

## Influence of Cartilage Interstitial Fluid on Gene Expression in Dermal Fibroblasts

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Cartilage interstitial fluid (CIF) containing bFGF, IGF1, TGF $\beta$ 1, BMP7, MCSF, GCSF, LIF and CIF-like cocktail, composed of agents found in CIF, changed the expression of many genes in cells of the synovial membrane. Because the main type of cells of the synovial membrane are fibroblast-like cells, in the present work we evaluated the response of dermal fibroblasts to CIF and CIF-like cocktail and compared it with the response of synovial membrane cells. In the presence of CIF, fibroblasts changed shape from roughly polygonal to spindle-like, elongated cells. Real-time PCR showed that CIF stimulated expression of HAS1, HAS2, MMP3, TGF $\beta$ 1, TNF and IL6 while expression of collagen type I, versican, aggrecan, MMP2, TIMP2, TIMP3, and IL1 $\beta$  was inhibited. The CIF-like cocktail stimulated expression of HAS1, HAS2, collagen type I, TGF $\beta$ 1 and TNF. Both exerted a similar effect on the expression of HAS1, TGF $\beta$ 1, TNF and had no influence on TIMP1 and lubricin expression. The CIF-like cocktail, contrary to CIF, did not influence versican, aggrecan, MMP2, MMP3, TIMP2, TIMP3, IL1 $\beta$  and IL6 expression.

Key words: Cartilage interstitial fluid, fibroblast, matrix gene expression, cytokine gene expression.

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We have previously demonstrated that cartilage interstitial fluid (CIF) rinsed out from the cartilage of immature rats contained basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF1), transforming growth factor  $\beta$ 1 (TGF $\beta$ 1), bone morphogenetic protein 7 (BMP7), macrophage colony stimulating factor (MCSF), granulocyte colony stimulating factor (GCSF) and leukemia inhibitory factor (LIF). In the synovial membrane of the knee joint from adult rats CIF stimulated expression of hyaluronan synthases 1 and 2 (HAS1, HAS2), lubricin, collagen type I, versican, aggrecan, matrix metalloproteases 2 and 3 (MMP2, MMP3), tissue inhibitors of metalloproteases 1, 2, 3 (TIMP1, TIMP2, TIMP3), interleukin 6 (IL6), TGF $\beta$ 1 and decreased expression of tumor necrosis factor (TNF) and interleukin 1 $\beta$  (IL1 $\beta$ ) (Hyc *et al.* 2016). The synovial membrane is, however, a complex tissue

formed by four main types of cells, namely synoviocytes (fibroblast-like cells), macrophages, adipocytes and endothelial cells (Hyc *et al.* 2011); thus, the response to CIF represents a summary of the activities of various cell types. Since one of the main types of cells in the synovial membrane are fibroblast-like synoviocytes, fibroblasts, the most common type of cells found in connective tissue, may also be a target for CIF action. Fibroblasts produce collagens forming a three-dimensional framework for other tissues. Moreover, they control and modulate the extracellular matrix, and thus indirectly influence the function of other cells within the given tissue or organ. Beyond their role in structural support, fibroblasts are able to secrete and respond to cytokines, chemokines, and growth factors (Van Lintout *et al.* 2014). Matrix metalloproteinases (MMPs), the tissue inhibitors of

metalloproteinases (TIMPs) and growth factors produced by fibroblasts, degrade or stimulate the synthesis of numerous extracellular components. Their orchestrated activity is vitally important to ensure successful tissue growth, remodeling and repair after damage (BALCERZAK *et al.* 2001; BIRKEDAL-HANSEN 1995). Fibroblasts from various tissues, however, considerably differ in morphology, proliferation, matrix production and MMP expression (CHANG *et al.* 2014; LINDNER *et al.* 2012; NOLTE *et al.* 2008). It is known that fibroblasts from the papillary and reticular layer of dermis differ in morphology, rate of growth, and secretory profile (DRISKELL *et al.* 2013; DRISKELL & WATT 2015; JANSON *et al.* 2014). Fibroblasts from upper dermis (papillary fibroblasts) regulate hair growth and the arrector pili muscle. Cells from the lower dermis (reticular fibroblasts) synthesize the bulk of the fibrillar extracellular matrix. Since papillary fibroblasts differentiate into reticular fibroblasts in monolayer cultures (JANSON *et al.* 2014), in our experiments fibroblasts prepared from the whole dermis were used.

The purpose of our work was to evaluate the influence of cartilage interstitial fluid (CIF) and CIF-like cocktail (prepared from all factors found in CIF) on gene expression in dermal fibroblasts. These experiments have an interesting prospective aspect. TAKEHARA (2000) suggested that cocktails of various growth factors may permit subtle growth regulation of skin fibroblasts and that such technology would have applications in the treatment of many skin diseases. Thus, recognition of how CIF, as the connective tissue intercellular fluid with evolutionary established composition probably optimal for tissue performance, influences gene expression may help to realize this goal.

## Materials and Methods

### Animals

Three-to five day-old inbred Lewis rats of both sexes served as cartilage donors for CIF preparation and culture of dermal fibroblasts. 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 (2011.11.29 No.34/2011).

### Cartilage interstitial fluid (CIF) preparation

CIF was prepared as described previously (HYC *et al.* 2016). Briefly, CIF was rinsed out from the articular-epiphyseal cartilage complexes dissected from newborn rats. After clearing from the sur-

rounding 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 Hamilton PTFE Body Two-Way Valve (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. CIF from 10-20 rats was pooled to obtain more uniform material. The lyophilisate was dissolved in RPMI (Gibco) medium and the protein content was determined. The total amount of protein in CIF harvested from cartilage obtained from one animal varied from 0.87 to 1.1 mg. The working solution of CIF was standardized to contain 1 mg/ml of protein.

### Preparation of the CIF-like cocktail

The CIF-like cocktail contained commercial, recombined cytokines, used in concentration identical to that found in CIF, i.e. 25 pg/ml GCSF, 60 pg/ml MCSF, 25 pg/ml LIF, 80 pg/ml BMP7 and 2.3 ng/ml bFGF (PromoKine; PromoCell GmbH, Heidelberg, Germany), 0.5 ng/ml TGFβ1 (Sigma) and 2.0 ng/ml IGF1 (R&D Systems Inc., Minneapolis, MN, USA) (HYC *et al.* 2016).

### Isolation and culture of dermal fibroblasts

Three-to five-day old rats were killed by cervical dislocation and put into 10% Antibiotic-Antimycotic Solution (Sigma) at 37°C for 1h. The skin was cut into small fragments. The enzyme solution used for cell liberation contained 0.25% collagenase (Type I), 0.05% DNase, 17.5 μM Nα-p-tosyl-l-lysine chloromethyl ketone and 1% Antibiotic-Antimycotic Solution (Sigma) in RPMI medium, (GIBCO). Skin fragments were stirred on a magnetic stirrer at 37°C for 60 min. The cells were filtered through a 40-μm mesh nylon filter and seeded into 25cm<sup>2</sup> flasks (Corning Inc., Corning, NY, USA) in the RPMI medium, supplemented with 10% FCS and antibiotics at a density of 10<sup>6</sup> cells per flask. After the cells reached subconfluency, they were detached with 0.25% trypsin-EDTA (Sigma), rinsed and seeded into 24 well flat-bottomed plates (Corning) at a density of 5x10<sup>4</sup> per well. Control medium contained RPMI supplemented with 2% of FBS, 1% Antibiotic-Antimycotic Solution, and 1 mg/ml of bovine albumin (Sigma). Experimental groups contained 1 mg/ml of CIF or 1 mg/ml of BSA with CIF-like cocktail. 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: HAS1, lubricin, collagen type I, aggrecan, versican, MMP3, TIMP1, IL1 $\beta$ , IL6, TNF and TGF $\beta$ 1 was examined.

Some fibroblasts in control or experimental medium were also seeded onto 12 mm glass slides placed in 24 well plates (Corning) and after 24 h of culture cells were stained with hematoxylin and eosin.

#### Total fibroblast RNA isolation

RNA was isolated with the NucleoSpin®RNA II kit (Macherey-Nagel, Duren, Germany), according to the manufacturer's protocol (using of DNase digestion). The quantity and quality of the isolated total RNA was evaluated spectrophotometrically using ND-2000 – Spectrophotometer NanoDrop2000 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 an Eppendorf Mastercycler gradient (10 min 25°C, 120 min 37°C, 5 sec 85°C). Briefly, 2  $\mu$ l of 10 $\times$  RT buffer, 0.8  $\mu$ l of 25 $\times$  dNTP Mix, 2  $\mu$ l of 10 $\times$  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, probe was stained with FAM and both primers and probe map within a single exon). For gene expression analysis, the proper TaqMan expression assays were used. (Rn00597231\_m1 for HAS1, Rn00565774\_m1 for HAS2, Rn01526721\_m1 for collagen type I, Rn00573424\_m1 for aggrecan, Rn01493763\_m1 for versican, 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 and all span exons (Applied Biosystems). Reactions were run in 25  $\mu$ l volume with TaqMan Universal Master Mix, the

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 ver. 1.2 (Applied Biosystems).

#### Statistical analysis

Data were analyzed by the Mann-Whitney U test (Statistica12 software, StatSoft Polska) (LIVAK & SCHMITTGEN 2001). A p-value <0.05 was considered to indicate a statistically significant difference.

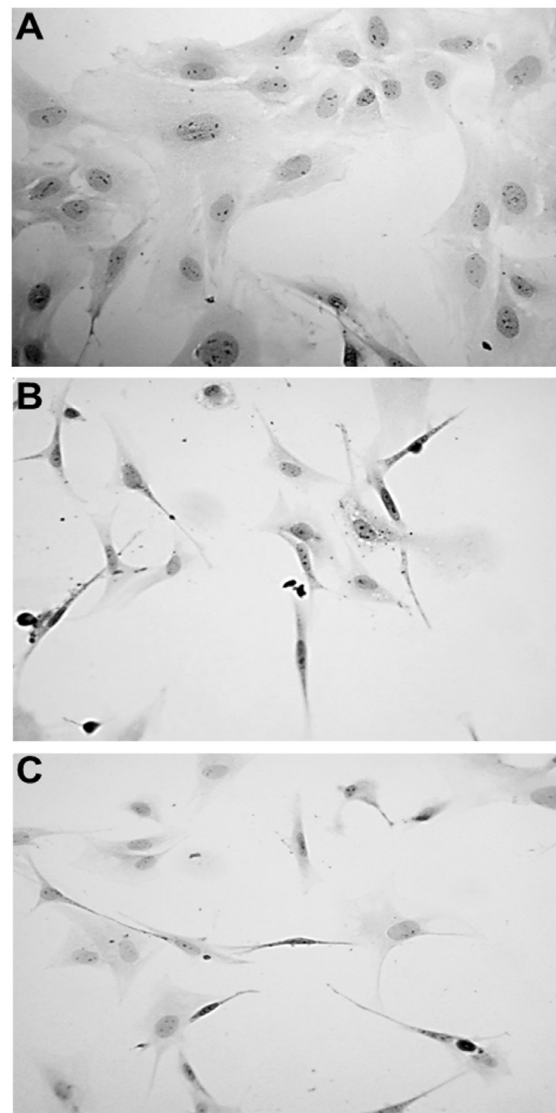


Fig. 1 A, B, C. Fibroblasts incubated 24 h: A – in the control medium; B – in the medium with CIF; C – with CIF-like cocktail; hematoxylin and eosin, x400.

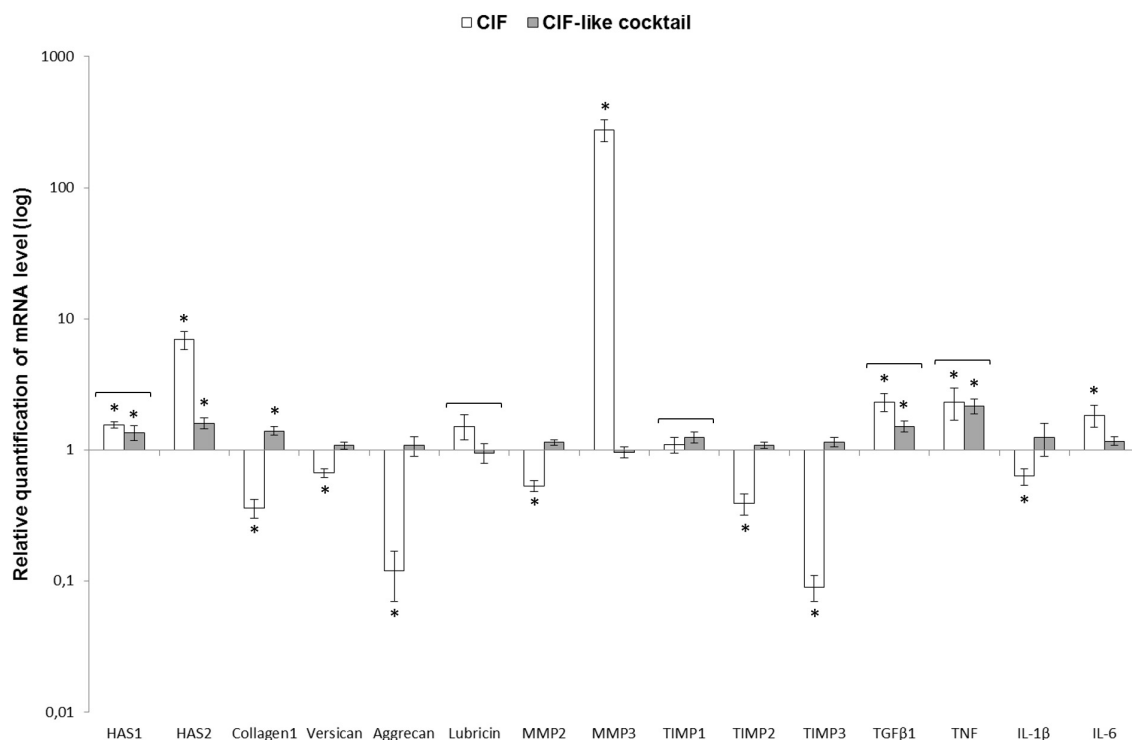


Fig. 2. Mean  $\pm$  SE of HASs, matrix proteins, MMPs, TIMPs and cytokine mRNA levels in the dermal fibroblasts after 24 h of incubation with CIF or CIF-like cocktail. In each group  $n=12$ . Relative expression was calculated against the reference gene, GAPDH. All data were analyzed by a Mann-Whitney U test. Statistically significant differences between control and experimental groups are marked with asterisks. 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.

## Results

The influence of CIF was observed at the level of morphology of cultured cells. Control fibroblasts were flattened and had multiform appearance with scanty, usually short processes (Fig. 1A). In CIF treated cultures cells were smaller, assumed a spindle-like form with long, thin processes or were triangular with slim, very long extensions. (Fig. 1B). The influence of CIF-like cocktail on the morphology of cultured fibroblasts was similar to the influence of CIF but less pronounced (Fig. 1C).

At the molecular level, CIF stimulated expression of HAS1, HAS2, MMP3, TGFβ1, TNF and IL6. Expression of collagen type I, versican, aggrecan, MMP2, TIMP2, TIMP3, and IL1β was inhibited. The CIF-like cocktail stimulated expression of HAS1, HAS2, collagen I, TGF and TNF. Both agents exerted a similar effect (according to the Mann-Whitney test) on the expression of genes encoding HAS1, TGFβ1, TNF and had no influence of TIMP1 and lubricin expression. The CIF-like cocktail, contrary to CIF, did not influence versican, aggrecan, MMP2, MMP3, TIMP2, TIMP3, IL1β and IL6 expression (Fig. 2).

## Discussion

In previous work (HYC *et al.* 2016) the influence of CIF and CIF-like cocktail on gene expression in cells of synovial membranes cultured as whole organs was studied. Although the synovial membrane response was tested after 4 hours of incubation and exposition of dermal fibroblasts lasted 24h, there is a partial similarity in action of CIF in both types of cultures, stimulatory in the case of HAS1, HAS2, MMP3, TGFβ1, IL6 and inhibitory in the case of IL1β (Fig. 2, Table 1). On the other hand, CIF stimulated expression of TNF in dermal fibroblasts and inhibited it in synovial membrane. CIF stimulated expression of collagen type I, aggrecan, versican, MMP2, TIMP2 and TIMP3 in the synovial membrane and inhibited their expression in dermal fibroblasts although both IGF1 (BLACKSTOCK *et al.* 2014) and TGFβ1 (CHU & PRASAD 1999; TAKEHARA 2000; VARGA *et al.* 1987; VERRECCHIA & MAUVIEL 2004) enhance collagen type I expression, as well as proteoglycan synthesis (PATIL *et al.* 2011; SAH *et al.* 1994; SCHALKWIJK *et al.* 1989). Thus, the inhibitory effect of CIF in dermal fibroblasts, which contains both these factors in considerable concentra-



Table 1

Comparison of cartilage interstitial fluid (CIF) and CIF-like cocktail influence on gene expression in dermal fibroblasts and synovial membrane cells. Increased gene expression (+), decreased gene expression (-)

Gene	Dermal fibroblasts		Synovial membranes (by HYC <i>et al.</i> 2016)	
	CIF	CIF-like cocktail	CIF	CIF-like cocktail
HAS1	+	+	+	+
HAS2	+	+	+	not studied
Collagen type I	-	+	+	+
Versican	-	no effect	+	not studied
Aggrecan	-	no effect	+	+
Lubricin	no effect	no effect	+	+
MMP2	-	no effect	+	not studied
MMP3	+	no effect	+	no effect
TIMP1	no effect	no effect	+	+
TIMP2	-	no effect	+	not studied
TIMP3	-	no effect	+	not studied
TGFβ1	+	+	+	+
TNF	+	+	-	no effect
IL1β	-	no effect	-	not studied
IL6	+	no effect	+	no effect

tions (IGF1 2 ng and TGFβ1 0.5 ng/ml) (HYC *et al.* 2016) is unexpected. Fibroblasts, however, secrete a variety of growth factors, cytokines, and chemokines, such as bFGF, HGF, KGF, GM-CSF, VEGFα, PDGF-BB, IL1α, IL6, IL8, TGFβ1, TNF and CC motif chemokine ligands CCL2, CCL5, CCL11 (GRØN *et al.* 2002; HAKENJOS *et al.* 2000; MAAROF *et al.* 2016; MAAS-SZABOWSKI & FUSENIG 1996; SCHWACHULA *et al.* 1994). It is therefore possible that the effects of CIF or CIF-like cocktail are influenced by endogenous cytokines produced in response to exogenous stimulation.

The action of CIF-like cocktail in both cultures was similar to that of CIF only for HAS1 and TGFβ1 (Table 1).

TGFβ1 inhibited MMP3 (stromelysin 1) expression (BARRIENTOS *et al.* 2008) while bFGF stimulated it in chondrocytes (KREJCI *et al.* 2005) and periodontal ligament cells (HAKKI *et al.* 2009; SCHIMAZU & MORISHITA 2003). In our experiments expression of MMP3 was dramatically augmented in CIF treated fibroblasts, but not after CIF-like cocktail stimulation. Since both TGFβ1 and bFGF were present in the same concentrations in CIF and the CIF-like cocktail, some other factors must participate in the induction of the CIF effect. Recently, PRIAM *et al.* (2013) found that the 14-3-3ε protein released from bone cells under influence of mechanical stress strongly raised MMP3 expression; it remains to be seen whether such protein is also produced by chondrocytes.

The differences in action of CIF and CIF-like cocktail probably result from the lack of some factors in the CIF-like cocktail, moreover, native LIF and hematopoietic growth factors are glycosylated (MATHIEU *et al.* 2012; MORSTYN & BURGESS 1988) while recombinant forms lack carbohydrate components and that could influence their activity.

In summary, CIF is a mixture of different agents produced by articular chondrocytes affecting a variety of cells and processes. The composition of CIF was naturally established during evolution and probably could be optimal for the proper function of chondrocytes, synoviocytes and also other connective tissue cells.

As a matter of historical interest, in the pre-cytokine and pre-prion era, pulverized calf cartilage was used as a stimulant for wound healing and treatment of chronic ulcers (PRUDDEN 1964). Glycosaminoglycans or other matrix components were considered to be the active agents, since the existence of growth factors, such as those present in CIF, was at that time unknown. Were they responsible for the therapeutic effects of powdered cartilage?

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