Influence of cartilage interstitial fluid on the mRNA levels of matrix proteins, cytokines, metalloproteases and their inhibitors in synovial membrane

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Abstract. Articular cartilage and the synovial membrane both ensure the smooth action of synovial joints; however, the influence of chondrocytes on synovial metabolism remains unclear. The secretory activity of chondrocytes is usually studied in cell cultures and may differ from that in intact cartilage. According to McCutchen's theory of 'weeping' joint lubrication, loading of the articular cartilage during motion squeezes the fluid with lubricating properties from the cartilage. The purpose of the study was to obtain cartilage interstitial fluid (CIF) from intact cartilage and to evaluate its influence on gene expression in the synovial membrane cells. CIF was rinsed out from the cartilage of newborn rats at a pressure of three bar. The chondrocytes survived rinsing and grew in culture. Cytokines in CIF were detected using the enzyme-linked immunosorbent assay (ELISA). The influence of CIF and CIF-like cocktail (all cytokines found in CIF) on gene expression in the synovial membrane cells was studied after a 4 h-incubation, by real-time PCR. Data were analyzed using the Wilcoxon matched-pair test or by the Mann-Whitney U test. CIF contained basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF)-1, transforming growth factor β1 (TGFβ1), bone morphogenetic protein 7 (BMP7), macrophage (M)-colony-stimulating factor (CSF), granulocyte (G)-CSF and leukemia inhibitory factor (LIF). CIF stimulated the expression of hyaluronan synthase (HAS)1 and 2, lubricin, collagen I, versican, aggrecan, matrix metalloproteinases (MMPs)2 and 3, tissue inhibitors of metalloproteinases (TIMPs) 1-3, interleukin (IL)-6 and TGFβ1, and decreased the expression of tumor necrosis factor (TNF) and IL-1β. Incubation of the synovial membrane with CIF-like cocktail partially imitated the effects of CIF. Analysis of CIF composition may help to characterize the secretory activity of chondrocytes in their natural environment under various physiological and pathological conditions and to understand the interactions between articular cartilage and the synovial membrane.

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Abbreviation: CIF, cartilage interstitial fluid

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Introduction

Articular cartilage and the synovial membrane (SM) are the main components of synovial joints. The SM produces hyaluronic acid and lubricin, which are important for articular cartilage lubrication (1,2). Synoviocytes also secrete factors which induce the synthesis of metalloproteinases by chondrocytes (3). Moreover, normal synovial fluid stimulates the synthesis of collagen type II and glycosaminoglycans in articular cartilage (4). The question remains, whether cartilage is a passive beneficiary of SM activity or whether chondrocytes can also influence the metabolism of cartilage by cytokines or other factors. Chondrocyte survival and differentiation require their interaction with extracellular matrix (5). The secretory activity of chondrocytes is usually studied *in vitro*, after their release from the matrix, but such an approach has some limitations. The yield of isolated cells is low in comparison with their content in cartilage, possibly resulting in the uncontrolled selection of certain chondrocyte subpopulations (6). Furthermore, the application of enzymes for the purpose of chondrocyte isolation changes their gene expression (7). Chondrocytes cultured as a monolayer down-regulate the expression of cartilage matrix molecules such as collagen type II and aggrecan as well as increase the expression of collagen type I and versican, which is typical of fibroblast-like cells (8-10). They also undergo other changes in phenotype expression with the upregulation of markers regarded as distinctive for mesenchymal stem cells (11).

Cultured *in vitro* articular chondrocytes, particularly after stimulation by proinflammatory agents, secrete numerous cytokines, matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinases (TIMPs) and other factors (12). These experiments, however, were performed on cells released from the cartilage and thus, it is difficult to estimate the type of cytokine and the rate of production by chondrocytes in their natural environment, without modifications imposed by enzymatic baths or culture conditions.

In this study, we aimed to establish which cytokines are produced by chondrocytes in the cartilage and also, to evaluate the influence of these cytokines on the SM as a possible target organ. The concept of the study emerged from the McCutchen (13) theory of 'weeping' lubrication in synovial joints. According to this study and others (14,15) cartilage matrix contains a fluid phase, representing about 70% of its volume. During joint loading, about 10% of this liquid is squeezed from the cartilage surface (which, in a molecular sense, is porous) into the intraarticular cavity, and is responsible for hydrostatic lubrication. It is, therefore, plausible that cartilage interstitial fluid (CIF) squeezed from cartilage during joint loading contains cytokines produced by chondrocytes.

We have previously demonstrated that rat SM dissected from the knee joint and incubated *in vitro* responded to stimulation with cytokines and lipopolysaccharide by increasing the production of hyaluronic acid and changing the mRNA expression of hyaluronan synthases (HASs), cytokines, MMPs and TIMPs (16,17). These findings suggested that the SM in this experimental model would also respond to factors present in the CIF. CIF was obtained by rinsing out the interstitial fluid from the dissected articular-epiphyseal cartilage complexes of newborn rats at a pressure of three bar and the cytokine content of the CIF was evaluated using an enzyme-linked immunosorbent assay (ELISA). SM exposed to CIF exhibited changes in the mRNA expression of cytokines, MMPs, TIMPs and components of the extracellular matrix. Incubating the SM with a cocktail of all factors found in CIF (CIF-like cocktail) demonstrated that this set of cytokines, to a considerable degree, imitates the effects of CIF.

Materials and methods

Animals. SMs were removed from the knee joints of specific pathogen-free, inbred, male Lewis rats (n=24; 3 months old) purchased from the Animal Unit of the Mossakowski Medical Research Centre at the Polish Academy of Sciences (Warsaw, Poland). Three-to-five day-old inbred Lewis rats (n=20) of both genders served as cartilage donors. The present study and the methods were approved by the Animal Ethics Committee of the Medical University of Warsaw (Warsaw, Poland).

Preparation of rat SMs. Rats were euthanized by inhalation of halothane. After opening the knee joint cavity, the SM was excised together with the patella, the patellar ligament and the joint capsule. The SM with the infrapatellar fat pad was then separated from the other tissues according to the method described previously (18).

Preparation of CIF. Newborn rats were euthanized by decapitation. CIF was rinsed out from the articular-epiphyseal cartilage complexes dissected from the newborn rats, with the exclusion of calcified fragments of the growth plate, which could be recognized and separated during dissection. After clearing from the surrounding tissues, cartilages from several animals were weighed. The mean weight of cartilage obtained from one animal was 110 mg. For CIF preparation, cartilage from 2 animals were placed in 2 ml phosphate-buffered saline (PBS; Gibco-BRL, Paisley, UK) and cut into small fragments. Since cutting involves the exertion of pressure on the cartilage, some CIF was probably already squeezed into PBS. The fluid together with the cartilage fragments was transferred into a 50 ml Luer Lock syringe closed with the PTFE Body Two-Way Valve from Hamilton (Sigma-Aldrich Chemie, Steinheim, Germany) and the plunger was pressed to compress the air in the syringe so as to increase the pressure to three bar. Then, the plunger was slowly released. This procedure was repeated 20 times. Cutting the dissected cartilage and rinsing out the CIF lasted about 15-20 min. The fluid was separated from the cartilage fragments by centrifugation, and desalting was performed on PD-10 columns (Amersham Biosciences, Uppsala, Sweden) and lyophilized. CIF from 10-20 rats was pooled to obtain more uniform material. The lyophilisate was dissolved in RPMI-1640 (Gibco-BRL) medium and the protein content was determined. The total amount of protein in the CIF squeezed from the cartilage obtained from one animal varied from 0.87 to 1.1 mg. A working solution of CIF was standardized to contain 1 mg/ml protein. The presence and concentration of factors supposedly occurring in CIF [tumor necrosis factor (TNF), transforming growth factor β1 (TGFβ1), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF) , interleukin (IL)-1β, IL-6, IL-7, IL-10, granulocyte-macrophage (GM)-colony-stimulating factor (CSF) granulocyte-macrophage (GM)-CSF, macrophage (M)-CSF, granulocyte (G)-CSF, insulin-like growth factor (IGF)-1, leukemia inhibitory factor (LIF), bone morphogenetic protein (BMP)2, BMP7, lubricin and hyaluronic acid (HA)] were estimated using an ELISA.

Chondrocyte culture. Cartilage fragments used for harvesting CIF were digested with constant stirring in an enzymatic solution containing 0.25% collagenase (type I), 0.05% DNase, 17.5 μM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) and 1% antibiotic-antimycotic solution (all from Sigma, St Louis, MO, USA) in RPMI-1640 medium (Gibco-BRL) for 3 h at 37˚C. The viability of chondrocytes was checked using the trypan blue test (Sigma). Chondrocytes were seeded into 24-well plates (Corning, Inc., Corning, NY, USA) at a density of $5x10^5$ cells/well in 1 ml of culture medium and observed for 7 days (10).

Incubation of SM. As a standard procedure, dissected SMs were incubated in RPMI-1640 (Gibco-BRL) medium in flat-bottomed 24-well plates (Corning, Inc.) in a humidified atmosphere of 5% CO_2 in air at 37˚C with constant, slow motion, for 4 h. The SM from one knee joint served as the control to the SM from the opposite knee. The control medium was enriched by 0.1% bovine albumin (Sigma). Experimental SMs were incubated either in CIF or in the CIF-like cocktail with commercial cytokines identical in concentration with that present in CIF. The following cytokines were used: G-CSF, M-CSF, LIF, BMP7 and bFGF (PromoKine; PromoCell GmbH, Heidelberg, Germany), TGFβ1 (Sigma) and IGF1 (R&D Systems Inc., Minneapolis, MN, USA). After culture, total RNA from SM cells was isolated and the expression of genes encoding HAS1, HAS2, lubricin, collagen type I, aggrecan, versican, MMP2, MMP3, TIMP1, TIMP2, TIMP3, IL-1β, IL-6, TNF and TGFβ1 was examined.

Protein determination. Ten microliters of CIF dissolved in the medium (without serum) or medium alone (blank test) was

placed in a flat-bottomed 96-well plate (Corning, Inc.) and 0.2 ml BCA protein assay reagent (Pierce, Rockford, IL, USA) was added to each well. The plate was incubated at 37˚C for 30 min. Protein concentrations were determined spectrophotometrically at 550 nm in a microplate reader (model 550; SLT Spectra Labinstruments, Crailsheim, Germany).

Analysis of CIF by ELISA. Cytokine and extracellular matrix protein levels were evaluated using rat immunoassay kits for IL-6, IL-10, TNF, TGFβ1, IGF1, GM-CSF and BMP2 from R&D Systems, Inc., for IL-7, bFGF, M-CSF, G-CSF, LIF, BMP7, EGF and PDGF from Biotang, Inc. (Waltham, MA, USA), for HA and lubricin from Cusabio Biotech Co., Ltd. (Hubei, China), and for IL-1β from Life Technology (Frederick, MD, USA) according to the manufacturers' instructions.

Total RNA isolation from SM samples. RNA was isolated using a NucleoSpin® RNA II kit (Macherey-Nagel, Duren, Germany), according to manufacturer's instructions. The quantity and quality of the isolated total RNA was evaluated spectrophotometrically using a NanoDrop 2000 spectrophotometer (ND-2000) with software for the analysis of nucleic acids (both from Thermo Fisher Scientific, Wilmington, DE, USA).

Reverse transcription. Reverse transcription was performed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions in an Eppendorf gradient Mastercycler (Eppendorf AG, Hamburg, Germany). cDNA samples were stored at -20˚C.

Real-time polymerase chain reaction (PCR). Real-time PCR was performed in an ABI PRISM 7500 (Applied Biosystems) using 96-well optical plates. Each sample was run in triplicate and was supplied with an endogenous control [rat GAPDH endogenous control (VIC®/MGB Probe)]. For gene expression analysis, the appropriate TaqMan expression assays was used. All probes were stained with FAM (Applied Biosystems). The reaction was run in 25 μl mix of TaqMan Universal Master Mix, appropriate primer set, MGB probe and 50 ng cDNA template. Universal thermal conditions (10 min at 95˚C, 40 cycles of 15 sec at 95˚C and 1 min at 60˚C) were used. Data analysis was performed using sequence detection software ver. 1.2 (Applied Biosystems). The amount of RNA transcript in SMs maintained in the control medium was estimated by ΔCt.

Statistical analysis. Data were analyzed by the Wilcoxon matched-pair test or by the Mann-Whitney U test (Statistica software) (19). A p-value <0.05 was considered to indicate a statistically significant difference.

Results

More than 90% of chondrocytes isolated from cartilage fragments used for CIF production were viable and achieved 2 population doublings within 7 days of culture (data not shown), which were similar to the findings of a study examining chondrocytes isolated from intact cartilage (10).

Table I. Concentration of cytokines in CIF.

Cytokine	Mean concentration (pg/ml) ± SD	Cytokine concentration (pg/1 mg of cartilage)
bFGF	2320±210	21.1
IGF1	2054±246	18.7
TGFβ1	517±96	4.7
BMP7	80.5±29	0.73
M-CSF	61±15	0.55
LIF	24±3	0.22
G-CSF	23±5	0.21

CIF, cartilage interstitial fluid; bFGF, basic fibroblast growth factor; IGF1, insulin-like growth factor-1; TGFβ1, transforming growth factor β1; BMP7, bone morphogenetic protein 7; M-CSF, macrophage-colony-stimulating factor LIF, leukemia inhibitory factor; G-CSF, granulocyte-colony-stimulating factor.

Seven cytokines were detected in the CIF following ELISA analysis (Table I). bFGF, and IGF1 predominated with the value >2,000 pg/ml, TGFβ1 reached 500 pg/ml whereas BMP7, M-CSF, G-CSF and LIF were <100 pg/ml. Nine cytokines (IL-1β, IL-7, IL-6, IL-10, PDGF, EGF, TNF, GM-CSF and BMP2) were either absent or below the sensitivity level of the assay. The matrix proteins lubricin and HA were not detected. Cytokine concentrations present in the CIF and calculated per mg of wet weight of cartilage from which CIF was obtained are listed in Table I.

CIF stimulated the mRNA expression of HAS1 ($p<0.005$), HAS2 ($p<0.005$), lubricin ($p<0.003$), collagen type I ($p<0.005$), versican ($p<0.007$), aggrecan ($p<0.005$), MMP2 ($p<0.003$), MMP3 ($p<0.008$), TIMP1($p<0.008$), TIMP2($p<0.008$), TIMP3 ($p<0.008$), IL-6 ($p<0.03$) and TGFβ1 ($p<0.005$), whereas the expression of TNF ($p<0.002$) and IL-1β ($p<0.03$) was inhibited (Fig. 1). Observations regarding the influence of factors present in CIF (CIF-like cocktail) on the SM were limited to nine selected genes (Fig. 2). CIF-like cocktail stimulated the mRNA expression of HAS1 ($p<0.01$), lubricin ($p<0.01$), collagen type I ($p<0.02$), aggrecan ($p<0.02$), TIMP1 ($p<0.02$) and TGFβ1 ($p<0.01$) genes. There was no statistical difference between the expression levels of these genes after CIF and CIF-like cocktail treatment (for HAS1 $p>0.05$, for the rest of genes $p>0.1$). Contrary to CIF, CIF-like cocktail did not change the expression of MMP-3 ($p>0.5$), IL-6 ($p>0.25$) and TNF ($p>0.35$) and therefore the mRNA levels of these genes were different from the mRNA levels following CIF treatment (for MMP3 and IL-6 $p<0.01$, for TNF $p<0.05$).

Discussion

While presenting the first study detecting cytokines in CIF and its effects on the SM, to the best of our knowledge, we have to consider several limitations of this approach. The amount of available cartilage in newborn rats is low, and it is necessary to collect articular cartilage from many joints together with non-calcified fragments of growth plates which cannot be separated during dissection. It is, however, important that

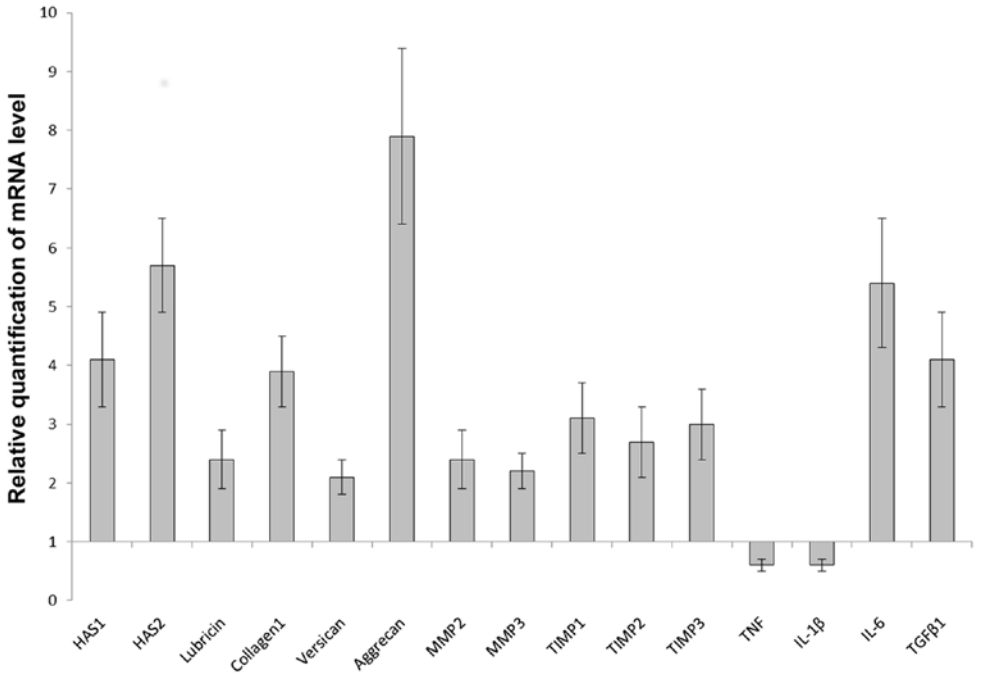

Figure 1. mRNA expression of hyaluronan synthase (HAS)1 and HAS2, extracellular matrix proteins, matrix metalloproteinases (MMPs), tissue inhibitors of metalloproteinase (TIMPs) and cytokines in the synovial membrane after 4 h of incubation with 1 mg/ml cartilage interstitial fluid (CIF) measured by real-time PCR. Values are expressed as the means ± SE. In each group, n=12. Relative expression was calculated against the reference gene, GAPDH. Analysis was conducted as a relative quantification study, using control synovial membrane gene expression as a calibrator (value, 1). Differences in the expression of all genes were significant, according to the Wilcoxon matched-pair test at $p<0.05$. TNF, tumor necrosis factor; IL-1β, interleukin-1β; IL-6, interleukin-6; TGFβ1, transforming growth factor β1.

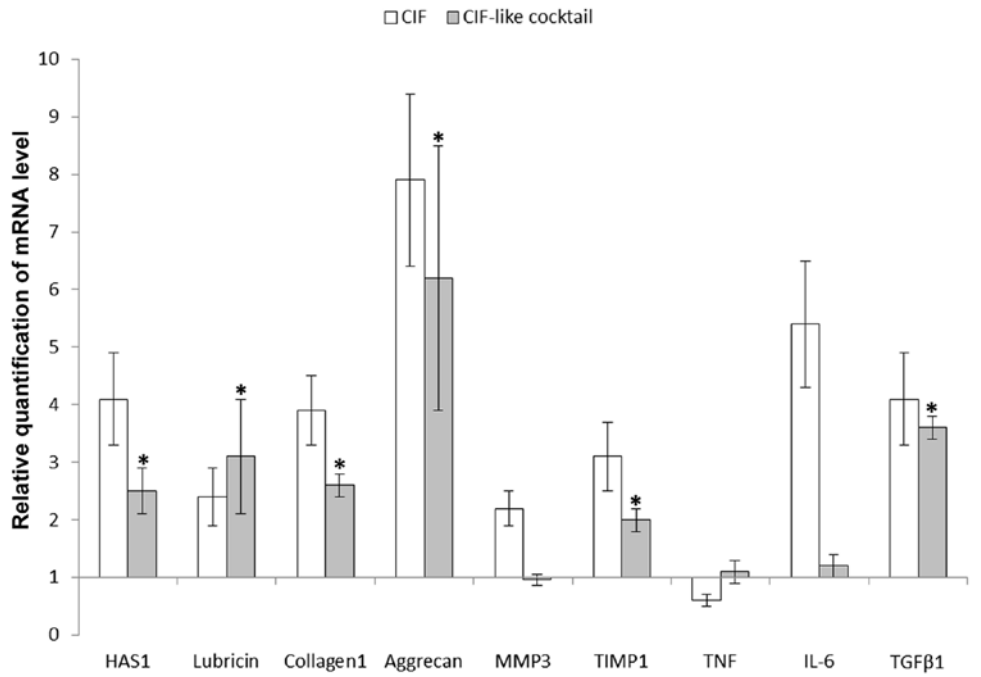

Figure 2. mRNA expression of hyaluronan synthase 1 (HAS1), matrix proteins, matrix metalloproteinase 3 (MMP3), tissue inhibitor of metalloproteinase 1 (TIMP1) and cytokines in the synovial membrane after 4 h of incubation with cartilage interstitial fluid (CIF) or CIF-like cocktail, measured by real-time PCR. Values are expressed as the means ± SE. In each group, n=12. Relative expression was calculated against the reference gene, GAPDH. Lack of differences between the influence of CIF and CIF-like cocktail are marked with asterisks (Mann-Whitney U test). TNF, tumor necrosis factor; Il-6, interleukin-6; TGFβ1, transforming growth factor β1.

CIF is prepared without damage to the chondrocytes, which after CIF harvesting, survived enzymatic isolation and grew in culture (data not shown). The concentrations of various factors in CIF probably represent their average value in the whole cartilage, without taking into consideration zonal chondrocyte distribution (20) and gradients between chondrocytes and

territorial or interterritorial matrix. Cytokines are probably released, as we expected, following McCutchen's (13) theory of 'weeping' lubrication, during each loading of cartilage. Thus, their concentrations in the synovial fluid may vary depending on physical activity. Agents present in CIF (Table I) may act on chondrocytes in an auto- or paracrine fashion, or after being squeezed from the cartilage, during loading of the synovial cells. They may influence both the formation of cartilage matrix components and the production of cytokines.

The SM is formed from four main types of cells, namely synoviocytes (fibroblast-like cells), macrophages, adipocytes and epitheliocytes. Each of these cell types produces a panel of cytokines (17); thus, CIF may stimulate their secretion and they, in turn, may affect expression of particular genes.

Numerous studies describe the effects of particular factors detected in CIF on the expression of connective tissue matrix components. Thus, IGF1 stimulates the synthesis of cartilage matrix proteins (21,22) and collagen type I (23). IGFs are also presumably the major regulatory factors of cartilage proteoglycan synthesis present in human synovial fluid (24). TGFβ1 is involved in the control of differentiation and dedifferentiation of chondrocytes, the synthesis of collagen type II and proteoglycans, and maintaining the homeostasis of cartilage (25). It also enhances the mRNA expression of type I collagen (26).

CIF and CIF-like cocktail stimulated the mRNA expression of collagen type I and aggrecan, a proteoglycan specific for cartilage (Fig. 2) in the SM. The presence in CIF of both IGF1 and TGFβ1 may be important for keeping chondrocytes in the differentiated state, since these factors, acting jointly, reexpressed aggrecan and type II collagen genes in dedifferentiated articular chondrocytes (27). A similar synergistic action of both factors was observed by Seifarth *et al* (28), who found that chondrocytes dedifferentiated by IL-1 regained a chondrocyte-like phenotype after treatment with IGF1 and/or TGFβ1 alone, but co-treatment with IGF1 and TGFβ1 exerted additive anabolic effects.

TGFβ1 stimulated HAS1 expression in fibroblasts (29) and hyaluronan synthesis in rat SM (16). The expression of lubricin and HAS1 was also stimulated by CIF and CIF-like cocktail (Fig. 2) suggesting that the factors present in CIF may influence joint lubrication.

TGFβ1 inhibited MMP3 synthesis and stimulated TIMP1 production in various tissues (30). CIF, however, increased the mRNA expression of MMP3 and TIMP1 whereas CIF-like cocktail had no effect on MMP3 but stimulated the expression of TIMP1.

Since TGFβ1 can induce its own gene expression (31), CIF and CIF-like cocktail could stimulate TGFβ1 expression through a similar mechanism (Fig. 2).

bFGF is synthesized by chondrocytes and functions as an autocrine/paracrine mitogen via its deposition into the cartilage extracellular matrix and subsequent release depending on the biological activity of cartilage (32). It may, depending on the dose and age of cartilage, stimulate or inhibit the synthesis of matrix proteins and accelerate proteoglycan degradation (21,33). bFGF was present in CIF at a relatively high concentration but in view of the above-mentioned reports, its effect on the SM is difficult to estimate.

The low content in CIF of G-CSF and M-CSF is in agreement with observations that they were absent or present at low levels in unstimulated cultures of articular cartilage or chondrocytes (34,35).

LIF, a member of the IL-6 family of cytokines, displays pleiotropic effects on various cell types and organs (36). It was not detected in non-stimulated, short-term chondrocyte cultures, but appeared after stimulation with IL-1 or TNF (37). The small amount of LIF detected in CIF is in agreement with these observations (Table I).

Comparing the effects of CIF with those evoked by a CIF-like cocktail indicates that in the latter some factors were missing. Particularly, CIF-like cocktail did not contain factors responsible for the stimulation of IL-6 gene expression and inhibition of TNF gene expression (Fig. 2).

IL-6 is a multifunctional cytokine with well-defined pro- and anti-inflammatory properties (38). It is produced by articular chondrocytes (39), and by the four main cell types in the SM (17). TGFβ1 increased IL-6 production by chondrocytes (39) and human fibroblasts (40) but IGF1 had no significant effect (39). In the present study, CIF strongly increased the mRNA expression of IL-6, but CIF-like cocktail had no effect.

TNF is a major proinflammatory mediator with a marked functional duality, being strongly engaged both in tissue regeneration/expansion and destruction (41). TNF is expressed in macrophages (42) which are presumably its main source in the SM (17). The administration of CIF inhibited TNF expression, whereas CIF-like cocktail had no statistically valid effect (Fig. 2). The reason for differences in the expression of TNF in the SM under the influence of CIF and CIF-like cocktail remains unclear, since exposing macrophages to IGF1 at a dose similar to that present in CIF and CIF-like cocktail enhanced TNF release and its mRNA level (43).

To sum up, the stimulatory effect of CIF on collagen type I and aggrecan expression observed in this study is in accord with previously published data demonstrating increased expression under the influence of IGF1 and TGFβ1. It is also interesting that in the case of MMP3, TIMP1 and IL-6, CIF exerted stimulatory effects whereas CIF-like cocktail stimulated only the expression of TIMP1. On the other hand, CIF inhibited TNF expression and CIF-like cocktail had no effect. It suggests a need for further, more thorough studies on the content of CIF, and also indicates that the influence of CIF on the SM may not only depend on the activity of particular factors but also on their interactions.

Harvesting CIF from the cartilage of larger animals and humans in order to determine its contents may require more sophisticated equipment than a syringe. Once, however, technical problems have been resolved, studies on CIF may provide valuable information regarding the relationship between cartilage and the SM in physiological and pathological states.

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