alterations were observed in SW1353 cells under the experimental conditions of this study (Fig. 4b).

In keeping with those observations, the results obtained with human OA primary chondrocytes ($n = 3$; PC1,2,3) further confirmed those obtained in SW1353 cells, as shown in Table 3. Namely, despite minor inter-individual differences, IL1β significantly increased mRNA levels of MMP-1, MMP-3, MMP-7, MMP-9, and MMP-13 in all 3 cell populations. At 5 µg/ml, CSE significantly suppressed the IL1β-mediated upregulation of MMP-3, MMP-7, MMP-9, and MMP-13 in all cell populations and that of MMP-1 in 2 out of 3 donors. Similar to the results in SW1353 cells,

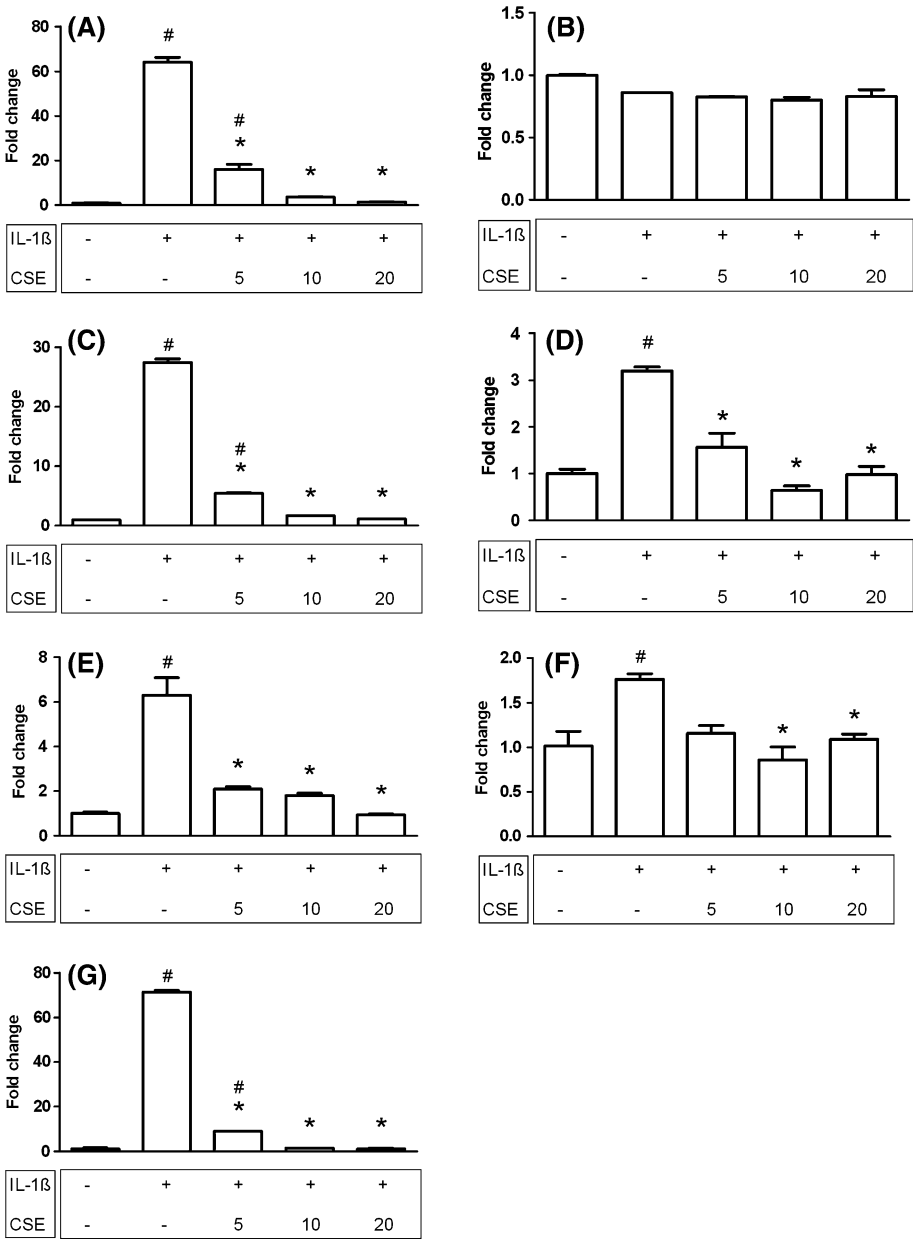

Fig. 4 CSE inhibits IL1β-mediated MMP transcription in SW1353 cells. Serum-starved SW1353 cells were pretreated with 5, 10, or 20 µg/ml of CSE followed by the addition of 10 ng/ml IL1β. After 6 h of incubation, total RNA was isolated and mRNA levels of **a** MMP-1, **b** MMP-2, **c** MMP-3, **d** MMP-7, **e** MMP-8, **f** MMP-9, and **g** MMP-13 were quantified using RT-qPCR and expressed as fold change with respect to untreated controls. Mean values and standard deviations are given. *indicates significant differences with respect to cells treated with IL1β only ($P < 0.05$). #indicates significant differences with respect to untreated controls ($P < 0.05$)

10 and 20 µg/ml CSE reduced the IL-1β-induced MMP mRNA expression to basal levels. Expression of MMP-2 and MMP-8 were only slightly altered in the presence of IL1β and CSE ($P < 0.05$ in 1 out of 3 donors).

Since the balance between MMP and TIMP activities determines cartilage degradation in OA [Nagase and Woessner 1999), we examined the effect of 5 µg/ml CSE on the gene expression of TIMP-1, TIMP-2, and TIMP-3 in IL1β-treated chondrocytes (Fig. 5). In primary cells, neither IL1β nor 5 µg/ml CSE regulated the mRNA levels of any of the TIMPs (Fig. 5a, b, c). In SW1353 cells, however, TIMP-1 and TIMP-3 mRNA levels were up-regulated by IL1β 2.1 ± 0.3 fold and 3.0 ± 0.1 fold, respectively. Treatment with 5 µg/ml CSE reduced only TIMP-3 mRNA levels significantly in SW1353 cells (Fig. 5d).

These results suggest that CSE is a potent inhibitor of IL1β-mediated MMP transcription in human chondrocytes, whereas it appears not to interfere with TIMP gene expression.

CSE inhibits MMP-13 promoter activation

It has been shown that IL1β-induced MMP gene expression is mediated by diverse signaling pathways and transcription

Table 3 Regulation of MMP transcription by IL1β and CSE in primary chondrocytes

		Ctrl	IL-1β			
				+CSE 5 μg/ml	+CSE 10 μg/ml	+CSE 20 μg/ml
MMP-1	PC1	1.00 ± 0.10	5.76 ± 0.26*	1.83 ± 0.03**	0.91 ± 0.07**	0.78 ± 0.06**
	PC2	1.00 ± 0.18	15.4 ± 0.01*	10.12 ± 0.45**	2.21 ± 0.22**	0.91 ± 0.10**
	PC3	1.00 ± 0.15	4.24 ± 0.28*	3.63 ± 0.18	3.10 ± 0.06**	1.27 ± 0.09**
MMP-2	PC1	1.00 ± 0.06	1.46 ± 0.06*	1.05 ± 0.09**	0.97 ± 0.05**	0.83 ± 0.02**
	PC2	1.00 ± 0.02	1.17 ± 0.05	1.08 ± 0.15	1.06 ± 0.13	0.82 ± 0.02
	PC3	1.00 ± 0.09	1.24 ± 0.03	1.23 ± 0.04	1.55 ± 0.08**	1.06 ± 0.08
MMP-3	PC1	1.00 ± 0.01	3.16 ± 0.01*	1.35 ± 0.02**	1.08 ± 0.01**	0.84 ± 0.01**
	PC2	1.00 ± 0.01	8.37 ± 0.25*	3.71 ± 0.09**	1.12 ± 0.01**	1.05 ± 0.04**
	PC3	1.00 ± 0.01	4.74 ± 0.02*	3.12 ± 0.02**	3.48 ± 0.02**	1.15 ± 0.01**
MMP-7	PC1	1.00 ± 0.46	10.40 ± 1.06*	5.56 ± 0.92**	1.97 ± 1.60**	3.41 ± 0.02**
	PC2	1.00 ± 0.47	8.19 ± 0.26*	2.41 ± 0.27**	3.15 ± 1.33**	2.14 ± 0.82**
	PC3	1.00 ± 0.19	4.45 ± 0.17*	3.02 ± 0.12**	2.78 ± 0.06**	3.04 ± 0.11**
MMP-8	PC1	1.00 ± 0.57	1.29 ± 0.02	1.92 ± 0.22	0.65 ± 0.35	1.32 ± 0.58
	PC2	1.00 ± 0.06	2.11 ± 0.33*	1.69 ± 0.35	1.02 ± 0.02**	0.57 ± 0.11**
	PC3	1.00 ± 0.02	1.44 ± 0.13	1.14 ± 0.03	2.04 ± 0.26	0.89 ± 0.17
MMP-9	PC1	1.00 ± 0.01	1.89 ± 0.28*	1.11 ± 0.04**	0.85 ± 0.16**	1.03 ± 0.10**
	PC2	1.00 ± 0.04	10.29 ± 1.28*	2.64 ± 0.36**	0.66 ± 0.16**	0.54 ± 0.06**
	PC3	1.00 ± 0.37	7.65 ± 1.48*	3.76 ± 1.15**	1.22 ± 0.14**	2.15 ± 0.18**
MMP-13	PC1	1.00 ± 0.17	13.22 ± 0.07*	1.39 ± 0.03**	0.72 ± 0.04**	0.64 ± 0.05**
	PC2	1.00 ± 0.01	10.42 ± 0.37*	2.71 ± 0.23**	1.11 ± 0.03**	0.85 ± 0.01**
	PC3	1.00 ± 0.02	4.65 ± 0.40*	2.80 ± 0.24**	4.42 ± 0.54	1.20 ± 0.01**

Primary chondrocytes were isolated from 3 donors (PC1, PC2, PC3) and cultured and analyzed separately. Serum-starved cells were pretreated with 5 to 20 μg/ml CSE followed by stimulation with 10 ng/ml IL1β for 6 h. mRNA levels of MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13 were quantified using RT-qPCR and expressed as fold change with respect to untreated controls. Mean values and standard deviations are given. * indicates significant differences with respect to untreated controls ($P < 0.05$). ** indicates significant differences with respect to cells treated with IL1β only ($P < 0.05$)

factors, including NF-kB (Vincenti and Brinckerhoff 2002; Marcu et al. 2010). To further explore the mechanism underlying suppression of MMP transcription by CSE, human T/C-28a2 chondrocytes were co-transfected with a MMP-13 reporter construct and p65/p50 expression vectors in the presence or absence of CSE. As shown in Fig. 6, p65/p50 overexpression transactivated the pGL2B(−1007/+27)MMP-13 construct by 7.1 ± 1.5 fold, and CSE reversed the p65/p50-driven MMP-13 promoter transactivation in a concentration-dependent manner. Whereas 5 μg/ml CSE significantly inhibited MMP-13 promoter activity by about 65%, 10 μg/ml CSE completely blocked the p65/p50-mediated promoter activation, re-establishing levels comparable to those in the untreated control ($P > 0.05$).

For comparison, additional experiments using CAPE were performed (Fig. 6). CAPE dose-dependently reduced the p65/p50-dependent promoter activation at a concentration of 0.5 μg/ml ($P < 0.05$) and re-established control levels at 2.5 μg/ml.

Discussion

The present study shows for the first time that CSE inhibits MMP expression in human chondrocytes in vitro. As our data demonstrate, pretreatment of primary chondrocytes with CSE effectively suppressed the IL1β-mediated upregulation of MMP-1, MMP-3, MMP-7, MMP-9, and MMP-13 at the level of gene expression involving NFkB signaling.

Since exacerbated production and activation of MMPs is associated with rheumatoid arthritis and OA, MMPs represent promising pharmacological targets for the treatment of arthritic diseases (Jacobsen et al. 2010). Strategies for MMP inhibition include direct MMP inhibition (by TIMPs, small molecule MMP inhibitors, or antibodies) and indirect MMP inhibition targeting signaling pathways that lead to MMP expression. Although considerable progress has been made in the development of direct MMP inhibitors, severe off-target effects, poor bioavailability and efficacy are frequently associated with their use in clinical trials

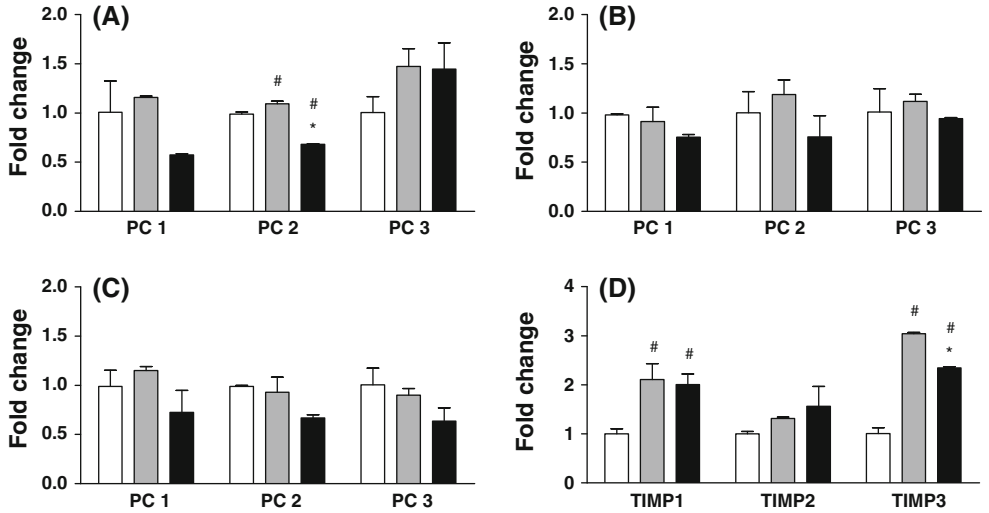

Fig. 5 Regulation of TIMP transcription by IL1β and CSE in SW1353 cells and primary chondrocytes. **a–c** Primary chondrocytes were isolated from 3 donors (PC1, PC2, PC3) and cultured and analyzed separately. Serum-starved cells were pretreated with 5 µg/ml CSE followed by the addition of 10 ng/ml IL1β. After 6 h of incubation, the mRNA levels of TIMP-1 (**a**), TIMP-2 (**b**), and TIMP-3 (**c**) were quantified using RT-qPCR and expressed as fold change with respect to untreated controls (*white bars*). *Gray bars* represent IL1β-treated cells, whereas *black bars* represent cells pretreated with CSE. **d** TIMP-1, TIMP-2, and TIMP-3 mRNA levels were quantified in SW1353 cells under exposure to IL1β and CSE as described. Mean values and standard deviations are given. *indicates significant differences with respect to cells treated with IL1β only ($P < 0.05$). #indicates significant differences with respect to untreated controls ($P < 0.05$)

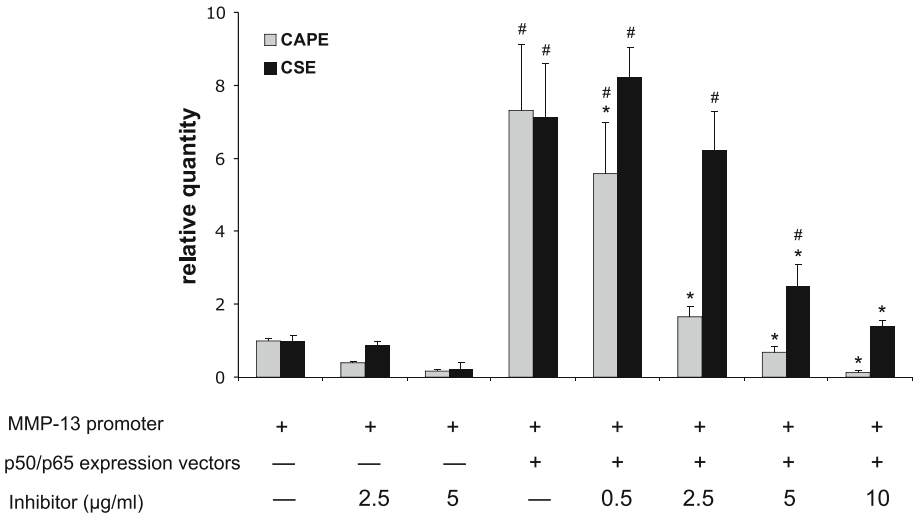

Fig. 6 Effect of CSE or CAPE on MMP-13 promoter activity. T/C28a2 chondrocytes, cotransfected with a MMP-13 luciferase reporter construct and p50 and p65 expression vectors, were treated with CAPE (*gray bars*) or CSE (*black bars*) in a dose-dependent manner. Both CAPE and CES inhibited the NF-kB(p65/p50)-driven MMP-13 promoter activation. #indicates significant differences with respect to cells transfected with the MMP-13 reporter construct and the pCI empty vector ($P < 0.05$). *indicates significant differences with respect to cells co-transfected with the MMP-13 promoter and the p65/p50 expression vectors ($P < 0.05$)

(Clutterbuck et al. 2009). Thus, indirect MMP inhibition using nutraceuticals or plant-derived phytopharmaceuticals might provide a promising alternative to present therapeutic approaches. In this context, curcumin, resveratrol, or green tea catechins have already been investigated regarding their MMP-inhibitory actions (Shakibaei et al. 2007; Lee and Moon 2005; Annabi et al. 2007).

The results presented here suggest that *Caesalpinia sappan*-derived phytochemicals might be developed as nutraceuticals for the management of arthritic diseases. In this context, the strong inhibitory action of CSE on the IL1β-mediated overexpression of MMP-1, MMP-3, and MMP-13 appears of particular interest. Exacerbated production and activation of the collagenases, MMP-1 and

MMP-13, in articular chondrocytes are known to promote the cleavage of collagen triple helices allowing further degradation by other MMPs (Nagase and Woessner 1999). Supporting the role of MMP-13 in connective tissue degradation, Neuhold et al. (2001) reported that transgenic mice overexpressing MMP-13 show signs of articular cartilage degradation and exhibit joint pathology similar to that found in OA. Our results confirm that IL1β represents a potent inflammatory stimulus that leads to overexpression of MMP-1, MMP-3, and MMP-13 mRNA in primary and SW1353 chondrocytes. Interestingly, the upregulation of these genes by IL1β was more pronounced in SW1353 cells than in primary OA chondrocytes; however, when cultured in the presence of 5–20 μg/ml CSE, the effect of IL1β was suppressed in both cell models and the expression of MMP-1, MMP-3, and MMP-13 mRNA was downregulated to levels found under control conditions. The relevance of the RT-qPCR data was further verified by ELISA, demonstrating reduced MMP-13 protein levels in chondrocytes treated with CSE. In contrast, however, the transcription of TIMPs was not affected by the presence of CSE. Of note, CSE treatment did not totally block the expression of MMPs but rather inhibited IL1β-induced MMP overexpression. In this regard, CSE might be advantageous over other MMP inhibitors, since total MMP inhibition not only impairs physiological ECM remodeling, but may also affect cellular signaling to which MMPs contribute (Clutterbuck et al. 2009). In this context, it is noteworthy that CSE did not directly influence the gelatinolytic activity of MMP-2 under the experimental conditions of this study.

In a first attempt to elaborate the mechanism underlying the MMP-inhibitory action of CSE, we focused on the role of NF-kB in MMP-13 promoter activation. It is well established that IL1β, once bound to its type 1 receptor, activates NF-kB dimers by triggering phosphorylation and subsequent degradation of the inhibitory kB proteins (Daheshia and Yao 2008). The most prevalent activated NF-kB dimer is the heterodimer RelA (p65)/p50, which can bind to sites in NF-kB-dependent promoters regulating the transcription of response genes (Roman-Blas and Jimenez 2006). Mengshol et al. have assessed the role of NF-kB in MMP-13 transcription in chondrocytes and found that, besides JNK and p38, NF-kB signaling is required for the IL1β-mediated induction of MMP-13 (Mengshol et al. 2000). Although the promoter of MMP-13 does not contain consensus NF-kB binding sites (Burrage et al. 2006), the link between NF-kB activation and MMP-13 induction has been further demonstrated in a previous study on the effect of hyaluronan oligosaccharides in articular chondrocytes (Ohno et al. 2006). We show here that, indeed, NF-kB p65/p50 overexpression leads to MMP-13 promoter transactivation and that CSE suppresses the p65/p50-driven MMP-13 promoter activation, thereby suggesting that CSE's MMP-inhibitory actions are related to its blockade of the NF-kB signaling. Furthermore, the comparison to CAPE, a chemically defined specific inhibitor of NF-kB (Natarajan et al. 1996), revealed that CSE was only about 4 times less active in our assay. Our results go along with previous reports demonstrating that CSE and its constituents interfere with NF-kB signaling in different cell types. Namely, Bae et al. (2005) showed that brazilin, the main component of CSE, inhibited the DNA binding activity of NF-kB and AP-1 in LPS-stimulated mouse macrophages. In addition, protosappanin A was shown to target the NF-kB pathway in rat heart tissue (Wu et al. 2010). Therefore, our results suggest that CSE contains one or more active components that may act as potent inhibitors of NF-kB in human chondrocytes.

To date, the bioavailability of specific components of CSE has not been elaborated and it remains elusive whether the doses of CSE used in this study are representative for physiologically active concentrations. Despite this lack of specific knowledge, the efficacy of CSE in diverse in vivo models supports the apparent bioavailability of CSE components at therapeutically effective doses (Sun et al. 2011; Oh et al. 2001). Moreover, the present study demonstrates that CSE affects human chondrocytes in vitro at concentrations that are comparable to those of well-known dietary antioxidants including ascorbic acid, curcumin, or resveratrol (Csaki et al. 2009; Graeser et al. 2009). Similar to curcumin and resveratrol, CSE appears to exert its effects on chondrocytes via mechanisms targeting NFkB signaling. Interestingly, recent data have explored the different molecular targets of curcumin and resveratrol as well as their synergistic chondroprotective effects (Csaki et al. 2009). In this context, it might be of interest to further investigate the synergism between CSE components and other dietary antioxidants regarding the modulation of biomarkers of inflammation in cultured chondrocytes.

Conclusion

In summary, this study suggests that CSE or CSE-derived compounds may be beneficial as indirect MMP inhibitors for the future management of degenerative or inflammatory joint diseases. We present evidence that CSE effectively suppresses IL1β-mediated overexpression of major MMPs in human chondrocytes in vitro. More specifically, CSE inhibits the de novo synthesis of MMP-1, MMP-3, MMP-7, MMP-9, and MMP-13 at the gene expression level without affecting the expression of TIMPs. Further, we showed that the molecular mechanism underlying MMP-13 inhibition by CSE involves, at least in part, the interference with the

NF-kB (p65/p50)-driven MMP-13 promoter transactivation. Future studies should therefore aim to define CSE's actions in the context of arthritic diseases and to further delineate the molecular mechanisms evoked by CSE and its specific constituents both in vitro and in vivo.

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Conflict of interest The authors declare that they have no conflict of interest.

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Anti-inflammatory activity of an ethanolic *Caesalpinia sappan* extract in human chondrocytes and macrophages

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ABSTRACT

Ethnopharmacological relevance: Caesalpinia sappan is a common remedy in Traditional Chinese Medicine and possesses diverse biological activities including anti-inflammatory properties. Osteoarthritis (OA) is a degenerative joint disease with an inflammatory component that drives the degradation of cartilage extracellular matrix. In order to provide a scientific basis for the applicability of *Caesalpinia sappan* in arthritic diseases, the present study aimed to assess the effects of an ethanolic *Caesalpinia sappan* extract (CSE) on human chondrocytes and macrophages.

Materials and methods: Primary human chondrocytes were isolated from cartilage specimens of OA patients. Primary cells, SW1353 chondrocytes and THP-1 macrophages were serum-starved and pre-treated with different concentrations of CSE prior to stimulation with 10 ng/ml of interleukin-1beta (IL-1β) or lipopolysaccharide (LPS). Following viability tests, nitric oxide (NO) and tumor necrosis factor-alpha (TNF-α) were evaluated by Griess assay and ELISA, respectively. Using validated real-time PCR assays, mRNA levels of IL-1β, TNF-α, inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2) were quantified. SW1353 cells were cotransfected with a COX-2 luciferase reporter plasmid and nuclear factor-kappa-B (NF-κB) p50 and p65 expression vectors in the presence or absence of CSE.

Results: CSE dose-dependently inhibited the expression of pro-inflammatory cytokines IL-1β and TNF-α in IL-1β-stimulated chondrocytes and LPS-stimulated THP-1 macrophages. CSE further suppressed the synthesis of NO in primary OA chondrocytes by blocking iNOS mRNA expression. The inhibition of COX-2 transcription was found to be related with the CSE inhibition of the p65/p50-driven transactivation of the COX-2 promoter.

Conclusions: The present report is first to demonstrate the anti-inflammatory activity of CSE in an in vitro cell model of joint inflammation. CSE can effectively abrogate the IL-1β-induced over-expression of inflammatory mediators at the transcriptional level in human chondrocytes and macrophages, most likely by inhibiting NF-κB (p65/p50) signaling. Blockade of IL-1β-induced NF-κB signaling and its down-stream pro-inflammatory targets by CSE may be beneficial for reducing cartilage breakdown in arthritis.

1. Introduction

Osteoarthritis (OA) ranks among the major causes of physical disability of elderly patients, thus representing a critical factor in health economics. In contrast to rheumatoid arthritis, OA is conventionally not considered a classical inflammatory arthropathy, but thought to develop from chronic overuse or injury of the joint. However, evidence has accumulated that, besides mechanical and genetic factors, inflammatory processes within joint tissues contribute to the OA onset and progression. Chondrocytes, the unique cell component of articular cartilage, are embedded in a highly organized extracellular matrix (ECM), comprising collagen type II fibrils and proteoglycans, which confer to the cartilage structural rigidity and protective resiliency (Goldring and Marcu, 2009). In response to mechanical or biochemical stress, chondrocytes over-express pro-inflammatory mediators such as interleukin-1beta (IL-1β) and tumor necrosis factor-alpha (TNF-α) that will, in an autocrine/paracrine manner, stimulate their own production and

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Nomenclature

OA	Osteoarthritis
CSE	ethanolic *Caesalpinia sappan* extract
IL-1β	interleukin-1 beta
LPS	lipopolysaccharide
NO	nitric oxide
TNF-α	tumor necrosis factor- alpha
iNOS	inducible nitric oxide synthase
COX-2	cyclooxygenase-2
NF-κB	nuclear factor-kappa-B
ECM	extracellular matrix
MMPs	matrix metalloproteinases
ADAMTS	a disintegrin and metalloproteinase with thrombospondin motifs
PGE_2	prostaglandin E_2
NSAIDs	nonsteroidal anti-inflammatory drugs
DMEM	Dulbecco's modified Eagle's medium
FCS	fetal calf serum
PMA	phorbol 12-myristate 13-acetate
RT-qPCR	quantitative real-time RT-PCR
ITS	insulin–transferrin–selenium
RIN	RNA integrity numbers
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
ACTB	beta actin
HPRT1	hypoxanthine phosphoribosyl-transferase I
SDHA	succinate dehydrogenase complex, subunit A
B2M	beta-2-microglobulin
PC	primary chondrocytes

induce the expression of matrix-degrading proteinases, including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) (Bonnet and Walsh, 2005; Goldring and Goldring, 2007). Recently, it has been demonstrated that mediators such as prostaglandin E_2 (PGE_2) (Gosset et al., 2010) or nitric oxide (NO) (Tsuruda et al., 2004) play key roles in the induction of MMPs in an inflammatory context. In addition to the chondrocytes, macrophages that have infiltrated the OA synovium contribute to inflammation and matrix degradation in OA tissues (Benito et al., 2005; Bondeson et al., 2006). Therefore, inflammatory mediators represent potential targets for OA disease modification.

The mechanism of cartilage degradation in OA is known to be a multifactorial process. However, standard pharmacological interventions, such as the use of nonsteroidal anti-inflammatory drugs (NSAIDs), often act in a monomodal way that is frequently associated with significant adverse effects (Fendrick and Greenberg, 2009). Although considerable progress has been made in the development of novel strategies, such as the use of direct MMP inhibitors, no clinically effective inhibitor exists to date. Thus, novel, safe and effective anti-inflammatory agents are demanded for the therapy of arthritic diseases. Nutraceuticals and phytopharmaceuticals that usually contain a range of active compounds targeting multiple pathways could provide an alternative to conventional treatment of OA (Clutterbuck et al., 2009). A comprehensive review on current nutraceuticals for the management of OA has been published elsewhere (Ameye and Chee, 2009).

Sappan Lignum, the heartwood of *Caesalpinia sappan* L. (Leguminosae), is the source of a natural red dye. In traditional Chinese medicine, Sappan wood has sweet, salty, and neutral characteristics, and is associated with the heart, liver and spleen meridians. Traditionally, it is applied as an aqueous decoction and prescribed to invigorate the blood system, promote menstruation, reduce pain and swelling (Chinese Pharmacopoeia, 2010). Additionally, Sappan wood has been medicinally recommended due to its astringent or diuretic properties, as well as for certain skin diseases (Sireeratawong et al., 2010). Recently, it has also been listed in the 15th edition of the Japanese Pharmacopoeia (Japanese Pharmacopoeia, 2006). Different extracts and active compounds isolated from Sappan lignum have been reported to possess diverse biological activities including antioxidative (Budami et al., 2003), anti-inflammtory (Jeong et al., 2008), antibacterial (Xu and Lee, 2004) and anticonvulsive (Baek et al., 2000) effects. Brazilin, the major compound of Sappan Lignum, was reported to have anti-inflammatory (Hikino et al., 1977; Washiyama et al., 2009), antibacterial (Batubara et al., 2010), and antihepatotoxic effects (You et al., 2005). In addition, numerous other compounds from Sappan Lignum such as prostosappanins A–E or sappanchalcone have also been shown to display some of the different biological activities (Liu et al., 2009). To our knowledge, however, *Caesalpinia sappan* has not been evaluated in the context of OA, and the effects of *Caesalpinia sappan* derived isolates on OA chondrocytes and synovial macrophages are unknown.

The present study, therefore, aimed to elucidate the anti-inflammatory activity of an ethanolic *Caesalpinia sappan* extract (CSE) in lipopolysaccharide (LPS)-stimulated human macrophages and IL-1β-stimulated human chondrocytes. Particular focus was on the inhibition of gene expression of proinflammatory factors such as IL-1β and TNF-α, and on the interference with NO production and NF-κB-mediated COX-2 promoter activation.

2. Materials and methods

2.1. Plant material and extraction

The heartwoods of *Caesalpinia sappan* were collected in June 2006 in San-Pa-Thong district, Chiang Mai province, Thailand, and identified by comparison with the voucher specimen (No. 87-1631) at the Herbarium Section, Northern Research Center for Medicinal Plants, Faculty of Pharmacy, Chiang Mai University, Thailand. Powdered heartwood (30 g) was extracted with 95% ethanol (350 ml) for 24 h using a Soxhlet apparatus and the resulting extract was concentrated under vacuum to yield a solid extract (5.5 g).

2.2. Characterization of the extract

Four batches of CSE were characterized by HPLC (ICS-3000, Dionex, USA) with a Nucleodur C-18 column (Macherey-Nagel, D) and a PDA-100 detector (Dionex) set at 280 nm. The eluent system consisted of a 45 min gradient program from 10% to 100% methanol (containing 2.5% acetic acid) at a flow rate of 0.8 ml/min. All 4 batches yielded congruent chromatograms, which were also comparable to that of Natural Red 24 (MP Biomedicals, USA, LOT #: R25179), a commercially available Sappan wood extract (Supplementary Figs. 1A and B).

Brazilin was isolated from CSE using preparative HPLC (Shimadzu, Japan) with a reversed-phase C18 column (Polygoprep C18, 12 μ, 25 mm × 250 mm, Austria) monitored at 280 nm. The eluent system consisted of a 45 min gradient program from 10% to 100% methanol (containing 1.25% acetic acid) at a flow rate of 22 ml/min and a column temperature of 50 °C. The fraction at a retention time of about 20 min was collected in repeated preparative HPLC separations. These fractions were further concentrated under reduced pressure and then freeze-dried to give a white compound which rapidly oxidized to a red-brown powder. In accordance with Chen et al. (Chen et al., 2008), the isolated compound was identified as brazilin ((6aS,11bR)-7,11b-dihydro-6H-indeno[2,1-c]chromene-3,6a,9,10-tetrol) using ^{1}H NMR (Bruker Avance 500

Table 1
Details on RT-qPCR primers.

Gene symbol	Primer sequences	Efficiency	Amplicon size	NM-number
IL-1β	Fw: CCTATTACAGTGGCAATGAGGATG Rv: AGTGGTGGTCGGAGATTCG	95.1%	132bp	NM_000576
TNF-α	Fw: TCAGCAAGGACAGCAGAGG Rv: CAGTATGTGAGAGGAAGAGAACC	96%	126bp	NM_000594
iNOS	Fw: GTTCTCACGGCACAGGTCTC Rv: GCAGGTCACTTATGTCACTTATC	94.6%	127bp	NM_000625
COX-2	Fw: CCGAGGTGTATGTATGAGTGTG Rv: TGTGTTTGGAGTGGGTTTCAG	96.5%	136bp	NM_000963

instrument, Bruker, USA). The HPLC chromatogram of brazilin is shown in Supplementary Fig. 1C.

2.3. Cell cultures

In accordance with the terms of the ethics committee of the Medical University Vienna (EK-Nr.: 081/2005) and following the guidelines of the Declaration of Helsinki and Tokyo, primary human chondrocytes were enzymatically isolated from articular cartilage of OA patients undergoing knee replacement surgery following established protocols (Toegel et al., 2009, 2010). Isolated chondrocytes were seeded at a density of $10^5/cm^2$ and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, A) containing 10% fetal calf serum (FCS; Biochrom AG, D) and 2 μl/ml gentamycin (Biochrom AG, D) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. For all assays, only freshly isolated and seeded cells without subculturing were used. The human chondrosarcoma cell line SW1353 (ATCC, USA) was cultured at a density of $10^4/cm^2$ under the same conditions as described above and passaged by incubation with 0.25% trypsin-EDTA. The human monocytic cell line THP-1 (ATCC, USA) was maintained in RPMI 1640 medium supplemented with 10% FCS, 4 mM L-glutamine, and 104 U/ml penicillin (Gibco, A) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Cells were differentiated into macrophages in the above medium containing 5 ng/ml phorbol 12-myristate 13-acetate (PMA) over 24 h. After washing once, cultures were changed to medium without PMA for 24 h and then used for further studies.

2.4. Cytotoxicity assay

The cytotoxicity of CSE was assessed in primary human chondrocytes and THP-1 cells using an MTT-based test (EZ4U, Biomedica, A) following the manufacturer's instructions. Briefly, 3×10^3 cells/well were seeded in quadruplicate in 96-well microplates (Iwaki, J) and cultured with various concentrations of CSE (0.625–40 μg/ml) for 3 days. The absorbance was recorded by a Spectrafluor Fluorometer (Tecan, A) at 450 nm with 620 nm as reference wave length.

2.5. Griess assay

Primary human OA chondrocytes ($n = 3$ donors) were seeded in 96-well microplates and analyzed separately. Upon confluency, cells were preincubated with CSE at three concentrations (10 μg/ml, 5 μg/ml or 2.5 μg/ml) for 1 h prior to coincubation with 10 ng/ml IL-1β (Miltenyi Biotec, D) for 24 h. The amount of accumulated nitrite derived from NO metabolism was determined in the cell culture supernatant by mixing with an equal volume of Griess reagent (1% sulphanilamide in 0.1 N HCl and 0.1% N-1-naphthylethylenediamine dihydrochloride in equal parts). Absorbance was measured at 544 nm with reference to 690 nm with a Spectrafluor Fluorometer. Nitrite concentrations were calculated using a $NaNO_2$ standard curve (0–100 μM in cell culture medium). A nitrite standard reference curve was established for each assay.

2.6. Quantitative real-time RT-PCR (RT-qPCR)

4×10^5/well primary human OA chondrocytes or 4×10^4/well SW1353 cells were seeded in 12-well microplates (Iwaki, J). Upon 90% confluency, medium was changed to DMEM containing 2% insulin–transferrin–selenium (ITS; Gibco, A) and incubated overnight prior to treatment. Chondrocytes were preincubated with CSE (5, 10, or 20 μg/ml) or 10 μM dexamethasone (Sigma, A) for 1 h and then incubated for a further 6 h in the presence of 10 ng/ml IL-1β. In a different set of experiment, cells were pretreated with 10 μg/ml Natural Red 24 (NR24). Cells without any treatment were used as control. THP-1 cells were seeded in 12-well microplates at 3×10^5 per well. After differentiation with PMA (see above), cells were preincubated with 5 μg/ml CSE for 1 h and then co-treated for an additional 2 h with 10 ng/ml LPS.

Total RNA was extracted from each sample using the NucleoSpin RNA II Kit (Macherey-Nagel, D) according to the manufacturer's instructions. To quantify and control for the integrity of the isolated RNA preparations, each sample was run on the Agilent 2100 Bioanalyzer Nano LabChip prior to reverse transcription of equal RNA quantities into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, A). RNA integrity numbers (RIN) were found to range from 7.8 to 10 for all samples.

qPCR analyses of IL-1β, TNF-α, iNOS and COX-2 mRNA were performed using primer sets described in Table 1. Primers for reference genes were used as previously described (Toegel et al., 2007). Amplification efficiencies of all primers were evaluated using dilution series of cDNA prepared from chondrocyte mRNA. All qPCR reactions were performed in 25 μl reaction mixtures containing 1 μl cDNA, 12.5 μl SensiMix SYBR Green Mix (GenXpress, A), 100 nM primers (Metabion, D), and nuclease-free water to 25 μl, and run in duplicate on an Mx3000P QPCR system (Stratagene, USA). Melting curves were generated to confirm a single gene-specific peak and no-template-controls were included in each run to control for contaminations.

The regulation of target genes was calculated as quantities relative to the untreated control group using the MxPro real-time QPCR software, considering both amplification efficiencies and normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as reference gene. In preliminary experiments, the expression stabilities of 5 candidate reference genes (GAPDH, beta actin (ACTB), hypoxanthine phosphoribosyl-transferase I (HPRT1), succinate dehydrogenase complex, subunit A (SDHA), beta-2-microglobulin (B2 M)) were determined under the experimental conditions described above. Then, the expression stabilities were evaluated using the geNorm software (Vandesompele et al., 2002) and GAPDH was selected as a stable reference gene under the experimental conditions of this study.

We declare that the current study has adhered to the minimal guidelines for the design and documentation of qPCR experiments as recently outlined by Bustin et al. (2010). A qPCR checklist listing all relevant technical information has been provided for the reviewers to assess the technical adequacy of the used qPCR protocols (Supplementary Table 1).

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2.7. TNF-α ELISA

4×10^5/well primary human OA chondrocytes from donors ($n = 2$ donors) or 4×10^4/well SW1353 cells were seeded in 12-well microplates. Upon 90% confluency, culture medium was changed to DMEM containing 2% ITS and cultured overnight. After 1 h preincubation with 5 μg/ml CSE, the cells were treated with 10 ng/ml IL-1β for an additional 48 h. Chondrocytes without any treatment were used as control. The TNF-α concentration in the cell culture supernatants was measured using the Human TNF alpha ELISA Ready-SET-Go! Kit (eBioscience, USA), as described by the manufacturer.

2.8. Transfections and reporter assays

The pXP2-*COX2*-Luc reporter construct, containing −170/+103 bp of the human *COX2* promoter, and the NF-kB p50 and p65 expression vectors were described previously (Grall et al., 2005). The sequences of all constructs were confirmed by DNA sequencing.

Transient transfection experiments were performed in SW1353 cells using LipofectAMINE PLUS Reagent (Invitrogen, A). Cells were seeded in 24-well plates at 10^4 cells/cm^2 in DMEM/F12 containing 10% FCS. Transfections were carried out in serum-free medium with a total of no more than 325 ng of plasmid DNA, including 300 ng of luciferase reporter construct and 25 ng per well of expression vector or empty backbone (control). Briefly, plasmid DNA was incubated for 15 min at room temperature with 6 μl Plus Reagent and 94 μl serum-free DMEM/F12. Following incubation, LipofectAMINE reagent (4 μl) in 96 μl serum-free medium was added to each reaction mixture and the incubation was continued for an additional 30 min at room temperature. Then, the transfection mixture was combined with 300 μl of serum-free medium and the lipid-nucleic acid complex (500 μl) was added drop-wise to the cell monolayer yielding a total volume of 1 ml. After incubation for 1 h at 37 °C, 1 ml of serum-free medium containing CSE was added to each well and incubation was continued for 23 h. Cell lysates were prepared using the Reporter Lysis Buffer (Promega, USA) and luciferase activities were determined by chemiluminescence assay using the LMaxII384 luminometer (Molecular Devices, USA) and the Luciferase Assay Substrate (Promega). Data are representative of two independent experiments performed in triplicate and expressed as fold-change versus unstimulated control.

2.9. Statistical analysis

Data were exported to the GraphPad Prism statistics software package (GraphPad Prism Software, USA). The Gaussian distribution of data was verified using the Kolmogorov–Smirnov test. Statistics were performed using one-way analysis of variance (ANOVA) with post hoc Tukey tests, cross-comparing all study groups (95% confidence interval). p-Values < 0.05 were considered significant.

3. Results

3.1. Effect of CSE on cell viability

As shown in Fig. 1, significant cytotoxicity of CSE was observed at concentrations higher than 40 μg/ml and 10 μg/ml in primary chondrocytes and THP-1 cells, respectively. The subsequent experiments were therefore performed using CSE concentrations not higher than 20 μg/ml for experiments carried out using human primary chondrocytes and at 5 μg/ml for treatment of THP-1 cells.

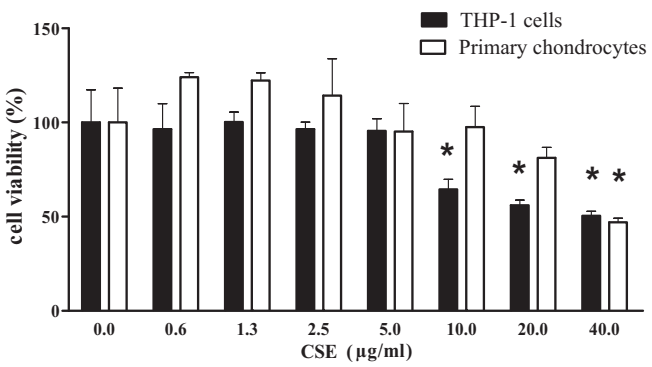

Fig. 1. Cytotoxic effect of CSE in differentiated THP-1 cells and primary chondrocytes. Cells were incubated for 72 h with various concentrations of CSE (0.625–40 μg/ml) and the viability was determined using MTT-based assays. Each value represents the mean ± SD. *Significant differences as compared to untreated cells ($p < 0.05$).

3.2. Effect of CSE on IL-1β and TNF-α mRNA in THP-1 cells

In THP-1 macrophages, 10 ng/ml LPS induced an 11.9 ± 0.6 fold and 60.2 ± 7.2 fold upregulation of IL-1β (Fig. 2A) and TNF-α (Fig. 2B) mRNA levels, respectively. Pretreatment with 5 μg/ml CSE significantly inhibited the upregulation of both cytokines in THP-1 macrophages. In the case of TNF-α, CSE pretreatment down-regulated the mRNA expression to levels comparable to control cells (Fig. 2B).

3.3. Effect of CSE on IL-1β and TNF-α expression in chondrocytes

As shown in Fig. 3A, IL-1β gene expression in SW1353 chondrocytes was upregulated by 45.3 ± 2.5 fold following stimulation with 10 ng/ml recombinant IL-1β. CSE, however, markedly suppressed the effect caused by IL-1β in a concentration-dependent manner. At 10 and 20 μg/ml, CSE completely blocked the IL-1β-induced IL-1β mRNA level, re-establishing expression levels comparable to untreated cells. The TNF-α mRNA level (Fig. 3B) was strongly increased 325 ± 9 fold by 10 ng/ml IL-1β, whereas pre-treatment with 10 or 20 μg/ml CSE significantly reduced this effect. In addition, comparable results regarding the impact of CSE on IL-1β and TNF-α mRNA expression were obtained in primary chondrocytes (Table 2). Interestingly, the upregulation of IL-1β and TNF-α mRNA expression caused by IL-1β was considerably higher in primary chondrocytes than in SW1353 cells. Nevertheless, the stimulated expression of IL-1β and TNF-α mRNA was clearly abrogated by CSE treatment in a dose-dependent manner. The validity of the assay was verified using 10 μM dexamethasone, a widely used anti-inflammatory glucocorticoid, as positive control. Dexamethasone substantially suppressed the IL-1β-mediated upregulation of IL-1β and TNF-α mRNA levels in primary chondrocytes isolated from 3 donors ($p < 0.05$; data not shown).

Interestingly, also NR24 down-regulated TNF-α mRNA levels in IL-1β stimulated primary chondrocytes (Supplementary Fig. 2). Even more importantly, the inhibitory effects mediated by NR24 were significantly less pronounced than those mediated by CSE ($p < 0.05$). This fact might be explained by the lower concentration of brazilin found in NR24 as compared to CSE (Supplementary Fig. 1).

The production of TNF-α was measured in the culture supernatants of chondrocytes using ELISA. As shown in Fig. 4, 24 h treatment with IL-1β (10 ng/ml) caused a 3.6 fold and 9.1 fold increase of TNF-α expression in SW1353 chondrocytes and primary human chondrocytes, respectively. Pretreatment of both cell types with 5 μg/ml CSE significantly suppressed the IL-1β-induced TNF-α

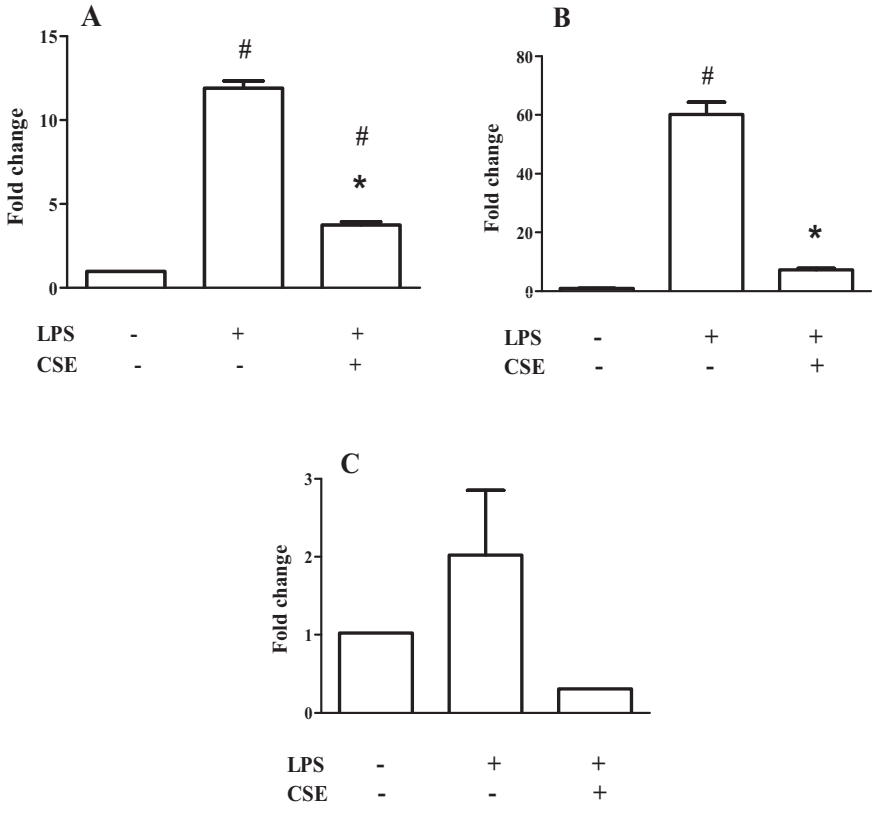

Fig. 2. CSE regulates IL-1β (A), TNF-α (B) and iNOS (C) mRNA in THP-1 cells. THP-1 cells were differentiated to macrophages using PMA and then incubated with 10 ng/ml IL-1β alone or following pretreatment with 5 μg/ml CSE. The alterations of mRNA levels were analyzed using RT-qPCR. Results are expressed as fold changes (mean ± SD) versus controls. *Significant differences compared to cells treated only with IL-1β ($p < 0.05$). #Significant differences compared to untreated cells ($p < 0.05$).

production. As such, 5 μg/ml CSE reduced the IL-1β-induced TNF-α production in primary cells from 19.1 ± 1.1 pg/ml to 5.4 ± 3.8 pg/ml. In both SW1353 and primary cells, the TNF-α production was down-regulated to basal levels.

3.4. Effect of CSE on NO production and on iNOS expression

Nitrite, the stable metabolite of NO, was measured using the Griess assay in primary chondrocytes. Stimulation with

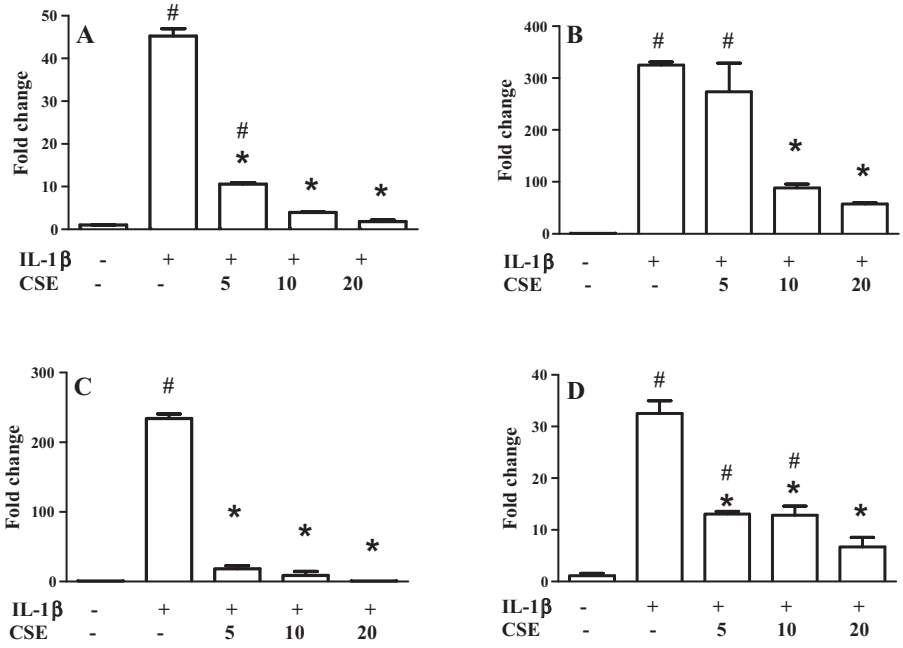

Fig. 3. CSE regulates IL-1β (A), TNF-α (B), iNOS (C) and COX-2 (D) mRNA in SW1353 chondrocytes. Cells were preincubated with 5, 10 or 20 μg/ml CSE for 1 h prior to stimulation with 10 ng/ml IL-1β for 6 h. The alteration of mRNA levels was analyzed using RT-qPCR. Results are expressed as relative quantities (mean ± SD) compared to controls. *Significant differences compared to cells treated only with IL-1β ($p < 0.05$). #Significant differences compared to untreated cells ($p < 0.05$).

Table 2
CSE regulates IL-1β, TNF-α, iNOS and COX-2 mRNA in primary chondrocytes. Cells were preincubated with 5, 10 or 20 μg/ml CSE for 1 h prior to stimulation with 10 ng/ml IL-1β for 6 h. The alteration of mRNA levels was analyzed using RT-qPCR. Data from 3 donors (PC1, 2, 3) are presented as relative quantities (mean ± SD) as compared to controls.

		Control	IL-1β	IL-1β+5 μg/ml CSE	IL-1β+10 μg/ml CSE	IL-1β+20 μg/ml CSE
IL-1β	PC1	1.00 ± 0.04	218.94 ± 6.95#	2.66 ± 0.01*	0.43 ± 0.06*	0.52 ± 0.11*
	PC2	1.00 ± 0.03	8359.31 ± 113.81#	752.09 ± 68.18 *	15.29 ± 1.73*	No Cq
	PC3	1.00 ± 0.01	910.01 ± 8.26#	229.19 ± 41.38	255.77 ± 2.32*	12.93 ± 1.06*
TNF-α	PC1	1.00 ± 0.78	468.07 ± 27.37#	4.82 ± 0.63*	3.68 ± 0.69*	7.64 ± 0.86*
	PC2	1.00 ± 0.03	1013.48 ± 136.44#	128.52 ± 4.63*	22.13 ± 0.30*	37.0 ± 5.16*
	PC3	1.00 ± 0.02	1786.13 ± 200.57#	501.60 ± 51.84*#	154.04 ± 16.61*#	28.88 ± 4.92*
iNOS	PC1	1.00 ± 0.04	659.17 ± 28.86#	48.86 ± 4.04*	1.22 ± 0.01*	0.19 ± 0.02*
	PC2	1.00 ± 0.10	20014.19 ± 4442.74#	3297.77 ± 96.27*#	163.84 ± 18.30*	0.88 ± 0.05*
	PC3	1.00 ± 0.11	7363.67 ± 644.13#	2952.28 ± 14.37#	4047.04 ± 666.50*#	123.64 ± 9.61*
COX-2	PC1	1.00 ± 0.01	96.10 ± 2.23#	21.39 ± 2.28*#	9.61 ± 0.53*#	2.93 ± 0.40*
	PC2	1.00 ± 0.04	121.84 ± 36.81#	71.07 ± 7.26#	23.19 ± 0.32*	9.07 ± 1.26*
	PC3	1.00 ± 0.13	304.77 ± 11.33#	134.24 ± 9.35*#	128.23 ± 3.58*#	28.36 ± 0.92*#

* Significant differences compared to cells treated only with IL-1β ($p < 0.05$).
# Significant differences compared to untreated cells ($p < 0.05$).

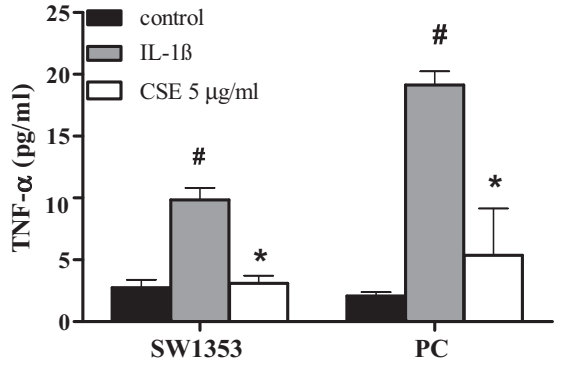

Fig. 4. CSE regulates IL-1β-induced TNF-α production in SW1353 and primary chondrocytes. After 1 h preincubation with 5 μg/ml CSE, cells were treated with 10 ng/ml IL-1β for additional 48 h. TNF-α levels in the cell culture medium were analyzed using ELISA assay. Primary cells were isolated from 2 donors and analyzed separately. Data are presented as relative quantities (mean ± SD) compared to controls. *Significant differences compared to cells treated only with IL-1β ($p < 0.05$). #Significant differences compared to untreated cells ($p < 0.05$).

IL-1β resulted in a 16.3 ± 4.9 fold increase of nitrite accumulation whereas pretreatment with CSE caused a concentration-dependent reduction of nitrite accumulation (Fig. 5). 5 μg/ml and 10 μg/ml CSE significantly suppressed the IL-1β-induced NO production to

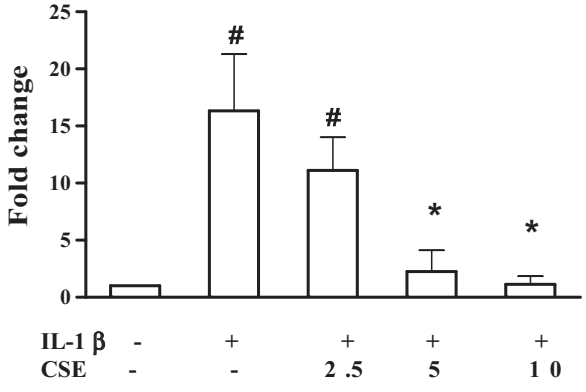

Fig. 5. CSE regulates IL-1β-stimulated NO production in primary chondrocytes. Primary cells were isolated from 3 donors and analyzed separately. Cells were incubated with 2.5, 5 and 10 μg/ml CSE for 1 h prior to coincubation with 10 ng/ml IL-1β for 24 h. The nitrite concentration in the culture medium was measured using the Griess assay. Data are presented as relative quantities (mean ± SD) compared to controls. *Significant differences compared to cells treated only with IL-1β ($p < 0.05$). #Significant differences compared to untreated cells ($p < 0.05$).

basal levels. At 2.5 μg/ml, CSE did not exert a statistically significant effect in this assay. For correlation, RT-qPCR was performed to quantify iNOS mRNA levels in IL-1β-stimulated SW1353 and primary chondrocytes as well as in LPS-stimulated THP-1 cells. As shown in Fig. 3C and Table 2, 10 ng/ml IL-1β markedly stimulated iNOS expression in SW1353 cells and primary chondrocytes, respectively. Pretreatment with CSE dose-dependently suppressed iNOS expression to control levels, supporting the results obtained with the Griess assay. A similar, albeit not significant, trend was also observed in THP-1 cells (Fig. 2C).

3.5. Effect of CSE on COX-2 transcription via p65/p50-mediated COX-2 promoter activation

As shown in Fig. 3D, IL-1β-induced COX-2 expression was significantly reduced by CSE pretreatment in SW1353 chondrocytes. Accordingly, IL-1β-induced COX-2 mRNA levels were reduced in a dose-dependent manner by CSE in human primary chondrocytes (Table 2). Previous studies have shown that IL-1β activates COX-2 expression via NF-κB signaling in different cell types, including chondrocytes (Crofford et al., 1997; Allport et al., 2000; Thomas et al., 2000). Therefore, in order to elucidate the mechanism underlying COX-2 suppression by CSE, SW1353 cells were co-transfected with a COX-2 reporter construct and NF-κB p65/p50 expression vectors in the presence or absence of CSE. As shown in Fig. 6, the basal COX-2 promoter activity was reduced by 2.5 and 5 μg/ml CSE, although the differences were not statistically significant. Overexpression of the NF-κB subunits p50 and p65 significantly enhanced the COX-2 promoter activity (3.4 ± 0.7 fold, $p < 0.05$) whereas the p65/p50-driven COX-2 transactivation was dose-dependently suppressed by CSE (0.5–10 μg/ml), indicating that the anti-inflammatory effects of CSE are, at least in part, derived from its actions on NF-κB signaling.

4. Discussion

It is now accepted that inflammatory processes play a role in the pathophysiology of OA. Indeed, different studies have shown that joint tissue cells, including synovial fibroblasts, synovial macrophages and chondrocytes, produce proinflammatory cytokines, chemokines and other proinflammatory mediators that will in turn result in an inflammatory environment that drives the upregulation of cartilage-degrading MMPs and ADAMTSs (Tsuruda et al., 2004; Bondeson et al., 2006).

Using human OA chondrocytes and THP-1 macrophages, the present study demonstrates for the first time that CSE is a potent inhibitor of proinflammatory mediators in the context

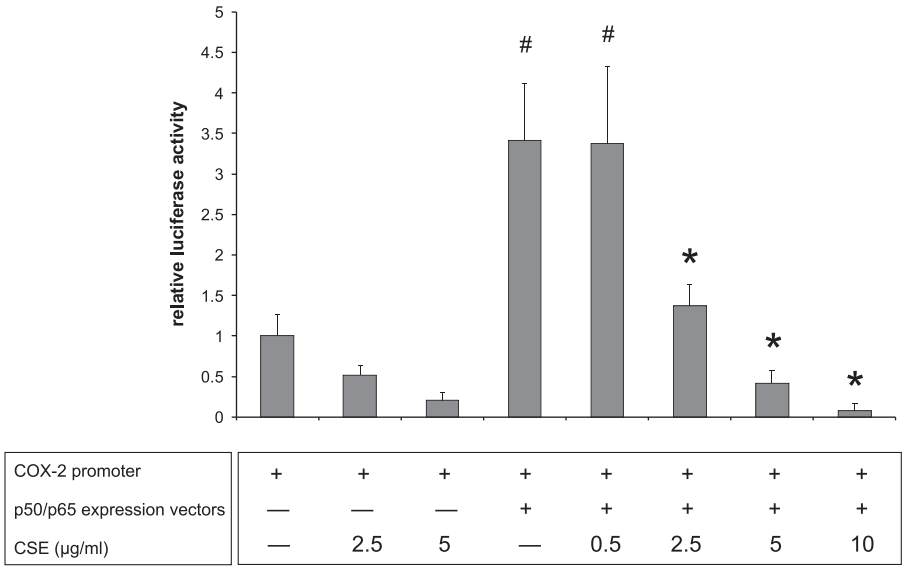

Fig. 6. CSE inhibits p65/p50-mediated COX-2 promoter activation in chondrocytes. SW1353 chondrocytes were transfected with the pXP2 luciferase construct containing the −170/+103 COX-2 promoter or cotransfected with NF-κB p50 and p65 expression vectors. Data shown are means of triplicate measurements of luciferase activity from two independent experiments. #Significant differences with respect to cells transfected with the COX-2 reporter construct and the pCI empty vector ($p < 0.05$). *Significant differences with respect to cells co-transfected with the COX-2 promoter and the p65/p50 expression vectors ($p < 0.05$).

of joint inflammation. Several lines of evidence have previously demonstrated the anti-inflammatory potential of *Caesalpinia sappan* extracts and their isolated compounds. In LPS-stimulated RAW264.7 mouse macrophages, Hu and coworkers found that brazilin, the main constituent of CSE, suppressed the release of IL-1β, TNF-α, NO, and PGE_2 and they suggested that these effects are mediated by Heme oxygenase-1 (Hu et al., 2009). Washiyama et al. compared seven compounds isolated from a methanolic extract of Sappan lignum in mouse macrophage-like J774.1 cells and demonstrated inhibitory effects on the expression of inflammatory mediators (Washiyama et al., 2009). More importantly, in vivo data from mouse and rat models further support our findings on the anti-inflammatory activity of *Caesalpinia sappan* isolates (Shen et al., 2007; Washiyama et al., 2009). Only recently, a 70% ethanolic extract of *Caesalpinia sappan* has been evaluated in a human cell model of TNF-α-stimulated umbilical vein endothelial cells (Lee et al., 2010). Our present study extends these results to human macrophages and shows that CSE down-regulates IL-1β and TNF-α mRNA levels in activated THP-1 cells. This result is of interest since synovial macrophages are known to contribute to the pathogenic cascade that leads to OA and rheumatoid arthritis as a major source of inflammatory cytokines and reactive oxygen intermediates (Bondeson et al., 2006). Targeting macrophages using anti-inflammatory phytopharmaceuticals may, therefore, represent a strategy to alleviate synovitis and deleterious mediators in the synovial fluid.

Besides synovial macrophages, OA chondrocytes are a promising target of anti-inflammatory agents. Our present results clearly document that the addition of CSE effectively suppresses the overexpression of IL-1β and TNF-α in stimulated OA chondrocytes as well as SW1353 cells in vitro, indicating that CSE contains one or more active components that may act as potent anti-inflammatory agents in human chondrocytes. Most likely, brazilin ((6aS,11bR)-7,11b-Dihydro-6H-indeno[2,1-c]chromene-3,6a,9,10-tetrol) contributes to the described anti-inflammatory action of CSE. In the present study, we have isolated brazilin from CSE and identified its structure using ^1H NMR. Interestingly, we found that CSE suppressed the IL-1β-mediated upregulation of TNF-α mRNA levels to a significantly higher extent than NR24. This finding correlates with the lower content of brazilin in NR24 as demonstrated using HPLC analysis and supports the role of brazilin in the anti-inflammatory action of CSE in human chondrocytes.

One of the biological messengers associated with inflammation is NO. NO is biosynthesized endogenously from L-arginine by nitric oxide synthases and can activate transcription factors or protein kinases in a cGMP-dependent manner. Although NO might also regulate physiological processes in chondrocytes, including collagen type X expression and alkaline phosphatase activity, several reports have shown that OA chondrocytes overexpress iNOS and that the resulting excess NO production by OA chondrocytes is a contributing factor to OA cartilage degradative processes (Studer et al., 1999; Abramson, 2008). Experiments in other cell types have indicated that the anti-inflammatory activity of CSE involves the reduction of NO synthesis via inhibition of iNOS mRNA expression (Bae et al., 2005; Washiyama et al., 2009). The latter led us to explore the action of CSE in human OA chondrocytes where, in agreement with the aforementioned reports, we demonstrated that the presence of the extract abrogates IL-1β-mediated NO production by suppressing iNOS transcription. This suggests that CSE might alleviate deleterious effects associated with over-expression of NO such as the inhibition of collagen and proteoglycan synthesis and the induction of apoptosis (Abramson, 2008).

Furthermore, inflammatory cytokines increase the production of prostaglandins via the induction of COX-2 and microsomal PGE synthase 1 (Abramson and Attur, 2009). Attur and coworkers reported that PGE_2 decreased proteoglycan synthesis and enhanced matrix degradation in OA cartilage explants (Attur et al., 2008). RT-qPCR results reported in the present study indicate that CSE is able to attenuate IL-1β-induced COX-2 mRNA expression in SW1353 and primary chondrocytes. This result correlates well with studies on CSE and brazilin in mouse macrophages, demonstrating that the potential anti-inflammatory effects of the agents involve the inhibition of PGE_2 production (Hong et al., 2002; Sasaki et al., 2007). Aiming to further elucidate the mechanisms underlying the anti-inflammatory action of CSE in chondrocytes, we focused on the role of NF-κB in COX-2 promoter activation. The NF-κB signaling is activated by pro-inflammatory stimuli including IL-1β and TNF-α. Activated NF-κB subunits, such as the RelA (p65)/p50 heterodimer, are translocated to the nucleus, where they bind to target DNA sequences and regulate the expression of a number of

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genes, including COX-2, iNOS, IL-1β and TNF-α. The COX-2 promoter contains at least four putative NF-κB binding sites and it has been shown that NF-κB modulates COX-2 expression in different cell types, including epithelial cells, rheumatoid synoviocytes and articular chondrocytes (Crofford et al., 1997; Allport et al., 2000; Thomas et al., 2000). In the present study, co-transfection experiments utilizing a COX-2 promoter luciferase construct and p65/p50 expression vectors in the presence or absence of CSE, revealed that CSE strongly inhibited the p65/p50-driven COX-2 transactivation in a dose-dependent manner. This result suggests that CSE interferes with the DNA binding activity of NF-κB subunits, thereby blocking COX-2 transcription. This finding is in agreement with a previous study by Bae et al. showing that brazilin inhibited the DNA binding activity of NF-κB and AP-1 in LPS-stimulated mouse macrophages (Bae et al., 2005).

Finally, it should be noted that the present study is limited in terms of its in vitro experimental design and that extrapolations to in vivo conditions should be carried out with caution. Future studies should aim to define the in vivo actions of CSE using animal models of arthritic diseases, and to further delineate the molecular mechanisms evoked by CSE and its specific constituents. As any other phytochemical drug, CSE or CSE-derived compounds will also require rigorous toxicological and pharmacokinetic testing as well as clinical verification of the findings obtained in vitro.

5. Conclusion

In this study, we demonstrated that CSE possesses anti-inflammatory activity in human macrophages and OA chondrocytes. In these cells, CSE substantially suppresses the expression of inflammatory mediators including IL-1β and TNF-α at the mRNA level. In addition, we show that the anti-inflammatory actions of CSE involve, at least in part, the inhibition of NO production via iNOS downregulation as well as the inhibition of COX-2 promoter activation by interfering with the canonical NF-κB (p65/p50) signaling pathway. Therefore, we suggest that CSE or CSE-derived compounds should be further developed as therapeutically effective agents or lead structures for future treatment of inflammation in OA.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jep.2011.09.011.

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Brazilin from Caesalpina sappan inhibits pro-inflammatory mediators and metalloproteinase-3 expression in human chondrocytes

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1. Introduction

Osteoarthritis (OA) is one of the most frequent and cost-intensive chronic disabling diseases in the elderly. Characterized by progressive degradation of cartilage, OA is traditionally not considered as a classical inflammatory athropathy. However, overexpression of inflammatory mediators including interleukin 1ß (IL-1ß), tumor necrosis factor (TNF-α), nitric oxide (NO) and prostaglandins contributes to dysregulations of chondrocyte function and remodelling of ECM. The action of these mediators drives catabolic pathways, inhibits matrix synthesis via the inductions of degradative proteases (matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS)), and promotes cellular apoptosis [1, 2, 3]. To date, the aetiology of OA remains to be defined and the process of clinically detectable OA is irreversible. In general, pharmacological and non-pharmacological approaches, the operations, focus on the relief of symptoms, such as reduction of pain, inflammation, and stiffness. Nonsteroidal anti-inflammatory drugs (NSAIDs) are currently the most widely recommended pharmacological therapy. However, the application of NSAIDs is also well associated with the risk of gastro-intestinal or cardiovascular adverse effects [4]. As alternatives to conventional medicine, a new class of agents has been reported to improve joint health, including glucosamine sulphate, chondroitin sulphate, avocado/soybean unsaponifiables, curcumin, diacerein and numerous plant products [5]. Although the clinical efficacy of these agents remains to be confirmed [6], they are widely available and generally

well-tolerated.

Sappan Lignum, the heartwood of *Caesalpinia sappan* L. (Leguminosae), has been widely used in oriental traditional medicine for improvement of blood circulation, sprains, and emmeniopathy [7]. Different extracts and active compounds, such as brazilin, brazilein, prostosappanins A-E, sappanchalcone from extracts have been reported to possess diverse biological activities including antioxidative [8], antiinflammtory [9, 10], and antibacterial [11, 12] effects. Brazilin, the major constituent of Sappan lignum, has been reported to show anti-inflammatory [10], antibacterial [12], and antihepatotoxic effects [13] in diverse cell types. In a previous study, we performed assays to clarify the anti-inflammatory activities of an ethanolic extract of sappan lignum (CSE) in an osteoarthritic cell model containing human chondrocytes and macrophages [14]. We presented evidence that the CSE inhibits chondrocyte catabolism via suppression IL-1ß-induced MMPs mRNA and protein levels [15]. Moreover, we explored part of the mechanisms underlying the anti-inflammatory effects and supposed that the inhibitory effects on MMPs are mediated by inhibiting Nuclear factor –κB (NF-κB)(p65/p50) signalling.

The present study aims to proof the hypothesis that brazilin is responsible for the recently observed anti-inflammatory effects of CSE in chondrocytes. Therefore, we isolated brazilin from CSE, identified the chemical structure, and determined the effects of brazilin on chondrocytes in comparision with CSE.

2. Material and methods

2.1. Brazilin Isolation

CSE isolation from Sappan lignum was described in our previous study [14, 15]. 3.25g CSE were extracted by stirring in100ml water at room temperature for 24h. The supernatant was filtered and freeze-dried to give 1.69g extract as an orange powder. The resulting extract was dissolved in water in a concentration of 10 mg/ml. Further separation was conducted using preparative high-performance liquid chromatography (HPLC, Schimadzu, Japan) with a reversed – phase C18 column (Polygoprep C18, 12 µ, 25mm x 250mm, Austria) monitored at 280nm. The eluent system consisted of a 45 min gradient program from 10% to 100% methanol (containing 1.25% acetic acid) at a flow rate of 22 ml/min; column temperature 50 ℃. The fraction with retentions time at about 20min was collected in repeated preparative HPLC separation. These fractions were further concentrated under vacuum and then freeze-dried to give a white compound which easily oxidized to a red-brown powder.

2.2. Analytic HPLC

The fraction was characterized by HPLC (ICS-3000, Dionex, USA) with a Nucleodur C-18 column (Macherey-Nagel, D) and a PDA-100 detector (Dionex) set at 280 nm. The eluent system consisted of a 45 min gradient program from 10% to 100% methanol (containing 2.5% acetic acid) at a flow rate of 0.8 ml/min and column temperature 50 ℃.

2.3. Brazilin Identification –NMR (Nuclear magnetic resonance)

The ^{1}H NMR and ^{13}C NMR spectra were recorded from $CDCl_3$ solutions on a Bruker Avance 500 instrument (Bruker, USA)500 MHz for ^{1}H, 125 MHz for ^{13}C) at 25 ℃ using a 5 mm direct detection broadband observe probe and deuterium lock. The center of the solvent signal was used as an internal standard which was related to tetramethylsilane with 7.26 ppm (^{1}H) and 77.0 ppm (^{13}C). The recording conditions were the following: ^{1}H NMR: pulse angle 30 °, acquisition time 5 s, digital resolution 0.2 Hz/data point, spectral width 16 ppm, relaxation delay 5 s; broad-band decoupled ^{13}C-NMR spectra: pulse angle 30 °, acquisition time 2 s, digital resolution 0.5 Hz/data point, spectral width 220 ppm, relaxation delay 2 s, exponential multiplication with 1.0 Hz line broadening factor before FT. Full and unambiguous assignment of (almost) all ^{1}H and ^{13}C NMR resonances was achieved by combined application of standard NMR spectroscopic techniques[x] such as APT, COSY, TOCSY, NOESY (1 s mixing time), gs-HSQC[y] and gs-HMBC[z] using standard Bruker software.

2.4. Biological activity assays

2.4.1. Cell culture

In accordance with the terms of the ethics committee of the Medical University Vienna (EK-Nr.: 081/2005) and following the guidelines of the Declaration of Helsinki and Tokyo, primary human chondrocytes were enzymatically isolated from articular cartilage of OA patients undergoing knee replacement surgery following

established protocols [16]. Isolated chondrocytes were seeded at a density of $10^5/cm^2$ and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, A) containing 10% fetal calf serum (FCS; Biochrom AG, D) and 2 µl/ml gentamycin (Biochrom AG, D) in a humidified atmosphere of 5% CO_2/95% air at 37 ℃. For all assays, only freshly isolated and seeded primary cells without subculturing were used. The human chondrosarcoma cell line SW1353 (ATCC, USA) was cultured at a density of $10^4/cm^2$ under the same conditions as described above and passaged by incubation with 0.25% trypsin-EDTA.

2.4.2. Cytotoxicity assay

The cytotoxicity of Brazilin was assessed in primary human chondrocytes cells using an MTT-based test (EZ4U, Biomedica, A) following the manufacturer's instructions. Briefly, $3x10^3$ cells/well were seeded in quadruplicate in 96-well microplates (Iwaki, J) and cultured with various concentrations of brazilin 0.625-40 µg/ml) for 3 days. The absorbance was recorded by a Spectrafluor Fluorometer (Tecan, A) at 450 nm with 620 nm as reference wave length.

2.4.3. Quantitative real-time RT-PCR (RT-qPCR)

$4x10^5$ primary human OA chondrocytes or $4x10^4$ SW1353 cells were seeded in 12-well microplates (Iwaki, J). Upon 90% confluency, medium was changed to DMEM containing 2% Insulin-Transferrin-Selenium (ITS; Gibco, A) and incubated overnight prior to treatment. Chondrocytes were preincubated with Brazilin (5, 10, or

20 µg/ml) or 5 µg/ml CSE for 1 h and then incubated for a further 6 h in the presence of 10 ng/ml IL-1ß (Miltenyi Biotec, D). Untreated cells were used as control.

Total RNA was extracted from each sample using the NucleoSpin RNA II Kit (Macherey-Nagel, D) according to the manufacturer's instructions. To quantify and control for the integrity of the isolated RNA preparations, each sample was run on the Agilent 2100 Bioanalyzer Nano LabChip prior to reverse transcription of equal RNA quantities into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, A). RNA integrity numbers (RIN) were found to range from 7.8 to 10 for all samples.

qPCR analyses of TNF-α, COX-2 and MMP3 mRNA were performed as previously described[14,16]. Primer sets described in Table 1. We declare that the current study has adhered to the minimal guidelines for the design and documentation of qPCR experiments as recently outlined by Bustin et al [17].

2.4.4. ELISA

$4x10^5$ Primary human OA chondrocytes or 4×10^4 SW1353 cells per well were seeded in 12-well microplates. Upon 90% confluency, culture medium was changed to DMEM containing 2% ITS and cultured overnight. After 1 h preincubation with different concentrations of brazilin, the cells were treated with 10 ng/ml IL-1ß for an additional 48 h. Untreated chondrocytes were used as control. The TNF-α, PGE-2, and MMP3 concentrations in the cell culture supernatants were measured using the Human TNF alpha ELISA Ready-SET-Go! Kit (eBioscience, San Diego, CA), the

Parameter ™ PGE_2 Assay and the Quantikine® Human Total MMP-3 Immunoassay (R&DSystems, USA) as described by the manufacturer.

2.5. Statistical Analyses

Data were exported to the GraphPad Prism statistics software package (GraphPad Prism Software, USA). The Gaussian distribution of data was verified using the Kolmogorov-Smirnov test. Statistics were performed using one-way analysis of variance (ANOVA) with post hoc Tukey tests, cross-comparing all study groups (95% confidence interval). p-values < 0.05 were considered significant.

3. Results

3.1. HPLC analysis

As shown in Fig.1, the isolation processes were controlled by analytic HPLC at 280nm wave length. Fig.1A is the chromatogram of CSE, than following Fig.1B shown the similar chromatogram of lyophilisate from CSE water extract. In Fig.1C one prominent peak after preparative HPLC isolation was shown in the chromatogram, with retention time 14.4 min at 280nm wave length.

3.2. Identification of Brazilin

^{1}HNMR

δ7.23(H-1), 6.69(H-11), 6.65(H-8), 6.51(H-2), 6.28(H-4), 3.93(S, 1H, H-12), 3.86(d, 1H, $J_{6a,6b}$=11.5Hz, H-6a), 3.56(d, 1H, $J_{6a,6b}$=11.5Hz, H-6b), 2.93(d, 1H, $J_{7a,7b}$=16.0Hz, H-7a), 2.74(dd, 1H, $J_{7a,7b}$=15.5Hz, H-7b).

^{13}C-NMR

δ40.54(C-7), 48.82(C-12), 69.14(C-6), 76.9(C-6a), 103.14(C-4), 109.52(C-2), 111.81(C-11), 112.87(C-8), 114.48(C-1a), 131.27(C-7a or 11a), 131.69(C-1), 136.28(C-7a or 11a), 143.00, 143.34 (C-9, C-10), 153.37(C-4a), 153.16(C-3)

The ^{1}HNMR and ^{13}C-NMR spectra of isolated compound coincided with brazilin ((6a*S*,11b*R*)-7,11b-Dihydrobenz[b]indeno[1,2-d]pyran-3,6a,9,10(6H)-tetrol)(C16H14 O5) (Fig.2). In agreement with previous studies [12, 18], the isolated compound was identified as brazilin.

3.3. Effect of brazilin on cell viability

Prior to examining the impact of brazilin on cytokine expression in chondrocytes, we confirmed the cytotoxicity of this compound on the primary human chondrocytes. As shown in Figure.3, there was no significant cytotoxicity of brazilin up to concentrations of 40 μg/ml in primary chondrocytes. The subsequent experiments were therefore performed using brazilin concentrations not higher than 20 μg/ml.

3.4. Brazilin reduces TNF-α, COX-2 and MMP3 gene expression stimulated by IL-1ß in primary cells and SW1353 cells

From previous studies [14, 15], we confirmed the anti-inflammatory activities of CSE in an osteoarthritic human chondrocytes and presented evidence that CSE inhibits chondrocyte catabolism via suppression of IL-1ß-induced MMPs mRNA and protein levels. To determine if brazilin might be the responsible compound of this anti-catabolic effect of CSE in chondrocytes, we performed qRT-PCR studies to investigate the effect of brazilin on inhibiting TNF-α, COX-2 and MMP3 gene expression in chondrocytes. As shown in Figure 4A and 4B, TNF-α gene expression in primary chondrocytes and SW1353 was markedly upregulated following stimulation with 10 ng/ml IL-1ß. Brazilin, particularly at concentrations of 10 μg/ml and 20 μg/ml, completely blocked the IL-1ß-induced TNF-α mRNA levels, re-establishing expression levels comparable to untreated cells. The COX-2 mRNA level in primary chondrocytes (Figure 4C) and SW1353 cells (Figure 4D) were strongly increased 76.28±9.76 fold and 25.25±2.71 fold, respectively by 10 ng/ml

IL-1ß, whereas pre-treatment with 5-20 μg/ml brazilin significantly reduced this effect. Figure 4E and 4F demonstrate that brazilin dose dependently suppressed the IL-1ß-mediated upregulation of MMP3 mRNA levels in primary chondrocytes and SW1353 cells. 5 μg/ml CSE was added in each experiment and 5-20 μg/ml CAPE, one well known inhibitor of NF-kappa B activation were added in the experiments with primary chondrocytes as positive control. Pretreatments with CSE and CAPE reduced the IL-1ß stimulated expression of TNF-α, COX-2 and MMP3 mRNA expression in chondrocytes. In the previous studies, 5, 10, or 20 μg/ml CSE showed significant inhibitory effect on expression of TNF-α, COX-2 and MMP3 mRNA. And in present study, 5μg/ml CSE in the most instances significantly inhibited the IL-1ß-mediated upregulation of TNF-α, COX-2 and MMP3 mRNA levels in primary chondrocytes and SW1353 cells, but was less effective comparing with 5μg/ml brazilin (Fig.4A.B.C.D.F). A similar effect with different concentrations of CAPE was also observed in the present study (Fig.4A,C,E).

3.5. Brazilin reduces TNF-α and MMP3 production but not PGE-2 production

To quantify the effects of brazilin on secreted levels of TNF-α, PGE2 and MMP3, the culture supernatants of chondrocytes were determined using ELISA assays. As shown in Figure 5A, 48 h treatment with IL-1ß (10 ng/ml) caused an increase of TNF-α expression in primary human chondrocytes from 278.37±16.33 to 396±30.85 pg/ml. Pretreatment with 10 μg/ml brazilin significantly suppressed the IL-1ß-induced TNF-α production released in cell culture. Interestingly, PGE-2 release (Figure 5B)

was not augmented by IL-1ß stimulation in this study in spite of the increased COX-2mRNA levels in presence of IL-1ß. An increase of MMP3 release following by IL-1ß was not observed in Figure 5C. However, the MMP3 production in primary cells treated with IL-1ß was down-regulated by 10 μg/ml brazilin, even lower than basal levels.

4. Discussion

Overproduction of pro-inflammatory cytokines, notably IL-1ß and TNF-α is one characteristic feature of established OA. Evidence indicates that in response to these proinflammatory mediators, chondrocytes increase the synthesis of catabolic proteinases (such as MMPs 1, 2, 3, 8, 9, and 13), production of PGE_2 by stimulating the expression of COX-2, PGE synthase 1, and soluble phospholipase A2, and production of NO via incucible nitric oxide synthetase(iNOS, or NOS2) [19,20,21]. Thereby, reducing or preventing the inflammatory properties within joint tissue represents a promising therapeutic approach to relief OA symptoms, to prevent the progression of OA and to restore the structure of cartilage.

Brazilin, the major compound in CSE, is also known as a red dye (Natural Red 24) used since the middle ages in dye fabric. Although brazilin possesses defined chemical structure, its own IUPAC name and CAS-number, most commercial available “brazilin” is an extract of sappan wood consisting several undefined compounds, probably because of the long history of utilization of this name in dye fabric as dye name. To our knowlegment, pure brazilin is commercial difficult to available. Thus, to obtain pure brazilin, we isolated it from CSE and identified it using analytical HPLC and NMR methods.

As noted above, TNF-α is one of the predominant proinflammatory cytokines, which

exert catabolic effects on chondrocyte metabolism. Our present results clearly demonstrate that the addition of brazilin effectively suppresses the over-expression of TNF-α in stimulated OA chondrocytes as well as SW1353 chondrocytes. Of note, 5 μg/ml brazilin showed a higher inhibitory effect on TNF-α mRNA expression than same concentration of CSE, indicating that brazilin contributes to at least the major part of the inhibitory effect of CSE on TNF-α production stimulated by IL-1ß. This result correlates well with a study obtained in mouse macrophages by Hu and coworkers [22]. In contrast, Washiyama *et al*. compared seven compounds isolated from a methanolic extract of Sappan lignum in mouse macrophage-like J774.1 cells and demonstrated that brazilin does not significantly contribute to the inhibitory effects of CSE on TNF-α production [10].

Furthermore, inflammatory cytokines increase the production of prostaglandins via the induction of COX-2 and microsomal PGE synthase 1 [3]. Evidence indicated that PGE_2 decrease proteoglycan synthesis and enhanced matrix degradation in OA cartilage explants [23]. RT-qPCR results reported in the present study indicate that brazilin is able to attenuate IL-1ß-induced COX-2 mRNA expression in SW1353 and primary chondrocytes in a dose dependent manner. No significant regulation was observed in ELISA assay of PGE_2 production in present study probably because of amount of PGE_2 in supernatant of cell culture under detection limitation in our experiments. Experiments in other cell types have indicated that the anti-inflammatory activity of brazilin involves the reduction of PGE_2 synthesis [22,

23]. Interestingly, Washiyama et al found that brazilin minimally inhibited COX-2 mRNA expression and PGE_2 production [10].

MMP3 is one of the particularly effective metalloproteinases in cartilage degradation because it cleaves a wide variety of matrix proteins, e.g., aggrecan, fibronection and several collagens, and it activates other MMPs [19]. Our previous studies revealed for the first time that CSE inhibits chondrocyte catabolism via suppression of IL-1ß-induced MMPs at mRNA and protein levels [15]. In present study, we demonstrated that brazilin decreased MMP3 mRNA expression and consequently reduced the MMP-3 synthesis. To our surprise, MMP-3 release was not induced by IL-1ß stimulation in ELISA assay or at least statistically not significant in this study. While in several studies, IL-1ß was shown to upregulate MMP-1, -3, and 13 in normal human chondrocytes, OA chondrocytes, and chondrosarcoma cells using Westenblot [19, 25, 26]. The reason might be the primary chondrocytes used in our experiments, were obtained from OA patients. These chondrocytes were accustomed to the milieu presenting with high level of IL-1ß and are not able to produce MMPs in response to IL-1ß stimulation. Another explains might be that ELISA assay observed only the protein level secreted to the cell culture. Perhaps there were upregulation existed intracellular and should be determined using Western blot. However, the MMP3 production in primary cells was down-regulated by 10 µg/ml brazilin, even lower than basal levels. This result indicates that brazilin at least partly contribute to reduction of MMP-3 synthesis caused by CSE and might be able to slow down extracellular matrix degradation by inhibiting MMP activity.

One limitation of our study is the limited number of cartilage specimens and limited biological assays. Therefore, additional experiments on normal and OA chondrocytes are needed before definitive conclusions can be drawn. Further research to explore the mechanism underlying the anti-inflammatory effects and the inhibitory effects on MMPs of brazilin and CSE in cartilage is also necessary.

In summary, we isolated brazilin from CSE, identified its structure, and determined its biological activities on OA chondrocytes in comparison to CSE. Brazilin substantially suppresses the expression of inflammatory mediator TNF-α, COX-2 and proteinase MMP3 and production of TNF-α and MMP3. The study suggests that brazilin largely contributes to the anti-inflammatory and anti-catabolic action of CSE in human chondrocytes. The data suggest that brazilin may be beneficial for future management of OA.

Glossary

ADAMTS = A disintegrin and metalloproteinase with thrombospondin motifs

COX-2 = Cyclooxygenase-2

CSE = Ethanolic *Caesalpinia Sappan* Extract

DMEM = Dulbecco's modified Eagle's medium

ECM = Extracellular matrix

FCS = Fetal calf serum

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase

IL-1ß = Interleukin-1 beta

iNOS = Inducible nitric oxide synthase

ITS = Insulin-Transferrin-Selenium

MMPs = Matrix metalloproteinases

NF-κB = Nuclear factor-kappa-B

NO = Nitric oxide

NSAIDs = Nonsteroidal anti-inflammatory drugs

OA = Osteoarthritis

PGE_2 = Prostaglandin E_2

RIN = RNA integrity numbers

RT-qPCR = Quantitative real-time RT-PCR

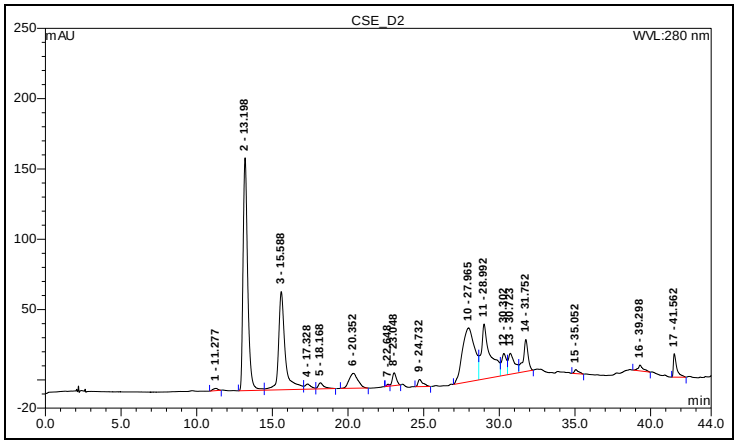
TNF-α = Tumor necrosis factor- alpha

5. Figures and Tables

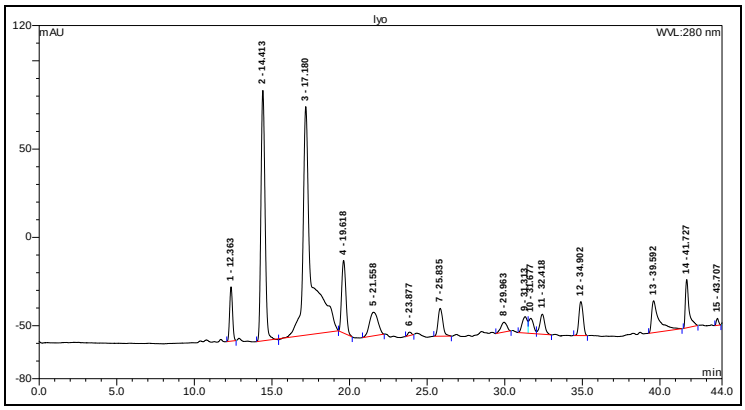
Gene symbol	Primer sequences	Efficiency	Amplicon size	NM-number
MMP 3	Fw: GGTGTGGAGTTCCTGATGTTG	99.9%	184bp	NM_002422
	Rv: AGCCTGGAGAATGTGAGTGG			
TNF-α	Fw: GCGGTGCTTGTTCCTCAG	96%	126bp	NM_000594
	Rv: GCTACAGGCTTGTCACTCG			
COX-2	Fw: CCGAGGTGTATGTATGAGTGTG	96.5%	136bp	NM_000963
	Rv: TGTGTTTGGAGTGGGTTTCAG			

Table 1. Details on RT-qPCR primers.

1A



1B



1C

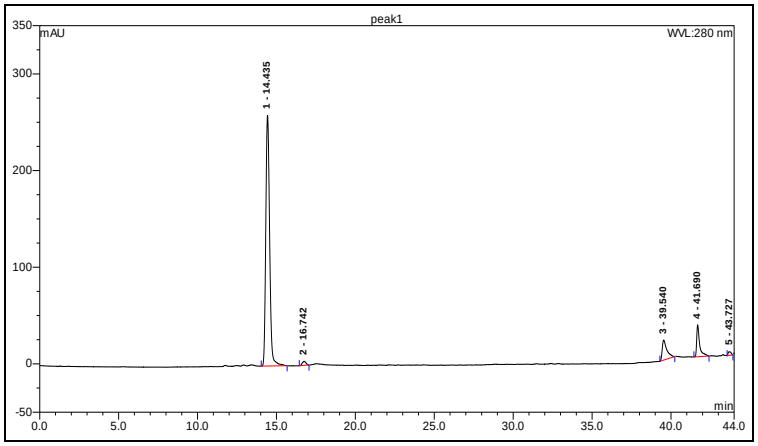


Figure 1. Analytic HPLC chromatogram at 280nm

(1A) 1mg/ml CSE (1B) 1mg/ml Lyophilisate from CSE water extract (1C) the major fraction collected in repeated preparative HPLC separation after lyophilisation

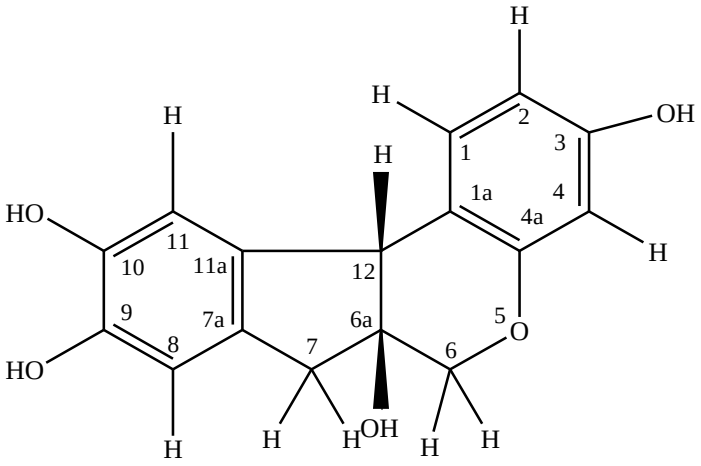


Figure 2. Structure of brazilin

The structure of brazilin conducted from NMR spectra.

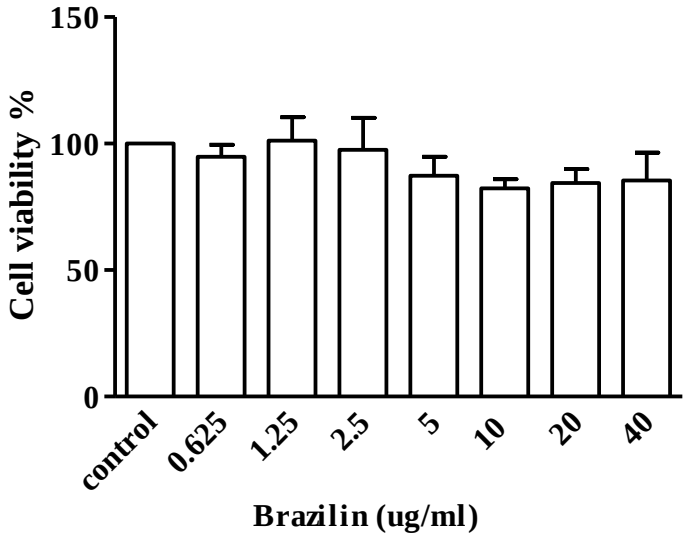


Figure3. Viability of primary chondrocytes in presence of brazilin.

Cells were incubated for 72 h with various concentrations of brazilin (0.625-40 μg/ml) and the viability was determined using MTT-based assays. Each value represents the mean ± SD. Significant differences as compared to untreated cells ($p < 0.05$).

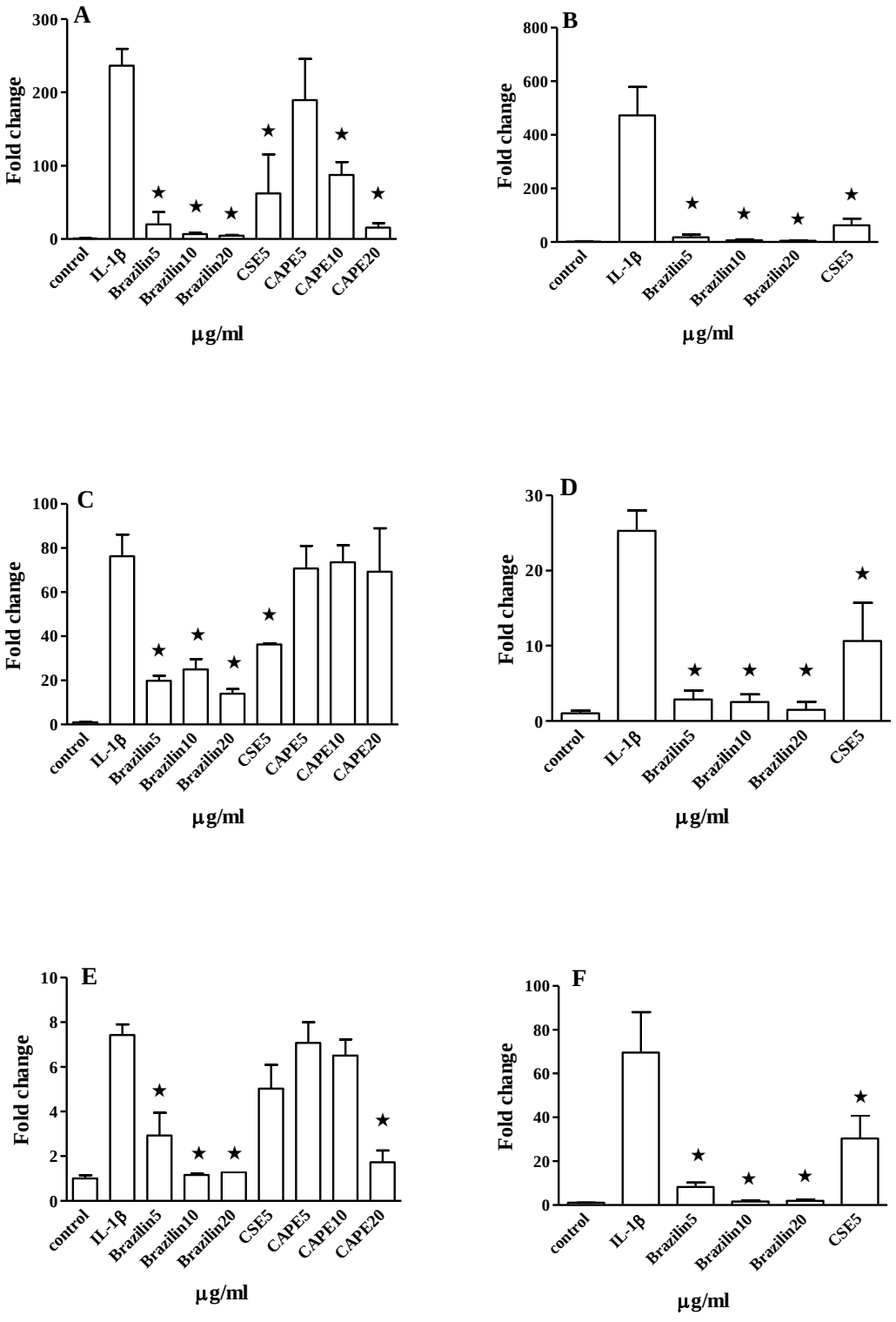


Figure 4. Effect of brazilin on TNF-α, COX-2 and MMP3 mRNA transcription in primary chondrocyte and SW1353 cells. (A) Effect of brazilin on TNF-α mRNA transcription in primary chondrocyte (B) Effect of brazilin on TNF-α mRNA

transcription in SW1353 cells (C) Effect of brazilin on COX-2 mRNA transcription in primary chondrocyte (D) Effect of brazilin on COX-2 mRNA transcription in SW1353 cells (E) Effect of brazilin on MMP-3 mRNA transcription in primary chondrocyte (F) Effect of brazilin on MMP-3 mRNA transcription in SW1353 cells. Cells were preincubated with various concentration of brazilin and CAPE for 1 h prior to stimulation with 10 ng/ml IL-1ß for 6 h. The alteration of mRNA levels was analyzed using RT-qPCR. Results are expressed as fold changes (mean ± SD) versus controls. Asterisk indicates significant differences compared to cells treated only with IL-1ß 10 ng/ml ($p < 0.05$).

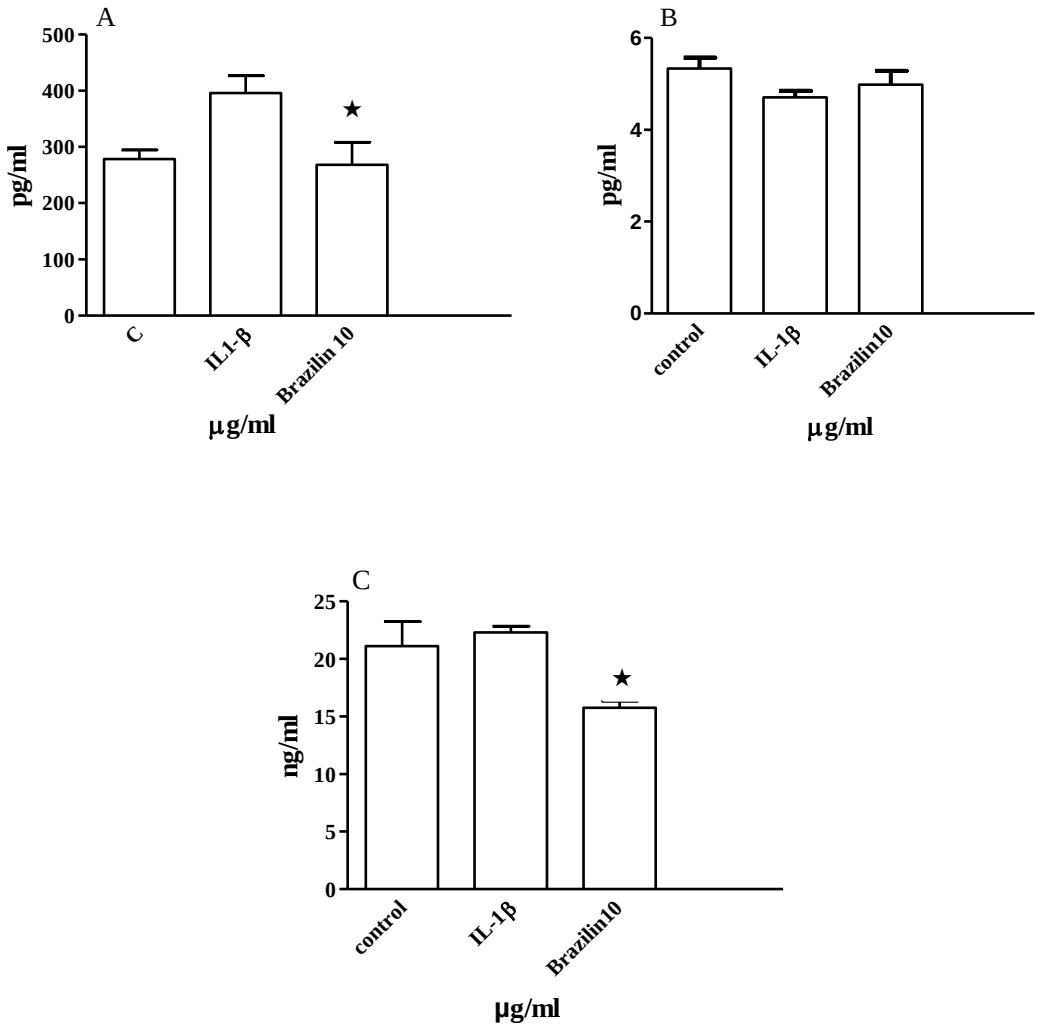


Figure 5. Effect of brazilin on TNF-α (A), PGE-2(B), and MMP3(C) production in primary chondrocytes. After 1 h preincubation with 10 µg/ml Brazilin, cells were treated with 10 ng/ml IL-1ß for additional 48 h. TNF-α, PGE_2, MMP3 levels in the cell culture medium were analyzed using ELISA assay. Data are presented as mean ± SD (n=1). Asterisk indicates significant differences compared to cells treated only with IL-1ß ($p < 0.05$).

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3. Conclusion

The present thesis aimed to investigate two separate aspects of inflammation in human chondrocytes, the unique cell component of articular cartilage.

First, we investigated the chondrocyte glycophenotype in different cell models and under inflammatory conditions in vitro (Study1-3). In the first study, using real-time qPCR, immunofluorescence, and glycosaminoglycan (GAG) staining, the glycoprofile of primary human chondrocytes and two chondrocyte cell lines was evaluated. The immortalized cell lines expressed and produced minor extracellular matrix genes and proteins in comparison with primary chondrocytes. Through the analysis of the glycosylation pattern via lectin-binding, a reduced level of high-mannose type and sialic acid-capped *N*-glycans as well as increased fucosylated *O*-glycosylation was obeserved in C-28/I2 and T/C-28a2 cell lines. Thus, it could be concluded that differences in the molecular chondrocyte phenotype are reflected in the glycophenotype of the cells. In the following 2 studies (Study 2 and 3), the regulation of the chondrocyte glycophenotype was monitored using quantitative PCR and LC–ESI-MS. Primary human chondrocytes predominantly express α-(2→6)-sialyltransferases (SiaT) and accordingly, α-(2→6)-linked sialic acid residues in glycoprotein N-glycans. In contrast, the preponderance of α-2,3-linked sialyl residues and, correspondingly, reduced levels of α-2,6-specific SiaTs are associated with the altered chondrocyte phenotype of C-28/I2 and SW1353 cells. Of

note, an overall shift towards α-(2→3)-sialylation of diantennary fucosylated N-glycans and towards α-(2→3)-SiaT mRNA levels was observed following stimulation of primary chondrocytes with IL-1ß or TNF-α. In addition, both cytokines increased overall sialylation of N- and O-glycans as well as α-(2→3)-linked sialic acid residues in chondrocyte glycoproteins. Moreover, a considerable shift from oligomannosidic glycans towards complex-type N-glycans was found in IL-1ß or TNF-α treated chondrocytes. In summary, inflammatory conditions induce specific alterations of the chondrocyte glycophenotype at mRNA and glycan levels, which might affect the maintenance of articular cartilage function.

As inflammatory events represent interesting targets for the treatment of OA, the second part of this thesis evaluated the activities of *Caesalpinia sappan* extracts and the isolated compound brazilin in stimulated human chondrocytes and macrophages (Studies 4-6). Using preparative HPLC, we isolated brazilin from the ethanolic extract of *Caesalpinia sappan* (CSE) and identified it by NMR and HPLC (Study 6). Following viability tests, ELISA, and validated real-time PCR assays, we found that CSE and brazilin can effectively abrogate the IL-1ß-induced overexpression of inflammatory mediators, such as IL-1ß, TNF-α, iNOS and COX-2. We further observed that the levels of IL-1ß-induced MMP-1, -3, -7, -9 and -13 mRNA, but not TIMP (tissue inhibitors of MMPs) mRNA levels, were down-regulated in chondrocytes in response to CSE. Co-transfection experiments with COX-2 and MMP-13 luciferase reporter constructs and NF-κB p50 and p65 expression vectors

further showed that CSE abrogated the NF-κB (p65/p50)-driven promoter activation. Blockade of IL-1ß-induced NF-κB signalling and its downstream pro-inflammatory targets by CSE and brazilin may be beneficial for reducing cartilage breakdown in arthritis.

In conclusion, this thesis (1) contributes to the elucidation of the chondrocyte glycophenotype under inflammatory conditions and (2) suggests that CSE or CSE-derived compounds should be further developed as therapeutically effective agents or lead structures for future management of inflammation in OA.

4. Appendix

German Abstract- Kurzfassung

Osteoarthrose ist definiert als eine progressive, degenerative Gelenkserkrankung im höheren Lebensalter. Im Gegensatz zur Rheumatoiden Arthritis beschreibt die Arthrose einen langsamen Verschleiß des Gelenksknorpels und ist als eine nicht entzündliche Gelenkserkrankung zu verstehen. Obwohl die entzündliche Komponente ein wesentliches Unterscheidungskriterium darstellt, kann auch eine Arthrose, insbesondere im fortgeschrittenen Stadium der Krankheit, von entzündlichen Prozessen begleitet sein und die klassischen Symptome einer Entzündung (Rötung, Schwellung und Schmerzen) aufweisen. Es ist bekannt, dass das Gelenksgewebe bei Arthrosepatienten eine deutlich erhöhte Produktion proinflammatorischer Zytokine, wie zum Beispiel IL-1ß und TNF-α, aufweist. IL-1ß und TNF-α steigern die Synthese matrixdegradierender Proteasen (MMPs) und inhibieren die Kollagen- und Aggrekansynthese.

In der vorliegenden Dissertation wurden zwei unterschiedliche Aspekte von Entzündungsprozessen in Chondrozyten untersucht.

Einerseits wurde der zelluläre Glykophänotyp in unterschiedlichen Chondrozytenmodellen unter Entzündungsbedingungen untergesucht. Die Studien 1-3 fokussieren den Blick auf (1) den Zusammenhang von Glykophänotyp und generellem Zellphänotyp in humanen Chondrozyten, (2) Sialylisierungsmuster von N- und O-Glykanen in Chondrozyten, und (3) den Einfluss inflammatorischer Zytokine

(IL-1ß und TNF-α) auf die Glykanexpression.

Weil Entzündungsprozesse immer wieder im Interesse der Erforschung neuer Behandlungsmöglichkeiten der OA stehen, evaluierte der zweite Teil dieser Dissertation die Effekte eines ethanolischen *Caesalpinia sappan* Extraktes (CSE) und einer daraus isolierten Reinsubstanz (Brazilin) auf stimulierte humane Chondrozyten und Macrophagen *in vitro*. In den Studien 4-6 konnte gezeigt werden, dass CSE und Brazilin anti-inflammtorische Effekte ausüben und knorpelabbauende Prozesse, welche durch IL-1ß induziert wurden, hemmen können.

Zusammenfassend trägt diese Arbeit zur Beschreibung des Chondrozyten-Glycophänotyps unter entzündlichen Bedingungen bei, und weist darauf hin, dass CSE oder aus CSE isolierte Wirkstoffe zukünftig als therapeutisch wirksame Agenten oder Leitstrukturen für die Behandlung entzündlicher Prozesse bei der OA weiterentwickelt werden sollten.

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CONGRESS CONTRIBUTIONS

S.Q. Wu, F.M. Unger, M.B. Goldring, H. Viernstein, S. Toegel, **Anti-inflammatory activity of an ethnolic Casalpinia Sappan extract in human chondrocytes *in vitro***. 2*010 OARSI World Congress on Osteoarthritis*, September, 2010, Brussels, Belgium.

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