IL-1β Regulates FHL2 and Other Cytoskeleton-Related Genes in Human Chondrocytes

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In osteoarthritis (OA), cartilage destruction is associated not only with an imbalance of anabolic and catabolic processes but also with alterations of the cytoskeletal organization in chondrocytes, although their pathogenetic origin is largely unknown so far. Therefore, we have studied possible effects of the proinflammatory cytokine IL-1β on components of the cytoskeleton in OA chondrocytes on gene expression level. Using a whole genome array, we found that IL-1β is involved in the regulation of many cytoskeleton-related genes. Apart from well-known cytoskeletal components, the expression and regulation of four genes coding for LIM proteins were shown. These four genes were previously undescribed in the chondrocyte context. Quantitative PCR analysis confirmed significant downregulation of *Fhl1*, *Fhl2*, *Lasp1*, and *Pdlim1* as well as *Tubb* and *Vim* by IL-1β. Inhibition of p38 mitogen-activated protein kinase (MAPK) by SB203580 counteracted the influence of IL-1β on *Fhl2* and *Tubb* expression, indicating partial involvement of this signaling pathway. Downregulation of the LIM-only protein FHL2 was confirmed additionally on the protein level. In agreement with these results, IL-1β induced changes in the morphology of chondrocytes, the organization of the cytoskeleton, and the cellular distribution of FHL2. We conclude that L-1β is involved in the regulation of various cytoskeletal components in human chondrocytes including the multifunctional protein FHL2. This might be relevant for the pathogenesis of OA.
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INTRODUCTION

Osteoarthritis (OA) is one of the most common joint diseases, severely restricting the mobility of affected patients. In OA, the balance between degradation and synthesis of the extracellular matrix (ECM) of articular cartilage is disturbed. Mechanical stress or joint inflammation results in enhanced production of degrading enzymes mediated by specific cytokines (1). One relevant cytokine in this context is the pleiotropic factor IL-1β, which is known to induce a set of cartilage-degrading enzymes like matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with thrombospondin motif (ADAMTS) (2,3) and to downregulate the synthesis of important matrix components such as collagen type II and aggrecan (4,5). Therefore, IL-1β stimulation of chondrocytes is a model widely used to investigate molecular events related to the development and progression of OA (6).

Further pathogenetic aspects of OA comprise alterations in the phenotype and cytoskeleton of chondrocytes (7). A disturbed cellular distribution of main cytoskeletal components in OA cartilage has been described (8). However, the underlying mechanisms are largely unknown. Several consequences of cytoskeletal changes in chondrocytes have been described so far. Beside an influence on viscoelastic properties (9), the cellular metabolism is affected (10). In particular, alterations in the cytoskeleton are known to modify nitric oxide- (NO-) induced cell reactions (11) and give rise to lytic enzyme expression (12). IL-1β signaling partially involves focal adhesion complexes (FACs), as IL-1-receptors are enriched at focal adhesion sites and the FAC-associated protein IRAK is required for IL-1-dependent ERK activation (13).

IL-1β controls mRNA-expression via different signaling pathways. A well-known key enzyme in one of these pathways in chondrocytes is the p38 MAP kinase, which has been found to be involved in IL-1β -induced gene regulation of several genes including aggrecan and NOS2A (14,15). These findings make

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p38 MAPK an interesting pharmaceutical target in inflammatory joint diseases.

We have conducted a whole human genome oligo-microarray to investigate the possible influence of IL-1β and p38 MAPK-inhibition on articular human chondrocyte gene expression and have found a high number of affected genes involved in cytoskeletal organization. To verify the microarray results, *Vim*, *Tubb*, and a group of genes encoding LIM proteins (*Fhl1*, *Fhl2*, *Lasp1*, and *Pdlim1*) not described previously in chondrocytes were analyzed by quantitative PCR. LIM proteins contain zinc-binding, cysteine-rich modules known as LIM domains. These conserved motifs mediate interactions with a variety of proteins in different cellular components (16). Several LIM proteins are associated with the actin cytoskeleton (17), as are those studied in this paper. The expression of FHL2 as the best studied member of the four-and-a-half-LIM-only protein family has been investigated further by Western blot. Finally, possible changes in the morphology of chondrocytes, the organization of their actin cytoskeleton, and FHL2 distribution induced by IL-1β have been analyzed.

MATERIALS AND METHODS

Cartilage Samples

Human osteoarthritic cartilage was obtained from donors undergoing total knee replacement due to osteoarthritis with informed consent of the patients according to the terms of the Ethics Committee of the University of Ulm. The age of the donors ranged from 58 to 72 y.

Cell Culture

Well-preserved cartilage from femoral condyles up to a Mankin score (18) of 7 was used for chondrocyte isolation. The cartilage was minced and digested 45 min with 9 U/mL Pronase (Sigma-Aldrich, Munich, Germany) and 14 h with 80 U/mL Collagenase (Sigma) in DMEM. After washing with DMEM and filtering through a 70 μm cell strainer (BD Falcon, Heidelberg, Germany), cells were cultivated in complete medium consisting of 1:1 DMEM/Hams F12 supplemented with 10% fetal bovine serum, 0.5% penicillin/streptomycin, 0.5% L-glutamine and 10 μg/mL 2-phospho-L-ascorbic acid trisodium salt (Sigma-Aldrich, Fluka, Seelze, Germany). After overnight adherence, the chondrocytes were frozen in complete medium containing 5% dimethyl sulfoxide (DMSO) (Roth, Karlsruhe, Germany) and stored in liquid nitrogen. All chemicals were obtained from Biochrom, Berlin, Germany, unless indicated otherwise.

Cell Stimulation and Treatment with p38 MAPK Inhibitor

For microarray and quantitative PCR experiments, thawed cells were cultivated for 1 to 3 days at a density of 5×10^4 cells/cm^2 in complete medium and silenced for 24 h in serum-free medium (DMEM containing 0.5% penicillin/streptomycin, 0.5% L-glutamine, 1% non essential amino acids 100-fold concentrate, 1mM Pyruvat, 0.1% SES1 Solution A, and 0.1% freshly added SES1 Solution B; Biochrom). Cells were stimulated for the indicated time with 10 ng/mL rh IL-1β (tebu-bio, Offenbach, Germany) in serum-free medium. Inhibitor-treated cells were preincubated for 15 min prior to stimulation and coincubated in parallel with 10 μM SB203580 (ratiopharm, Ulm) with a final DMSO-concentration of 0.1 % in the cultivation medium. For comparability, the same amount of DMSO was added to control cells. At the end of the stimulation period, cells were washed 2 times in sterile phosphate buffered saline (PBS) (PAA laboratories, Egelsbach, Germany) and lysed in 600 μl lysis buffer RLT (Qiagen, Hilden, Germany) per 10^6 cells. The overall cultivation time prior to cell lysis ranged from 7 to 9 days.

Microarray Experiment

To obtain enough RNA for the microarray experiment, cells of six different donors were pooled after thawing and split into three groups. One group served as control, one group was stimulated with IL-1β, and one group was incubated with SB203580 and IL-1β as described above. The stimulation time was 24 h. After cell lysis, a whole human genome oligo-microarray (Human Genome Oligo-Set-Version 2.0, Operon, Germany), representing 21,329 genes, was conducted at the Chip facility of Ulm according to Buchholz et al. (19). The experiment was performed three times with six different donors each. By using this experimental design with biological replication, we were able to assess biological variation despite the need to pool different donors (20).

Gene Ontologies

Genes that showed at least a two-fold regulation and a significance level of $P < 0.05$ in the microarray analysis were assigned to cellular components with the fatiGO program (21,22) using the level 5 filtering parameter. The affected cellular components were ranked according to the percentage of genes assigned to the respective component.

mRNA-Isolation and cDNA-Synthesis

For RNA-isolation from cultured cells, the RNeasy mini kit (Qiagen) was used according to the manufacturer's instructions. Briefly, the cell lysate was mixed 1:1 with 70% ethanol, loaded on a mini column, and, after several washing steps and DNase digestion, the RNA was eluted in 30 μl of RNase free water. cDNA was synthesized with Omniscript RT (Qiagen) in accordance with the manufacturer's instructions using 12 μl of RNA-solution.

Polymerase Chain Reaction (PCR)

Semiquantitative reverse transcriptase (RT) PCR was used to detect human *Col2* (collagen type II), *Acan* (aggrecan), *Comp* (cartilage oligomeric matrix protein), and *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase). Primers are given in Table 1. The PCR was performed using HotStarTaq *Plus* polymerase and buffer set (Qiagen) according to the manufacturer's instructions. Quantitative real-time PCR was used to detect human *Fhl1*, *Fhl2*,

Table 1. Primers
Sequences of primers used for quantitative real-time-PCR and semiquantitative RT-PCR.

	GenBank Accession No	Forward 5′-3′	Reverse 5′-3′
Primer for real-time PCR			
FHL1	NM_001449	CTGCGTGGATTGCTACAAGA	GTGCCAGGATTGTCCTTCAT
FHL2	NM_001450	GGTACCCGCAAGATGGAGTA	TTTGGGGATGAAACTCTTGG
LASP1	NM_006148	TGCGCTACAAGGAGGAGTTT	TGATCTGGTCCTGGGTCTTC
PDLIM1	NM_020992	AATGTGGCACTGGGATTGTT	GAAATGGCCCTTCTGTTTCA
TUBB	NM_001069	TGACTTGCAGCTGGAGAGAA	GTCTGAAGATCTGGCCGAAG
VIM	NM_003380	GAGAACTTTGCCGTTGAAGC	CTCAATGTCAAGGGCCATCT
18SrRNA	X03205	CGCAGCTAGGAATAATGGAATAGG	CATGGCCTCAGTTCCGAAA
Primer for RT-PCR			
COLII	NM_03315	CAGGGGTGAACGAGGTTTC	CACGAGCACCAGCACTTCC
ACAN	NM_001135	CACTGTTACCGCCACTTCCC	GACATCGTTCCACTCGCCCT
COMP	NM_000095	AGCAGATGGAGCAAACGTATTG	TGGAAGACTTCACAGCCTTG
GAPDH	NM_002046	GAGTCCACTGGCGTCTTCAC	GGTGCTAAGCAGTTGGTGGT

Lasp1, *Pdlim1*, *Tubb*, and *Vim* in human articular chondrocytes. The primers (Table 1) were designed using Primer 3 software (23). To obtain quantifiable results for all genes, specific standard curves using sequence-specific control probes were performed in parallel to the analyses. Therefore, a gene-specific cDNA fragment was amplified for each gene, using the specified primers (Table 1), and purified by using the NucleoSpin Extract Kit (Macherey-Nagel, Düren, Germany). The sequences were cloned into the pCR 2.1 TOPO vector using the TOPO TA Cloning Kit (Invitrogen, Karlsruhe, Germany) and plasmids were extracted using the Qiafilter Plasmid Midi Kit (Qiagen). The purified plasmids were quantified by measuring the absorbance at 260 nm in a spectrophotometer (GE Healthcare, München, Germany) and the inserts of the plasmids were sequenced for confirmation of correct amplification products of the specific primers. Expression of the genes was detected with the AbiPrism 7000 system (Applera, Darmstadt, Germany). For all analyzed genes, the Invitrogen Platinum SYBR qPCR SuperMix UDG (Invitrogen) was used according to the manufacturer's instructions, except *Vim*, for which Power SYBR Green PCR Master Mix (Applied Biosystems) was utilized. 18SrRNA was used as endogenous control, the applied concentration of all primers was 1 μM. For the standard curve, 120, 1,200, 12,000, 120,000 and 1,200,000 molecules per assay were employed (all in triplicate). For real-time PCR, six different donors were analyzed.

Cell Staining and Phase Contrast Microscopy

For FHL2 and actin filament staining, chondrocytes were seeded onto Permanox slides (Nunc GmbH, KG, Wiesbaden) and cultivated for 2 to 3 days in complete medium. Afterward, cells were stimulated as described above and subsequently fixed in 3.7% formaldehyde (Mallinckrodt Baker, Griesheim, Germany) in PBS for 5 min. Actin-labeling was performed with Phalloidin-FITC (Sigma-Aldrich, Fluka) for 40 min at room temperature protected from light. Bound FITC-conjugated Phalloidin was visualized by fluorescence microscopy (Zeiss Axioskop 2 mot plus, Oberkochen, Germany). For FHL2 staining, cells were incubated with anti-Fhl2 mAB clone F4B2 (dilution 1:2, gift of Victor Wixler, Muenster, Germany) for 1 h after permeabilization with 0.1% Triton X-100 (Merck, Darmstadt, Germany). Bound antibodies were visualized with DakoCytomation LSAB2 System-HRP (Dako, Hamburg, Germany). For morphological studies, cells were seeded in parallel on 12-well plates, treated equally and examined after the indicated time by inverse phase contrast microscopy.

Western Blotting

Cells of four different donors were pooled and stimulated as described, then trypsinized and lysed in SDS-buffer containing 12.5% 0.5 M Tris (pH 6.8), 1% sodiumdodecyl sulfate, 15% glycerine, and 2.3 g/L dithiothreitole. Equal protein amounts of the lysates were resolved by 12.5% SDS-PAGE. After electroblotting onto nitrocellulose membrane and over night blocking at 4°C in 5% nonfat dry milk, membranes were incubated with anti-Fhl2 mAB clone F4B2 (dilution 1:5) for 1 h at room temperature and developed with DakoCytomation LSAB2 System-HRP. Protein concentration of cell lysates was detected using Bradford reagent (Roti-Quant; Roth, Karlsruhe, Germany). Band densities of the immunoblots were quantified with the Geldoc XR System (Bio-Rad Laboratories GmbH, Munich, Germany) and percent intensities were calculated referring to the corresponding control.

STATISTICAL ANALYSIS

For the microarray results, a Print-tip LOESS-normalization according to Buchholz et al. (19) and a moderated *t*-test (24), were performed at the chip facility

of Ulm. For standardization of the gene expression levels determined by Taqman-analysis, mRNA-expression was normalized to 18SrRNA-expression. Differential regulation was determined by calculating the ratios of gene expression in different treatments. A two-tailed paired *t*-test of the calculated ratios was performed to evaluate significant differences from the relative control treatment as identical donors were always compared.

RESULTS

Control of Cell Phenotype and Viability

For the experiments, more than 40 donors provided OA cartilage with a macroscopically smooth surface and no severe osteoarthritic changes. The microarray experiment and real-time PCR experiments were performed with cells from different donors. A continuous check of collagen type II, aggrecan, and COMP (cartilage oligomeric matrix protein) expression during the experimental period confirmed a stable differentiation stage of the chondrocytes (Figure 1). Cell viability was specified by trypan blue staining and showed comparable results in the range of 80% to 90% with and without IL-1β stimulation.

Microarray Experiment

Chondrocyte-treatment with IL-1β induced numerous changes in the gene expression profile. The results of the microarray experiment confirmed many typical effects previously described in the literature. We observed that several ECM genes including *Col2* and *Col11* showed a downregulation by IL-1β, whereas genes related to the inflammatory cascade, for example, *Mmp1*, *Mmp13*, *Adamts10*, *Adamts3*, *Cox2*, *Nos2a*, *IL-1β*, *Il6*, and *Il8* were induced. Coincubation with SB203580 at least partially antagonized the IL-1β effect by downregulation of *Mmp13*, *Cox2*, and *Nos2a* in agreement with previous reports (14,25,26). Examination of the affected genes involved categorization of differentially expressed transcripts to diverse cellular components. Classification according to Gene Ontology annotations showed that the majority of the IL-1β- and SB203580-influenced genes were related to the cell membrane (24% and 22%), the nucleus (19% and 21%), and the plasma membrane (10% both). Though representing a rather small cellular component, the cytoskeleton (6% and 7%) was ranked fourth whereas other cell components were less represented. Table 2 and Table 3 present the list of IL-1β- and SB203580-regulated genes, respectively, involved in cytoskeletal context. We found many structural components of microfilaments, intermediate filaments, and microtubules, but also components of the FACs and integrins as well as factors that are involved in regulation and organization of the cytoskeleton. For further investigations by real-time PCR, a selection of regulated genes was made. The complete array data are deposited on ArrayExpress with the accession number E-MEXP-1434

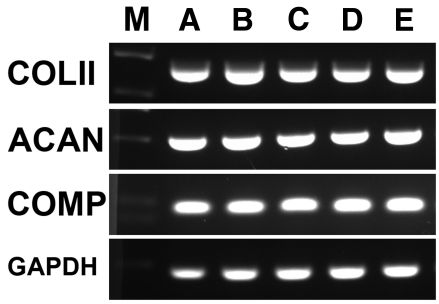

Figure 1. Preservation of the chondrogenic phenotype during the experiment. RT-PCR analysis for Collagen type II (COLII), Aggrecan (ACAN), and COMP is shown at different time points with GAPDH as housekeeping gene. M: Marker; A: directly after enzymatic preparation, passage 0 (P0); B: 1 day of monolayer culture (P0) prior to cryoconservation; C: 3 days of cultivation after cryoconservation (P1); D: 5 days of cultivation after cryoconservation (P1); E: 6 days of cultivation after cryoconservation (P1). The experiment was performed with three different donors and a representative example is shown.

Quantitative Gene Expression Analysis of Cytoskeletal Components

The cytoskeleton-related genes *Fhl1* (four and a half LIM domains 1, GenBank:NM_001449), *Fhl2* (four and a half LIM domains 2, GenBank:NM_001450), *Lasp1* (LIM and SH3 protein 1, GenBank:NM_006148), *Pdlim1* (PDZ and LIM domain 1 [elfin], GenBank:NM_020992), *Vim* (vimentin, GenBank:NM_003380), and *Tubb* (tubulin, β polypeptide, GenBank:NM_001069) were quantified by real-time PCR and normalized to 18SrRNA-expression. Gene expression in IL-1β-stimulated cells was compared with untreated cells and the effect of coincubation with SB203580 was investigated by comparison with IL-1β-stimulated cultures. In comparison to *Fhl2* and *Pdlim1*, the expression level of *Tubb* was about 10-fold and of *Vim*, *Fhl1*, and *Lasp1* about 100-fold higher.

Influence of IL-1β on Gene Expression Level

Figure 2A shows the effects of IL-1β on gene expression after 4 h and 24 h stimulation. All four genes containing LIM domains were downregulated by IL-1β stimulation. *Fhl1* showed a 2.5-fold after 4 h ($P = 0.013$) and a 44-fold downregulation ($P < 0.0001$) after 24 h of stimulation. For *Fhl2*, we found a highly significant downregulation after 4 h (6.5-fold, $P < 0.0001$) and 24 h (3.5-fold, $P < 0.0001$). *Lasp1* was 2.3-fold downregulated after 4 h ($P < 0.001$) and 2.5-fold after 24 h ($P < 0.001$) whereas *Pdlim1* showed significant downregulation after 24 h (2.4-fold, $P < 0.01$) but not after 4 h. *Tubb* and *Vim* were downregulated after 24 h (3-fold and 5-fold, respectively, $P < 0.0001$). In all cases, the direction and the magnitude of the gene expression changes of the 24 h data were consistent with the microarray data, though, in the case of *Vim*, the results of the microarray failed the significance level of $P < 0.05$ (array data: fold change = 0.34, $P = 0.077$).

Influence of p38 MAPK Inhibition by SB203580 on Gene Expression Level

The effect of SB203580 on gene expression in IL-1β-stimulated cells is il-

Table 2. IL-1β-regulated, cytoskeleton-related genes found in microarray experiment
The fold-change gives the ratio of expression levels comparing 24 h IL-1β-stimulated cells to control cells. The most strongly regulated genes showing *P*-values < 0.05 are listed. Also all genes further analyzed by quantitative real-time PCR are listed and highlighted. Three independent experiments using each a pool of six donors were conducted.

Gene Symbol	Gene Description	GenBank Accession No	Influence of IL-1β	
			Fold Change	*P*-Value
Downregulation				
FHL1	Four and a half LIM domains 1	NM_001449	0.04	0.001
ITGA11	Integrin, α 11	AF109681	0.09	0.000
SGCB	Sarcoglycan, β	NM_000232	0.09	0.002
EMAPL	Echinoderm microtubule-assoc. protein-like	NM_004434	0.10	0.035
SPTA1	Spectrin, alpha, erythrocytic 1	NM_003126	0.10	0.000
TUBB	Tubulin, β polypeptide	NM_001069	0.19	0.000
SNTG1	Syntrophin, γ 1	NM_018967	0.20	0.010
ARPC1B	Actin related protein 2/3 complex	NM_005720	0.26	0.004
FHL2	Four and a half LIM domains 2	NM_001450	0.28	0.001
CALD1	Caldesmon 1	NM_033138	0.29	0.041
MYO6	Myosin VI	NM_004999	0.30	0.036
CDC42BPB	CDC42 binding protein kinase β	NM_006035	0.31	0.014
MYO1D	Myosin ID	AK026920	0.31	0.002
VIM	Vimentin	NM_003380	0.34	0.077
PDLIM1	PDZ and LIM domain 1 (elfin)	NM_020992	0.35	0.025
ACTG1	Actin, γ 1	NM_001614	0.39	0.017
ITGB5	Integrin, β 5	NM_002213	0.41	0.004
LASP1	LIM and SH3 protein 1	NM_006148	0.41	0.001
CORO1C	Coronin, actin binding protein, 1C	AL162070	0.41	0.004
KRT1	Keratin 1	NM_006121	0.42	0.036
LMNA	Lamin A/C	NM_005572	0.43	0.003
PLS3	Plastin 3 (T isoform)	NM_005032	0.44	0.005
CORO1A	Coronin, actin binding protein, 1A	NM_007074	0.45	0.003
PFN2	Profilin 2	NM_053024	0.45	0.003
KRT9	Keratin 9	NM_000226	0.45	0.026
Upregulation				
SLC9A3R1	Solute carrier fam. 9, isoform 3 regul. factor 1	NM_004252	16.83	0.004
KRTHA7	Keratin, hair, acidic, 7	NM_003770	16.81	0.004
SNTG2	Syntrophin, γ 2	NM_018968	8.30	0.010
ITGB3BP	Integrin β 3 binding protein	NM_014288	6.14	0.037
ITGA2B	Integrin, α 2b (platelet glycoprotein IIb)	NM_000419	4.03	0.001
SPTAN1	Spectrin, alpha, non-erythrocytic 1	NM_003127	3.79	0.006
TEKT3	Tektin 3	NM_031898	3.22	0.006
VIL2	Villin 2 (ezrin)	NM_003379	3.14	0.001
MYO1A	Myosin IA	NM_005379	2.83	0.001
KIF4A	Kinesin family member 4A	NM_012310	2.74	0.006
MYO10	Myosin X	NM_012334	2.68	0.003
KIF3A	Kinesin family member 3A	NM_007054	2.51	0.041
VIL1	Villin 1	NM_007127	2.31	0.007
ITGB3	Integrin, β 3 (platelet glycoprotein IIIa)	NM_000212	2.27	0.031

lustrated in Figure 2B. *Fhl2* was 2.6-fold upregulated after 4 h ($P < 0.05$), a tendency to upregulation also could be seen after 24 h (1.7-fold, $P = 0.07$) and *Tubb* showed significant upregulation after 24 h (2.2-fold, $P < 0.05$). A trend of *Lasp1* upregulation was observed after 24 h (1.5-fold, $P = 0.05$). These changes were opposite to the effect of IL-1β stimulation without SB203580-treatment. The expression of the other IL-1β-regulated genes (*Fhl1*, *Pdlim1*, and *Vim*) was not modulated by SB203580. Comparison with the microarray data showed accordance of the gene expression of *Fhl1*, *Tubb*, and, in tendency, of *Lasp1*. In contrast, the significant upregulation of *Pdlim1* and *Vim*, as assessed in the mi-

Table 3. SB203580-influenced, cytoskeleton-related genes found in microarray experiment
The fold-change gives the ratio of expression levels comparing 24 h SB203580-treated and IL-1β-stimulated cells to IL-1β-stimulated cells without inhibition. The most strongly regulated genes showing *P*-values < 0.05 are listed. Also, all genes further analyzed by quantitative real-time PCR are listed and highlighted. Three independent experiments using each a pool of six donors were conducted.

Gene Symbol	Gene Description	GenBank Accession No	Influence of SB203580	
			Fold Change	*P*-Value
Downregulation				
MAPRE2	Microtubule-associated protein, RP/EB family	NM_014268	0.12	0.005
bf SLC9A3R1	Solute carrier fam. 9, isoform 3 regul. factor 1	NM_004252	0.13	0.013
MAP1LC3A	Microtubule-associated protein 1 light chain 3a	NM_032514	0.17	0.010
KRTHA7	Keratin, hair, acidic, 7	NM_003770	0.19	0.015
MAP7	Microtubule-associated protein 7	NM_003980	0.21	0.006
IQGAP2	IQ motif containing GTPase activating prot. 2	NM_006633	0.26	0.043
ITGA2B	Integrin, α 2b (platelet glycoprotein IIb)	NM_000419	0.32	0.027
KRTHB6	Keratin, hair, basic, 6 (monilethrix)	NM_002284	0.41	0.007
DOCK1	Dedicator of cyto-kinesis 1	NM_001380	0.42	0.010
EPB41	Erythrocyte membrane protein band 4.1	NM_004437	0.42	0.044
CYLC2	Cylicin 2	NM_001340	0.43	0.020
KRT7	Keratin 7	NM_005556	0.45	0.010
FN1	Fibronectin 1	NM_002026	0.45	0.03
MLF1	Myeloid leukemia factor 1	NM_022443	0.48	0.027
FHL1	Four and a half LIM domains 1	NM_001449	0.84	0.756
Upregulation				
HARP11	Hypothalamus protein HARP11	NM_018477	12.76	0.021
EMAPL	Echinoderm microtubule-assoc. protein-like	NM_004434	10.52	0.020
SGCB	Sarcoglycan, β	NM_000232	6.32	0.009
CDC42BPB	CDC42 binding protein kinase β	NM_006035	6.25	0.005
SGCG	Sarcoglycan, γ	NM_000231	6.22	0.011
NF2	Neurofibromin 2 (bilateral acoustic neuroma)	AF369658	5.19	0.007
HINT	Histidine triad nucleotide binding protein	AK054976	4.24	0.034
VIM	Vimentin	NM_003380	3.77	0.001
PKP3	Plakophilin 3	NM_007183	3.59	0.010
KNSL5	Kinesin-like 5 (mitotic kinesin-like protein 1)	NM_004856	3.56	0.015
TUBB	Tubulin, β polypeptide	NM_001069	3.21	0.014
ACTL7A	Actin-like 7A	NM_006687	2.82	0.030
RNF28	Ring finger protein 28	AF361946	2.55	0.007
LASP1	LIM and SH3 protein 1	NM_006148	2.33	0.018
MARKL1	MAP/microtubule affinity-regul. kinase like 1	AB058763	2.32	0.022
ITGB3BP	Integrin β 3 binding protein	NM_014288	2.26	0.020
PDLIM1	PDZ and LIM domain 1 (elfin)	NM_020992	2.23	0.028
FHL2	Four and a half LIM domains 2	NM_001450	1.23	0.773

croarray, could not be confirmed in real-time PCR.

Changes in Morphology and Cytoskeletal Organization of Chondrocytes by IL-1β

Figure 3 visualizes the effects of IL-1β on the cell morphology of chondrocytes. Control cells showed a flattened shape (A). Phalloidin staining indicated good adherence on the surface by focal adhesion sites and a meshwork of actin filaments with stress fibers (C). Compared with control cells, IL-1β-stimulated chondrocytes presented a completely different appearance. After 24 h, many cells had begun to shrink (B,D) and showed a spherical shape. No cytoskeletal structure could be observed in the spherical cells by phalloidin staining. Pretreatment with SB203580 could not prevent the morphological changes seen in IL-1β-stimulated cells (data not shown).

Regulation of FHL2 Protein Expression and Distribution

According to gene expression data, Western blot analysis (Figure 4A) confirmed downregulation of FHL2 by IL-1β on protein level. The rating of band intensity revealed a significant influence after 48 h of stimulation. Preincubation with SB203580 could not diminish this effect markedly. In addition, specific immunoreactivity could be observed for a 23 kDa Peptide, probably a proteolytic

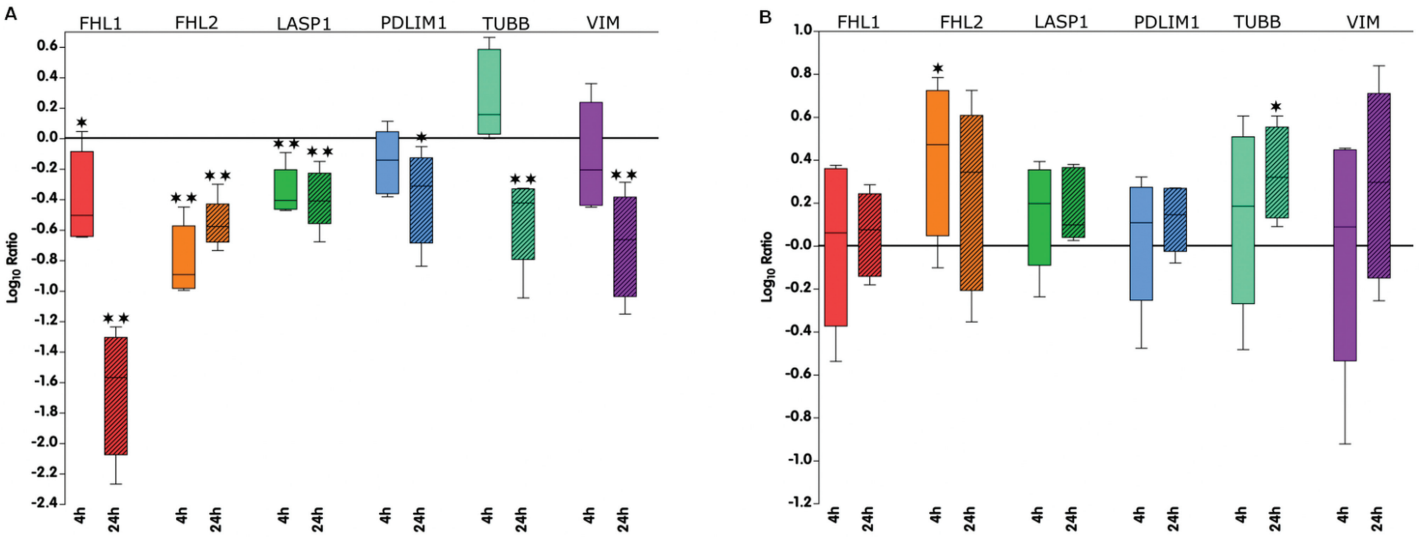

Figure 2. Influence of cell treatment on gene expression. Quantitative real-time PCR analysis for levels of mRNA expression of cytoskeleton-related genes in IL-1β-stimulated chondrocytes. Values are the $\log_{10}$-ratios of A: IL-1β stimulated to control gene expression levels and B: p38 MAPK-inhibited and IL-1β stimulated to IL-1β stimulated gene expression levels without inhibition. Six donors were analyzed. The stimulation times of 4 h and 24 h, respectively, are indicated. Significant changes are marked (*: $P < 0.05$, **: $P < 0.001$).

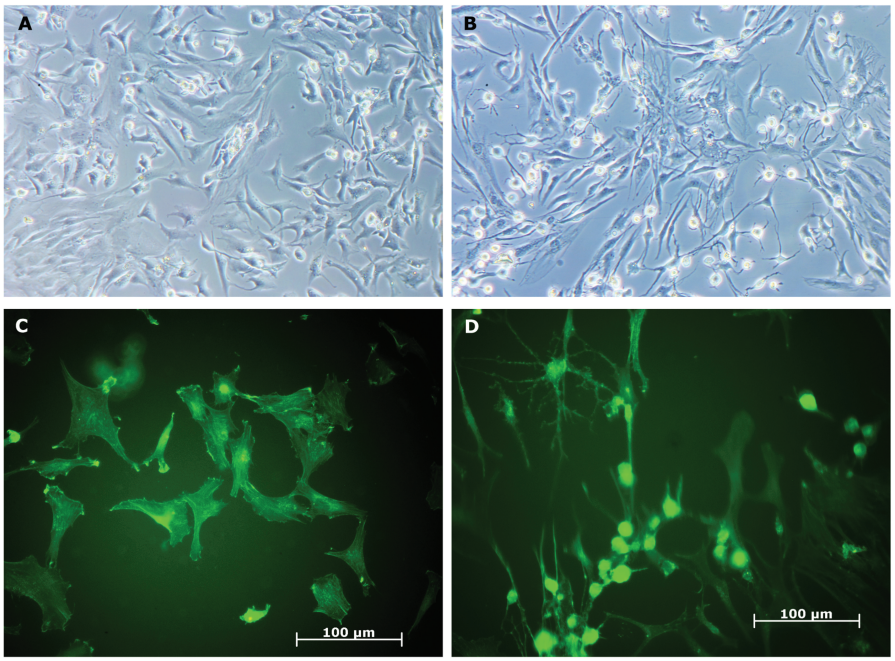

Figure 3. Influence of IL-1β on cell morphology. Chondrocytes with (B, D) and without (A, C) 24 h IL-1β stimulation (10 ng/mL). A + B: Phase contrast microscopy of chondrocytes grown on cell culture dishes (magnification 200x). C + D: Fluorescence microscopy of chondrocytes grown on Permanox slides and F-actin-stained with FITC-conjugated phalloidin.

fragment (27), that was upregulated by stimulation with IL-1β (data not shown). FHL2 immunostaining of untreated cells (Figure 4B) showed a typical localization of the protein at sites of adhesion, but also in and round the nucleus. IL-1β treatment mostly disturbed the flattened morphology and the described FHL2 distribution. Stimulated chondrocytes revealed a uniformly distributed strong staining in rounded cells, but diminished localization in focal adhesion sites in cells that still showed a flattened shape (Figure 4C).

DISCUSSION

By using a microarray approach in primary human osteoarthritic chondrocytes, we were able to identify a large group of cytoskeleton-related genes that showed changes in gene expression in response to IL-1β treatment. In real-time PCR analysis, we confirmed significant downregulation of four genes containing LIM domains and of *Tubb* and *Vim* gene expression by IL-1β treatment in good

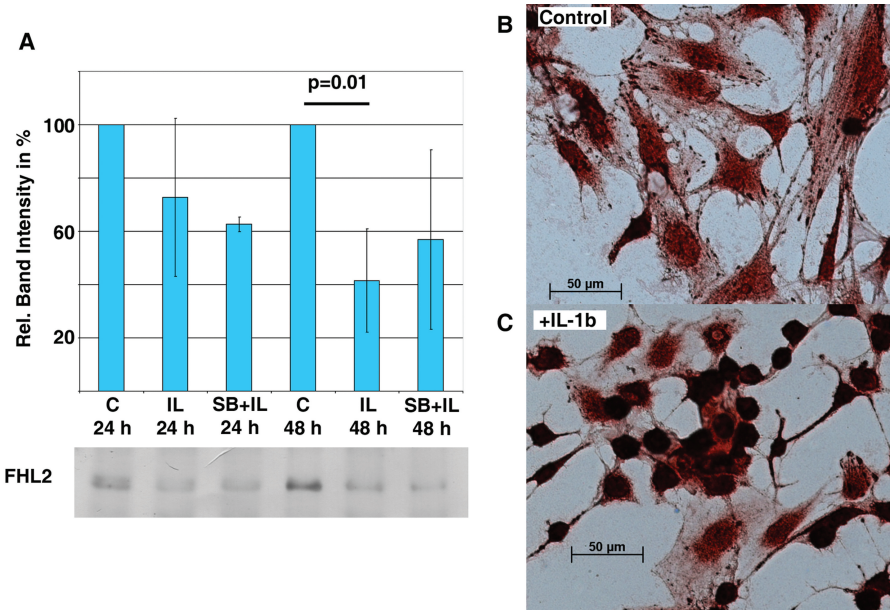

Figure 4. Influence of IL-1β on FHL2 protein expression and distribution. A: Western blot analysis from treated and untreated chondrocytes and corresponding analysis of band intensity of four (IL-1β treatment) and two (SB203580 and IL-1β treatment) independent experiments, respectively. B + C: Chondrocytes with (C) and without (B) IL-1β stimulation stained with αFHL2 antibody.

accordance to the microarray results. Both expression and regulation of the LIM protein related genes as well as regulation of *Tubb* and *Vim* by IL-1β are new aspects in cartilage research. The inhibition of p38 MAPK with SB203580 could significantly counteract the influence of IL-1β on *Fhl2* and *Tubb* expression, indicating an involvement of the p38 MAPK pathway in these cases. Corresponding to these results in gene expression analyses, we have demonstrated IL-1β regulation of FHL2 protein expression and effects on cell morphology and the distribution of FHL2 and the actin cytoskeleton of cultured chondrocytes. Therefore, we suggest an influence of IL-1β on the cytoskeletal organization of chondrocytes. A closer look at the biological functions of the proteins associated with the analyzed genes supports this notion.

FHL2 is a member of the four-and-a-half LIM family, that consists of five different proteins (17). It can interact with more than 50 different proteins including transcription factors, integrins, or structural cytoskeletal proteins such as actin. FHL2 lacks an obvious enzymatic activity, but a function as a scaffolding protein providing docking sites for other proteins was suggested (27). Its localization at adhesion complexes and in the nucleus observed in this work was also described for mouse fibroblasts (28). These findings support a role for this LIM protein as a signal transducer (27). It was shown that FHL2 acts as a molecular transmitter of the Rho signaling pathway, which is involved in the organization of the actin cytoskeleton (28). Moreover, FHL2 interacts with RUNX2, a transcriptional regulator not only involved in osteogenesis, but also in chondrogenesis. RUNX2 contributes to MMP13 expression (29) and is induced in early osteoarthritis (30). This may support the notion that FHL2 is involved not only in cytoskeletal changes but also in OA-associated transcriptional regulation in IL-1β-stimulated chondrocytes. The regulation of the *Fhl2* expression is understood only partially. Transcription of *Fhl2* is induced by serum (31), which can be seen as a model for anabolic processes. By contrast, IL-1β stimulation as a model for catabolic processes in chondrocytes (6) downregulated FHL2 gene and protein expression, as shown in this paper. Our finding that inhibition of the p38 MAPK diminishes the effect of IL-1β on *Fhl2* gene expression suggests at least partial regulation of *Fhl2* transcription via the p38 MAPK pathway. Indeed, there is a binding site for the p38 MAPK-regulated transcription factor MEF-2 in the *Fhl2* promoter (27,32). Because this effect could not be confirmed significantly on the protein level, further regulatory mechanisms on posttranscriptional level might be involved. Another reason could be the semiquantitative method of Western blot analysis that might not be sensitive enough to detect small changes on the protein level.

The other cytoskeleton associated LIM proteins FHL1, LASP1 and PDLIM1 also are associated with the actin cytoskeleton (33). Their specific roles are poorly understood, but they are suggested to fulfill functions in signal transduction. This is the first description of their expression and regulation in chondrocytes. FHL1, another member of the four-and-a-half LIM family, shows an integrin-dependent localization at focal adhesions, stress fibers, and the nucleus in myoblasts. It regulates integrin-mediated cellular functions such as cytoskeletal rearrangement, probably by regulating transcription factors (34). The LASP1 protein includes two actin binding sites and an SH_3 domain which is involved in binding to special focal adhesion proteins like zyxin and VASP. These binding properties make LASP1 a candidate for a scaffold protein that mediates formation of protein complexes. Stimulation with growth factors or ECM proteins facilitates its localization to focal complexes and actin polymerization sites, whereas the loss of LASP1 localization to FACs is associated with the induction of cell death (35). PDLIM1 belongs to the Enigma family and contains 1 PDZ and 1

LIM domain. It colocalizes with α-actinin and actin stress fibers in different cell types and may modulate the function of α-actinin 1 (36). Presuming comparable functions in chondrocytes, the downregulation of these LIM protein related genes may interfere with cytoskeletal arrangements and affect associated signaling pathways. The resulting total effect of IL-1β on actin polymerization and focal complex formation also may influence cell motility. There are some reports indicating the capacity of chondrocytes to migrate in vitro (37). This feature of chondrocytes also was addressed by new approaches for cartilage repair in vivo (38). An affection of cell migration by NO via the disruption of ECM adherence and actin assembly has been described for chondrocytes and an impairment of cartilage repair was suspected as a consequence (39).

Tubulin and Vimentin, components of microtubules and intermediate filaments, respectively, play major roles in intracytoplasmic transport and cell stability. The disruption of these structures in chondrocytes diminishes collagen type II-synthesis and, in case of tubulin, the synthesis of proteoglycans. It could promote an imbalance in cartilage homeostasis due to reduced anabolism (10). Disrupted intermediate filaments alter the viscoelastic properties of chondrocytes which might be associated to the increased cellular stiffness observed in OA cartilage (9). It has been shown that the number of tubulin- and vimentin-positive chondrocytes is significantly reduced in rat OA cartilage in comparison to normal cartilage (40). Accordingly, changes in cell morphology have been described in human OA chondrocytes in vivo, including disruption of the cytoskeleton. Especially the clustered cells from the upper zone cartilage displayed a disrupted distribution of actin, vimentin, and tubulin (7). The cells in the upper zone of articular cartilage are exposed mainly to inflammatory cytokines like IL-1β. These results agree with our new finding, that IL-1β significantly downregulates *Tubb* and *Vim* gene expression in chondrocytes and affects chondrocyte morphology in vitro.

The effects of IL-1β on various cytoskeletal components may be involved directly in OA pathogenesis as they could have complex effects on signal transduction to the nucleus, on anabolic and catabolic pathways, on viscoelastic properties, on cell motility, and on the responsiveness to extracellular stimuli. A deeper knowledge of the involved molecular processes also may help to set different pathogenetic mechanisms of degenerative joint diseases in a novel context.

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