

# Influence of cartilage interstitial fluid on gene expression in cruciate ligament fibroblasts

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**Abstract.** Loading of articular cartilage during motion squeezes the fluid from the cartilage, termed cartilage interstitial fluid (CIF), which was found to influence gene expression in synovial membrane cells. After crucial ligaments damage, these cells are exposed to synovial fluid containing factors released from articular cartilage; the aim of the present study was to establish the influence of CIF and factors present in CIF (CIF-like cocktails) on crucial ligament fibroblasts. CIF was squeezed from articular-epiphyseal cartilage complexes of newborn rats. Fibroblasts were obtained from crucial ligaments of adult rat knee joints. Cells were cultured in control medium, CIF and CIF-like cocktails, and the expression of selected genes was evaluated using quantitative PCR. CIF stimulated the expression of HAS1, HAS2, aggrecan, lubricin, MMP3, TIMP3 and TGF $\beta$ 1. Expression of collagen type I, versican, MMP2, TIMP2, TNF and IL1 $\beta$  was inhibited. The CIF-like cocktail stimulated HAS1, HAS2, collagen type I, versican, aggrecan, lubricin, TIMP1, TGF $\beta$ 1, IL1 $\beta$ , IL6 and inhibited of MMP3 and TNF expression. Both agents exerted similar effects on the expression of HAS2, aggrecan, lubricin, TGF $\beta$ 1 and TNF. CIF contains inhibitory and stimulatory factors affecting gene expression in crucial ligament fibroblasts and some of them were not included in the CIF-like cocktail. Due to the powerful influence of CIF on crucial ligament fibroblasts and the synovial membrane, further studies on its composition are needed. An improved CIF like-cocktail could be applied in the treatment of various joint or tendon ailments.

## Introduction

Both anterior and posterior cruciate ligaments are intracapsular but extrasynovial structures and are nourished by the middle genicular artery (1,2). Synovial vessels run obliquely and longitudinally over the entire length of the anterior cruciate ligament (ACL), beneath the synovial membrane. They arborize to form a web-like network of periligamentous vessels that ensheath the entire ligament (2).

Damage of cruciate ligaments may be accompanied by disruption of the overlying synovial sleeve. In consequence, fibroblasts forming ligaments may be exposed to the synovial fluid (3) and, by the same token, more accessible to biological stimulants introduced into the joint cavity. When the fibroblasts from the ACL were exposed to the synovial fluid, their plating efficiency was significantly reduced suggesting that their ability to proliferate in a milieu of synovial fluid is impaired (3). Biological stimulants, however, such as platelet rich plasma (PRP) preparations had beneficial influence on crucial ligaments healing in animal experiments (4,5). Moreover, platelets together with plasma proteins stimulated also collagen gene expression by cultured ACL fibroblasts (6). In studies with better defined growth factors, Lee *et al* (7) found that the stimulation of the cell outgrowth in explants of rabbit anterior cruciate by basic fibroblast growth factor (bFGF), insulin, transforming growth factor- $\beta$  1 (TGF $\beta$ 1), and platelet-derived growth factor-B (PDGF-B), was much greater in the presence of all four growth factors than the sum of the outgrowth with the individual factors. Stimulation with TGF $\beta$ 1 alone evoked strong proliferative response of cells from explants of the ACL (8). TGF $\beta$ 1 induced also dramatic elevation of metalloproteinase 2 (MMP2) activities and the MMP2/tissue metalloproteinase inhibitors (TIMPs) ratio in cells from ACL (9) and significantly increased mRNA level of lysyl oxidase family members (10) while tumor necrosis factor (TNF) downregulated it (11).

Analysing both synovial fluid and growth factors influence on the cruciate ligament fibroblasts (CLFs) it seems advisable to include also factors produced by chondrocytes from articular cartilage. McCutchen (12) and others (13) formulated the theory of 'weeping' lubrication in synovial joints. According to their studies cartilage matrix contains a fluid phase, representing ~70% of its volume. During joint loading, ~10% of this liquid is squeezed from the cartilage surface (which, in a molecular sense, is porous) into the intra-articular cavity, and is

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*Abbreviation:* CIF, cartilage interstitial fluid

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responsible for hydrostatic lubrication. Thus, it may be expected that cartilage interstitial fluid (CIF) squeezed from cartilage during joint loading contains cytokines produced by chondrocytes and affects tissues of the joint. We have previously found that CIF released from newborn rat cartilage contained bFGF, insulin-like growth factor 1 (IGF1), TGF $\beta$ 1, bone morphogenetic protein 7 (BMP7), macrophage colony-stimulating factor (M-CSF), granulocyte colony-stimulating factor (G-CSF) and leukemia inhibitory factor (LIF). We also demonstrated that CIF stimulated a number of genes in synovial membrane and dermal fibroblasts and these effects could be partially imitated by CIF-like cocktail composed of factors identified in CIF (14-16).

After crucial ligaments damage and tearing of synovial tissue cover, their cells would be exposed to synovial fluid, presumably containing factors not only produced by synovio-cytes but also released from articular cartilage. Thus, it appeared interesting to establish influence of CIF on the cells derived from the crucial ligaments, to see whether they react to CIF stimulation similarly to dermal fibroblasts, or display peculiarities which could be used in attempts to produce biological constructs replacing damaged ligaments.

## Materials and methods

**Animals.** Three- to five day-old inbred Lewis rats of both sexes served as cartilage donors for CIF preparation. Crucial ligaments were dissected from ten to twelve week-old male Lewis rats. The animals were obtained from the Animal Unit of the Warsaw Medical University. The study and the methods were approved by the Animal Ethics Committee of the Warsaw Medical University (Warsaw, Poland).

**Preparation of CIF.** CIF was prepared as described previously (14). Briefly, CIF was squeezed from the articular-epiphyseal cartilage complexes dissected from the newborn rats. After clearing from the surrounding tissues cartilages from 2 animals were put into 2 ml of PBS (Gibco BRL, Paisley, Scotland, UK) and cut into small fragments which, together with PBS, were transferred into a 50 ml Luer Lock syringe closed with the PTFE Body Two-Way Valve from Hamilton (Sigma-Aldrich Chemie, Steinheim, Germany). The air in the syringe was pressed with the plunger to increase pressure up to three bars. Then the plunger was slowly released. This procedure was repeated 20 times. The fluid was separated from cartilage fragments by centrifugation, desalted on PD-10 columns (Amersham Biosciences, Uppsala, Sweden) and lyophilized. The lyophilisate was dissolved in RPMI medium (Gibco). CIF from 10-20 rats was pooled to obtain more uniform material and the protein content was determined using BCA protein assay reagent (Pierce, Rockford, IL, USA). The total amount of protein in CIF squeezed from cartilage obtained from one animal varied from 0.87 to 1.1 mg. Working solution of CIF was standardized to contain 1 mg/ml of protein in RPMI supplemented with 2% of FCS and 1% of Antibiotic/Antimycotic Solution (all from Gibco).

**CIF-like cocktail preparation.** CIF-like cocktail contained commercial cytokines dissolved in RPMI medium with 0.1% of bovine serum albumin (BSA) and 2% of FCS (all from

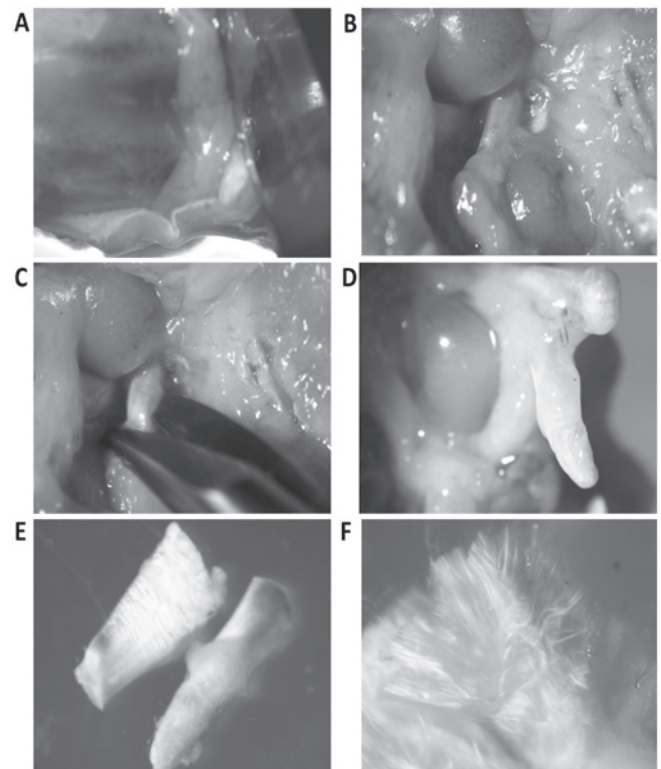


Figure 1. Dissection of crucial ligaments under control of the dissecting microscope. (A) Separation of femur from tibia. Crucial ligaments masked by fat deposits are joined to the femur. (B) Exposition of crucial ligaments. Ligaments are still joined to the femur and menisci. (C) Cutting of the ligament from the meniscus. (D) Separated from the menisci crucial ligaments are still joined with condyle of the femur. (E) Crucial ligaments after dissection. (F) Bundles of ligament collagen fibers separated with thin needle. Magnification, x60.

Gibco). Cytokines were used in concentration identical to that found in CIF: 25 pg/ml G-CSF, 60 pg/ml M-CSF, 25 pg/ml LIF, 80 pg/ml BMP7, 2.5 ng/ml bFGF (PromoKine; PromoCell GmbH, Heidelberg, Germany), 0.5 ng/ml TGF $\beta$ 1 (Sigma-Aldrich Chemie) and 2 ng/ml IGF1 (R&D Systems, MN, USA).

**Dissection of crucial ligaments.** Knee joints were cut off together with fragments of femur and tibia from 10-12 week old rats. Joint cavity was opened and the synovial membrane was excised together with patella, patellar ligament and joint capsule. Then all tissues covering the joint together with remnants of joint capsule were removed. The further preparation was done under dissecting microscope. Anterior and posterior crucial ligaments, together with menisci were separated from the tibia (Fig. 1A and B). The femur was grasped with sharp forceps and both crucial ligaments were cut off from the menisci and afterwards from the condyles of the femur (Fig. 1C-E).

**Isolation and culture of ligament fibroblasts.** Bundles of ligament collagen fibers were separated with thin needles to expose fibroblasts located between bundles (Fig. 1F). Ligaments were incubated in enzymic solution containing 0.25% collagenase (Type I), 0.05% DNase, 17.5  $\mu$ M N-a-p tosyl-L-lysine chloromethylketone (TLCK) and 1% antibiotic-antimycotic solution (all from Sigma-Aldrich Chemie) in RPMI medium, (Gibco).

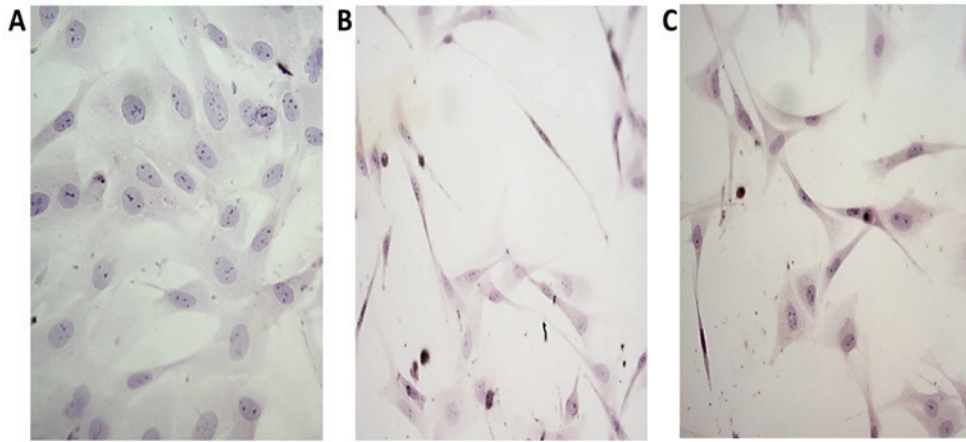


Figure 2. Ligament fibroblasts cultured 24 h. (A) In control medium. (B) In CIF working solution. (C) In CIF-like cocktail. Hematoxylin-eosin, magnification, x400.

Ligaments were delicately shaken for 40 min in humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C. The digested material was filtered through a 40- $\mu$ m mesh nylon filter. The liberated cells were rinsed 3 times with the RPMI medium (Gibco) supplemented with 10% FCS and 1% Antibiotic/Antimycotic solution (all from Gibco) and seeded into 25 cm<sup>2</sup> flasks (Corning Inc., Corning, NY, USA) in 5 ml of medium. After the cells reached subconfluency, they were detached with 0.25% trypsin-EDTA (Sigma-Aldrich Chemie), suspended in CIF, CIF-like cocktail or control medium (RPMI with 0.1% of BSA, 1% of Antibiotic/Antimycotic solution and 2% of FCS) and seeded into flat-bottomed 24-well plates (Corning) at the density 5x10<sup>4</sup> per well. The cells were incubated in humidified atmosphere of 5% CO<sub>2</sub> in air at 37°C for 24 h. After incubation the total RNA from cultured cells was isolated and the expression of genes encoding: Hyaluronan synthases (HAS1 and HAS2), extracellular matrix proteins (collagen type I, versican, aggrecan and lubricin), matrix metalloproteinases (MMP2 and MMP3), tissue inhibitors of metalloproteinases (TIMP1, TIMP2 and TIMP3), and cytokines (TGF $\beta$ 1, TNF, IL1 $\beta$  and IL6) was examined.

Some fibroblasts in control or experimental medium were also seeded onto 12 mm glass slides placed in 24 well plates and, after 24 h culture, were fixed and stained with hematoxylin/eosin.

**Total RNA isolation.** RNA was isolated with NucleoSpin®RNA II kit (Macherey-Nagel, Duren, Germany), according to manufacturer's protocol. The quantity and quality of the isolated total RNA was evaluated spectrophotometrically using ND-2000-Spectrophotometer NanoDrop 2000 with software for analysis of nucleic acids (Thermo Fisher Scientific, Wilmington, Delaware, USA).

**Reverse transcription.** Reverse transcription was performed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Cheshire, UK), according to the manufacturer's protocol in Eppendorf Mastercycler gradient (10 min at 25°C, 120 min at 37°C and 5 sec. at 85°C). Briefly, 2  $\mu$ l of 10X RT buffer, 0.8  $\mu$ l of 25x dNTP Mix, 2  $\mu$ l of 10x Random Primers, 1  $\mu$ l of Multiscribe Reverse Transcriptase, 4.2  $\mu$ l of

nuclease-free water and 10  $\mu$ l of mRNA (0.5  $\mu$ g) per one reaction. cDNA samples were stored at -20°C.

**Real-time PCR.** Real-time PCR was performed in the 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 Rn01775763\_g1). For gene expression analysis, the proper TaqMan expression assays was used. (Rn00597231\_m1 for HAS1, Rn00565774\_m1 for HAS2, Rn01526721\_m1 for collagen type I, Rn01493763\_m1 for versican, Rn00573424\_m1 for aggrecan, Rn01490812\_m1 for lubricin, Rn01538167 for MMP2, Rn00591740\_m1 for MMP3, Rn 00587558\_m1 for TIMP1, Rn00573232\_m1 for TIMP2, Rn00441826\_m1 for TIMP3, Rn00572010\_m1 for TGF $\beta$ 1, Rn99999017\_m1 for TNF, Rn00580432\_m1 for IL1 $\beta$  and Rn01410330\_m1 for IL6). All probes were stained with FAM (Applied Biosystems). Reactions was run in 25  $\mu$ l of volume with TaqMan Universal Master Mix, appropriate primer set, MGB probe and 50 ng of 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 done with sequence detection software version 1.2 (Applied Biosystems).

**Statistical analysis.** Data were analyzed by the Mann-Whitney U test (Statistical2 software; StatSoft Polska, Krakow, Poland). P<0.05 was considered to indicate a statistically significant difference (17).

## Results

Ligament fibroblasts cultured in the control medium had multi-form appearance and possess scanty, usually short processes, characteristic for typical fibroblasts in culture. (Fig. 2A). In CIF treated cultures numerous fibroblasts assumed spindle-like form, with long, thin processes or were triangular with slim, very long extensions. (Fig. 2B). The influence of CIF-like cocktail on the morphology of cultured fibroblasts was similar to the influence of CIF (Fig. 2C).

In crucial ligament fibroblasts (CLFs) CIF stimulated the expression of genes encoding HAS1, HAS2, aggrecan,



Table I. Influence of CIF on cruciate ligament and dermal fibroblasts.

Gene	Cruciate ligament fibroblasts	Dermal fibroblasts <sup>a</sup>
The same effect (no statistically significant differences)		
HAS2	7.10±1.60	6.92±1.06
Collagen1	0.27±0.05	0.36±0.06
MMP2	0.53±0.11	0.53±0.05
TIMP1	1.23±0.22	1.10±0.15
TIMP2	0.40±0.05	0.39±0.07
TGFβ1	1.78±0.24	2.32±0.36
IL-1β	0.45±0.13	0.63±0.09
Similar effect (statistically significant differences)		
HAS1	24.17±6.58	1.56±0.09
Versican	0.43±0.05	0.67±0.05
Lubricin	5.2±0.64	1.52±0.33
MMP3	9.37±2.65	276.91±53.35
The opposite effect		
Aggrecan	2.97±0.62	0.12±0.05
TIMP3	1.92±0.24	0.09±0.02
TNF	0.66±0.15	2.32±0.63
IL-6	1.0±0.16	1.84±0.35

<sup>a</sup>(15). Data are presented as the mean ± standard error. Data were evaluated by Mann-Whitney test. CIF, cartilage interstitial fluid; HAS, hyaluronan synthases; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases; TGF, transforming growth factor; TNF, tumor necrosis factor; IL, interleukin.

lubricin, MMP3, TIMP3 and TGFβ1. Expression of collagen type I, versican, MMP2, TIMP2, TNF and IL1β was inhibited. CIF-like cocktail stimulated expression of HAS1, HAS2, collagen type I, versican, aggrecan, lubricin, TIMP1, TGFβ1, IL1β and IL6 and inhibited of MMP3 and TNF expression. Both agents exerted the similar effect on the expression of genes encoding HAS2, aggrecan, lubricin, TGFβ1 and TNF (Fig. 3).

## Discussion

In the previous work (15) the influence of CIF and CIF-like cocktail on the gene expression in dermal fibroblasts was studied. In the present work we examined the influence of CIF and CIF-like cocktail on fibroblasts isolated from crucial ligaments. Morphological changes of ligament fibroblasts under the influence of CIF or CIF-like cocktail were similar to those occurring in dermal fibroblasts and probably were caused by the reorganization of the cytoskeleton (Fig. 2B and C). The effects exerted by CIF and CIF-like cocktail on gene expression in dermal and CLFs demonstrated several differences (Tables I and II).

In case of HAS1 expression, CIF and CIF-like cocktail influence was markedly stronger on CLFs than on dermal fibroblasts (DFs). Similarly, HAS2 expression under the influence CIF-like cocktail was stronger in CLFs than in DFs, however, CIF stimulation of HAS2 was within the same range (Tables I and II). Hyaluronic acid present in synovial fluid is synthesized in synovial membrane by fibroblast-like synoviocytes, which express hyaluronan synthases (18). The

same authors (18) demonstrated that TGFβ1 is the potent stimulus for HAS1 transcription. Thus, TGFβ1 which is present both in CIF and CIF-like cocktail could be responsible for raise in HAS1 expression. In short, lasting 4 h only stimulation of the synovial membrane, expression of HAS1 was, however, not stimulated by TGFβ1 or IGF1 acting alone, but both factors applied together exerted distinct stimulatory effect suggestive of certain synergism (14,16). Since platelet-rich plasma (PRP), used for the treatment of joint ailments, contains, among others, TGFβ1, IGF1 and bFGF (19), the same factors which are present in CIF, administration of PRP could also stimulate HAS1 expression in crucial ligaments.

CIF inhibited gene expression of versican and collagen type I both on CLF and DF while CIF like cocktail stimulated it in CLFs (Tables I and II). Cells from human cruciate ligaments expanded in culture and treated with TGFβ1 and bFGF increased expression and production of collagenous and noncollagenous extracellular matrix proteins (6) similarly as CIF-like cocktail in which both these factors were present. Thus the marked difference between CIF and CIF-like cocktail suggests that the CIF contains some, as yet unidentified, inhibitory factor(s). A decrease in versican content detected in ruptured ACLs of patients in comparison to normal controls (20) could also depend on the influence of the same or similar factor(s) which gained access to the torn ligaments.

Expression of lubricin was stimulated by both agents only in the CLFs (Tables I and II). Similar stimulation was also evident in the rat synovial membrane (14). In torn ACLs, lubricin was generally found as a discrete layer covering the torn surface. Lubricin was also found on the native surfaces of intact ACLs

Table II. Influence of CIF-like cocktail on cruciate ligament and dermal fibroblasts.

Gene	Cruciate ligament fibroblasts	Dermal fibroblasts <sup>a</sup>
The same effect (no statistically significant differences)		
Collagen1	1.82±0.19	1.40±0.10
MMP2	1.03±0.07	1.14±0.04
TIMP2	0.92±0.07	1.09±0.06
TIMP3	0.70±0.22	1.15±0.09
IL-1β	1.43±0.09	1.24±0.35
Similar effect (statistically significant differences)		
HAS1	6.85±1.19	1.36±0.19
HAS2	4.33±0.40	1.60±0.16
TIMP1	2.43±0.31	1.25±0.12
TGFβ1	2.57±0.17	1.52±0.14
The opposite effect		
Versican	1.60±0.15	1.08±0.07
Aggrecan	2.77±0.26	1.08±0.19
Lubricin	3.58±0.45	0.95±0.16
MMP3	0.42±0.10	0.96±0.10
TNF	0.52±0.09	2.16±0.35
IL-6	3.23±0.81	1.17±0.09

<sup>a</sup>(15). Data are presented as the mean ± standard error; CIF, cartilage interstitial fluid; HAS, hyaluronan synthases; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases; TGF, transforming growth factor; TNF, tumor necrosis factor; IL, interleukin.

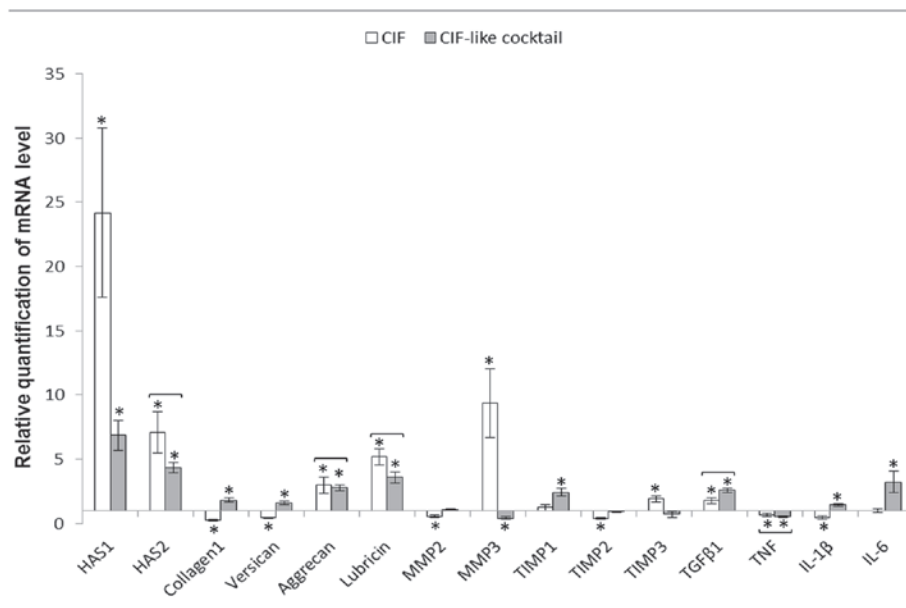


Figure 3. Mean ± standard error of HASs, matrix proteins, MMPs, TIMPs and cytokine mRNA levels in the cruciate ligament fibroblasts after 24 h of incubation with CIF or CIF-like cocktail (n=12). Relative expression was calculated against the reference gene, GAPDH. Analysis was conducted as a relative quantification study using fibroblasts cultured in control medium (RPMI medium containing 2% fetal calf serum) gene expression as a calibrator (value=1). \*P<0.05 between control and experimental groups. Statistically insignificant differences between the influence of CIF and CIF-like cocktail are joined by brackets, because the lack of differences suggests that CIF-like cocktail acts similarly to CIF. CIF, cartilage interstitial fluid; HAS, hyaluronan synthases; MMP, matrix metalloproteinase; TIMP, tissue inhibitors of metalloproteinases; TGF, transforming growth factor; TNF, tumor necrosis factor; IL, interleukin.

and on torn edges of ACLs. Thus, it may interfere with the integrative healing process needed for ACL repair (21).

Both CIF and CIF-like cocktail stimulated aggrecan expression in CLFs but not in DFs (Tables I and II). Presence

of aggrecan in fibrocartilaginous ligaments was also reported previously (22).

In synovial fluid samples of patients with crucial ligaments injury the average concentrations of MMP-3 was highly

elevated in comparison with normal controls. The concentration of MMP-3 remained high, independent of the duration since the injury (23). In our experiments stimulation of MMP3 by CIF was evident in the CLFs, although was many times stronger in DFs. CIF-like cocktail inhibited its expression in CLFs and had no influence in DFs (Tables I and II). Reactions of crucial ligaments may considerably differ from those of tendons, since downregulated expression of MMP3 mRNA have been found in injured or ruptured Achilles and supraspinatus tendons as compared to non-injured tendon groups (24).

The expression of TGF $\beta$ 1 was stimulated by CIF and CIF-like cocktail in DFs and CLFs (Tables I and II) and its secretion was also detected in human tendon fibroblasts (25).

Many articles refer to the fibroblast gene expression of the ligaments in damaged or pathological joints (20,21,23,24). Our study shows the influence of factors present in normal joint fluid on the gene expression in ACL fibroblasts. Therefore, our results may be the basis for evaluation the changes occurring in the pathological states of synovial joints.

It is evident from the presented data that CIF contains both inhibitory and stimulatory factors and some of them are not included in CIF-like cocktail. Melas *et al* (26) found that cultured chondrocytes may release up to 55 cytokines and other active agents either spontaneously or after stimulation by various ligands and list of growth factors produced by chondrocyte may still not be complete. Thus, in view of the powerful influence exerted by CIF both on the synovial membrane (14) and CLFs as evident in this work, further studies on its composition are needed, particularly that improved CIF like-cocktail could find application in treatment of various joint or tendon ailments.

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