Expression of pro-inflammatory markers by human dermal fibroblasts in a three-dimensional culture model is mediated by an autocrine interleukin-1 loop

Daniela KESSLER-BECKER, Thomas KRIEG and Beate ECKES¹

Department of Dermatology, University of Cologne, Joseph-Stelzmann-Strasse 9, D-50931 Cologne, Germany

In vivo, fibroblasts reside in connective tissues, with which they communicate in a reciprocal way. Such cell–extracellular matrix interactions can be studied *in vitro* by seeding fibroblasts in collagen lattices. Depending upon the mechanical properties of the system, fibroblasts are activated to assume defined phenotypes. In the present study, we examined a transcriptional profile of primary human dermal fibroblasts cultured in a relaxed collagen environment and found relative induction (> 2-fold) of 393 out of approx. 7100 transcripts when compared with the same system under mechanical tension. Despite down-regulated proliferation and matrix synthesis, cells did not become generally quiescent, since they induced transcription of numerous other genes including matrix metalloproteinases (MMPs) and growth factors/cytokines. Of particular interest was the induction of gene transcripts encoding pro-inflammatory mediators, e.g. cyclo-oxygenase-2

(COX-2), and interleukins (ILs)-1 and -6. These are apparently regulated in a hierarchical fashion, since the addition of IL-1 receptor antagonist prevented induction of COX-2, IL-1 and IL-6, but not that of MMP-1 or keratinocyte growth factor (KGF). Our results suggest strongly that skin fibroblasts are versatile cells, which adapt to their extracellular environment by displaying specific phenotypes. One such phenotype, induced by a mechanically relaxed collagen environment, is the ‘pro-inflammatory’ fibroblast. We propose that fibroblasts that are embedded in a matrix environment can actively participate in the regulation of inflammatory processes.

Key words: cell–matrix interaction, collagen lattice, differential gene expression, fibroblast, inflammation, transcriptional profiling.

INTRODUCTION

The interaction of cells with the surrounding extracellular matrix (ECM) is essential in many physiological and pathological processes, such as embryonic development, maintenance of tissue homeostasis, repair, fibrosis, tumour invasion and metastasis [1–3]. Besides providing tissues with structural integrity, ECM proteins form the three-dimensional scaffold needed for cell adhesion and migration.

The interaction of a three-dimensional scaffold with specific receptors at the cell surface, the correct composition and pliability of the ECM are prerequisites for the proper molecular structure and functioning of connective tissues. This also requires effective bi-directional signal transmission between ECM molecules, cell-surface receptors, e.g. integrins, and the cytoplasm [4,5]. Presence or absence of physical stimuli have been shown to trigger adaptive responses *in vitro* as well as *in vivo* (reviewed in [6]). Integration of these complex signalling networks leads to tight regulation of the cellular metabolism and controls a number of functional properties of various cell types.

Fibroblasts represent a not-very-well-characterized cell type present in most connective tissues. For a long time, this cell type was regarded mainly as a producer of ‘insoluble’ matrix proteins, but then it became apparent that fibroblasts are also capable of synthesizing many soluble signals, such as growth factors, cytokines and lipid mediators that influence cell–matrix and cell–cell interactions, as well as responding to many soluble signals via autocrine or paracrine regulatory mechanisms (reviewed in [7]). Thus fibroblasts not only orchestrate matrix deposition

and degradation, but are also involved in many biological and pathological processes. The functional variety of this cell type that is present nearly ubiquitously in connective tissues is also mirrored in fibroblast heterogeneity and different cell ‘phenotypes’ that can be induced in response to alterations of the extracellular environment, and/or growth factor and cytokine action. For example, upon mechanical stimulation due to physical resistance of the ECM, a significant percentage of the connective-tissue fibroblast population differentiates into contractile myofibroblasts, characterized by formation of stress fibres, fibronexus junctions and expression of ‘ α -smooth muscle actin’, an actin isoform that is naturally expressed by smooth muscle cells [8–10]. This myofibroblast conversion is accompanied by fundamental reprogramming of fibroblast gene expression towards enhanced ECM production, proliferation and inhibition of matrix degradation [11].

Collagen lattices are simple, but well-established, connective-tissue models that mimic three-dimensional properties of the ECM and allow the investigation of transcriptional changes of dermal fibroblasts upon tensile stimulation [12,13]. Brought into contact with a collagen type I matrix *in vitro*, fibroblasts adhere to the collagen fibres and contract the initially loose network to a dense tissue-like structure within 20 h. If contraction is prevented by fixing the matrix to a rigid support (e.g. a nylon or steel ring), fibroblasts develop self-generated tension [13]. Continuous mechanical stimulation results in phenotypic conversion into a matrix-producing fibroblast [11,13]. Regarding cell morphology, ‘stressed’ fibroblasts differ extensively from monolayer fibroblasts or cells grown in relaxed collagen lattices. In the latter, fibroblasts lack self-generated tensional forces and actin stress

Abbreviations used: Cdc, cell-division cycle; COX-2, cyclo-oxygenase-2; DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; FAP- α , fibroblast-activation protein- α ; FCS, foetal calf serum; ICAM-1, intercellular adhesion molecule-1; IGF, insulin-like growth factor; IGFBP, IGF-binding protein; IL, interleukin; IL-1ra, IL-1 receptor antagonist; KGF, keratinocyte growth factor; MMP, matrix metalloproteinase; MT1, membrane type 1; ROR α 1, RAR (retinoic acid receptor)-related orphan receptor α 1; TNF, tumour necrosis factor.

¹ To whom correspondence should be addressed (e-mail beate.eckes@uni-koeln.de).

fibres, and exhibit a reorganized actin cytoskeleton and a stellate morphology [11,14]. Single aspects of the reprogrammed gene expression pattern in such relaxed lattices have been described earlier, including arrest of cell division, reduced production of collagen and other extracellular macromolecules coincident with up-regulation of MMP (matrix metalloproteinase)-1 (collagenase) [15,16]. Experiments involving blocking of the collagen-binding integrin receptors $\alpha 1\beta 1$ and $\alpha 2\beta 1$ demonstrated that integrin $\alpha 2\beta 1$ is the major determinant in the contraction of collagen lattices [17,18] and up-regulation of MMP-1 [18,19] and MT1 (membrane type 1)-MMP [20], whereas integrin $\alpha 1\beta 1$ mediates down-regulation of collagen type I ($\alpha 1$) chains [18]. Decreased mechanical tension is also regarded as a signal that triggers programmed cell death in fibroblasts and other cell types [21–25]. Interestingly, an autocrine loop of IL (interleukin)-1 α and IL-1 β produced by relaxed dermal fibroblasts has been described previously [26], but its function remains unclear because IL-1 is not involved in the up-regulation of matrix-degrading MMP-1 in retracted collagen lattices.

In the present study, we examined a detailed transcriptional profile of fibroblasts grown in relaxed collagen lattices to broaden our understanding of matrix-induced changes in gene expression. Interestingly, upon stress relaxation, fibroblasts express a multitude of transcripts that are known as essential mediators in inflammatory tissue reactions, where they are produced by cell types such as monocytes, macrophages and lymphocytes. In relaxed fibroblasts, some of these mediators, such as COX-2 (cyclooxygenase-2) and IL-6 are directly regulated by an autocrine IL-1 loop. These findings demonstrate that fibroblasts in a mechanically relaxed collagenous environment are able to acquire a specialized cell phenotype: the inflammatory-activated fibroblast. Although these results have been generated in an *in vitro* system, they clearly document the striking versatility of fibroblasts that enables them to control a number of physiological and pathological processes *in vivo*.

EXPERIMENTAL

Cell culture

Cultures of human skin fibroblasts were established by outgrowth from skin biopsies of healthy donors in DMEM (Dulbecco's modified Eagle's medium; Life Technologies, Eggenstein, Germany) supplemented with 10 % (v/v) FCS (foetal calf serum; PAA Laboratories, Linz, Austria), 2 mmol/l glutamine (Biochrom, Berlin, Germany), 100 units/ml penicillin (Biochrom), 100 μ g/ml streptomycin (Biochrom) and 50 μ g/ml sodium ascorbate (Sigma-Aldrich, Deisenhofen, Germany), and were grown in the moist atmosphere of a CO₂ incubator (5 % CO₂) at 37 °C [11]. Cells were subcultured by trypsinization (0.1 % trypsin and 0.02 % EDTA; Biochrom) in PBS and reseeded at a ratio of 1:2. Absence of mycoplasma was checked routinely by bisbenzimidazole fluorochrome (Hoechst 33258) staining.

Preparation of collagen lattices

Acid-extracted, but not pepsinized, collagen I from newborn bovine skin was purchased from the Institut für Biomedizinische Forschung (IBFB, Leipzig, Germany). Freeze-dried collagen was redissolved at 3 mg/ml in sterile 17 mmol/l acetic acid. Mechanically stressed and relaxed three-dimensional collagen lattices were prepared as described previously [11]. Briefly, fibroblasts were seeded at 2×10^5 cells/ml into a solution containing 0.3 mg/ml collagen type I and grown at 37 °C in uncoated bacterial dishes of 100 mm or 60 mm diameter. Cells in

mechanically relaxed lattices were allowed to contract the gel matrix, whereas in stressed lattices, contraction was prohibited by a braided nylon thread (0.5 mm diameter) placed at the inner perimeter of the dish. In parallel, 3×10^6 fibroblasts were cultured as monolayers in 145-mm-diameter tissue-culture dishes under identical conditions.

Isolation of RNA and Northern blot analysis

Collagen lattices and cells from monolayer cultures were lysed and homogenized in TriFast reagent (Peglab, Erlangen, Germany). Total RNA was prepared according to the manufacturer's instructions, followed by a clean-up procedure employing RNeasy columns (Qiagen, Hilden, Germany). To isolate total RNA from monolayer cultures, RNeasy spin columns were used following the manufacturer's protocol. mRNA was prepared from total RNA and was twice purified employing an Oligotex mRNA kit (Qiagen).

For Northern blot analysis, 5 μ g of total RNA was separated by electrophoresis on 1 % agarose/0.66 M formaldehyde gels and transferred on to hybridization membranes (NEN Life Science Products, Boston, MA, U.S.A.). After UV-cross-linking (Stratalinker, Stratagene, La Jolla, CA, U.S.A.), membranes were stained with 0.04 % (w/v) Methylene Blue. The cDNA probes used were human KGF (keratinocyte growth factor; 0.75 kb *NorI* fragment [27]) and IL-6 (0.56 kb *EcoRI/PstI* fragment [28]). Additional probes for Northern blot analysis were generated by RT (reverse transcriptase)-PCR. The selection of gene-specific primers was based on published sequence information. COX-2 [29]: sense, 5'-TCATTCACCAGGCAAATTGCTGGCAGG-3' (bp 1328–1354); antisense, 5'-ACAGTTCAGTCGAACGTTCTTTAGTAGTA-3' (bp 1881–1910). Stanniocalcin [30]: sense, 5'-CACACAGCCTCTAAGCGTGC-3' (bp 2961–2980); antisense, 5'-AGACGAAGCTTTGGAAGTTTAAGG-3' (bp 3478–3501). FAP (fibroblast-activation protein) [31]: sense, 5'-GCTAACTTTCAAAAACATCTGGAAAAATG-3' (bp 183–211); antisense, 5'-GTAATATGTTGCTGTGTGAAGAGTATCTCC-3' (bp 573–601). IL-1 β [32]: sense, 5'-TGCTCTGGGATCTCTTCAGCC-3' (bp 44–65); antisense, 5'-AGCGGTTGCTCATCA-GAATGTGG-3' (bp 1216–1238). Lysyl hydroxylase 2 [33]: sense, 5'-CTAAAGTTTACATTGATCCACT-3' (bp 587–608); antisense, 5'-GGCTTCCGTTGACTTAGA-3' (bp 1095–1113). PCR fragments were subcloned using the TOPO-T/A-cloning kit (Invitrogen, Groningen, The Netherlands). Hybridization probes were radiolabelled by random priming (Amersham Biosciences, Freiburg, Germany) with [α -³²P]dCTP and hybridized overnight at 42 °C in ULTRAhyb hybridization solution (Ambion, Austin, TX, U.S.A.).

Filters were washed for 15 min in 2 $\times$ SSC (where 1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate), 0.1 % (w/v) SDS at room temperature (22 °C), followed by one wash at 42 °C in the same solution, thereafter at elevated stringency, and exposed to X-ray films for autoradiography. Equal loading of lanes was confirmed either by Methylene Blue staining of the nylon membranes or by hybridization of oligodeoxynucleotides complementary to 18 S rRNA (24 bases) labelled with ³²P [34]. Signal intensities were quantified densitometrically (Bio-Rad, Heidelberg, Germany).

Gene expression profiling by cDNA microchip array analysis

The cDNA microchip array experiment was performed using the public domain UniGem V 1.0 array (Incyte Genomics, CA, U.S.A.). This array contained cDNA fragments of approx. 7100 genes with known, as well as unknown, function. Data were analysed with GemTools software. According to the supplier's

information, the sensitivity of the assay was the detection of one within 100 000 transcripts.

Fibroblasts were cultured from the abdominal skin of an approx. 50-year-old Caucasian female and grown for 20 h under stressed and relaxed conditions in collagen lattices. mRNA was prepared as described above and was transcribed into cDNA following the chip manufacturer's instructions using fluorescent dyes to label cDNAs from different cell populations (Cy3, stressed lattice; Cy5, relaxed lattice). cDNA from both conditions was hybridized simultaneously to the identical array. Fluorescent ratios were calculated for all elements, including controls, for sensitivity, differential expression and reverse-transcription quality. To minimize false-positive elements, a threshold value was set at 2.0-fold balanced differential expression. In Table 1, differential expression coefficients are listed as *x*-fold induction in relaxed compared with mechanically stressed fibroblasts. Selected positive elements were confirmed by subsequent Northern blot analysis in independent experiments.

Functional blocking of the IL-1 receptor

To block IL-1 receptor function, dermal fibroblasts were detached from confluent monolayer cultures by trypsinization, resuspended in DMEM/10% (v/v) FCS at 0.55×10^6 cells/ml and IL-1 receptor antagonist (IL-1ra; R&D Systems, Wiesbaden, Germany) was added at 0.01–1 µg/ml. Cells were pre-incubated in this suspension for 1 h at 37 °C in a CO₂ incubator and then used for the preparation of collagen gels as described above.

RESULTS

Genes induced in relaxed fibroblasts

For a comprehensive description of transcriptional changes of human dermal fibroblasts in a three-dimensional environment without mechanical load, collagen lattices were employed as an *in vitro* model system. Skin fibroblasts were cultured for 20 h either under isotonic conditions in so-called relaxed collagen lattices, which were allowed to retract the collagenous matrix without significant mechanical resistance. Parallel cultures of mechanically stressed lattices were set up, in which the collagen matrix adhered to a circular nylon thread. In the latter system, cells developed self-generated tension against the matrix. After 20 h of culture, maximal contraction of the relaxed collagen gels was observed, and mRNA was isolated from both systems. After transcription into cDNA and labelling by Cy3 (stressed) or Cy5 (relaxed), gene-expression profiles were compared. The UniGem V1.0 array had been chosen because both cDNA populations were hybridized simultaneously in one reaction to the array containing approx. 7100 different cDNA fragments encoding known, as well as unknown, gene products (and controls). The comparison led to a detailed characterization of mechanically modulated gene expression of fibroblasts subjected to mechanical load resulting in a matrix-producing cell phenotype and identification of mechano-sensitive transcripts [11].

In analogy, this system can be employed to identify transcriptional changes in a model system mimicking cell–matrix interactions under mechanical relaxation. A full list of results from this analysis, including mechanically stressed and relaxed transcripts, is available at <http://www.BiochemJ.org/bj/379/bj3790351add.htm>. Considering two-fold or higher differences in gene expression as a significant threshold, induction by lack of mechanical tension was observed in 393 transcripts at the mRNA level coding for known, as well as unknown, gene products. Annotated transcripts were grouped according to their specific

Table 1 Differentially expressed sequences in fibroblasts cultured for 20 h in stressed or relaxed collagen lattices

ADAM-17, a disintegrin and metalloproteinase-17; Bcl2, B-cell leukaemia/lymphoma 2; DEC1, differentiation of embryo chondrocytes 1; FADD, Fas-associated death domain; FGF, fibroblast growth factor; IκB, inhibitory κB; SH3, Src homology 3; TBP, TATA-box-binding protein; TRAIL, TNF-related apoptosis-inducing ligand.

Differential expression*	Transcript	GenBank [®] accession number
A. Cell-cycle regulators and proliferation-associated genes		
8.0	Cdc-like kinase	A1056204
7.4	B-cell translocation gene, anti-proliferative	A1185071
4.3	RORα1	U04897
2.1	Cyclin G2	AA281779
B. DNA synthesis/modification/transcription and nucleoside metabolism		
6.3	TBP-associated factor 172	AF038362
4.5	RNA polymerase elongation factor (ELL2)	U88629
C. Transcription factors and DNA-binding proteins		
4.3	DEC1 (basic helix–loop–helix protein 2)	A1274584
3.6	Chromodomain helicase DNA-binding protein 2	AF006514
2.0	Zinc-finger protein 136	U09367
D. Intracellular modulators/ion channels/signal transducers		
15.5	Protein tyrosine phosphatase type IVa, member 1	AA872475
6.0	Serine/threonine kinase 2	L20321
5.9	Human glucose transporter-like protein-III	AA171601
5.1	TNF-α induced protein (A20)	U09278
4.3	Serum-inducible kinase	T09264
3.7	Protein tyrosine phosphatase, non-receptor type 1	R93274
3.7	Solute carrier family 6	Z18956
3.6	RhoB homologue member B	AA808550
3.5	Ras-like protein gem	AA298157
2.0	IκB	R80854
E. Receptors and cell-surface proteins		
3.9	Endothelin receptor type B	A1248993
2.4	Platelet-activating factor receptor	D10202
2.4	Integrin α1	X68742
2.3	ICAM-1	X06990
2.2	Fibroblast growth factor receptor 1	AA419611
F. Cytokines, growth factors and chemokines		
14.2	IL-6	AA381892
12.2	Stanniocalcin	U25997
7.2	Inhibin, βA (activin AB, α-polypeptide)	M13436
4.0	KGF (FGF-7)	AA094160
3.6	IGFBP-3	AA375301
2.9	IGFBP-5	A1472963
G. Apoptosis and general stress-response genes		
20.0	Prostaglandin synthase (COX-2)	A123006
8.2	SH3-domain-binding protein 5 (SH3BP5)	AB005047
5.2	Superoxide dismutase 2, mitochondrial (SOD2)	AA370569
3.9	Bcl2/adenovirus E1B-interacting protein 3 (BNIP3)	AA503316
2.4	FADD-like anti-apoptotic molecule	AA296229
2.2	Apoptosis inhibitory protein	U19251
2.2	Heat shock 60 kDa protein 1 (chaperonin)	AA330429
2.0	Apoptotic protease-activating factor (APAF1)	W38879
2.0	TRAIL/Apo2 (TNF ligand family, member 10)	NM003810
2.0	Caspase 2	AA285285
H. Proteases		
4.7	Fibroblast-activation protein α	U09278
2.1	MMP-20	AJ003147
2.0	ADAM-17	AA824363
I. Extracellular proteins		
5.2	Lysyl hydroxylase	U84573

* Differential expression by > 2.0-fold as compared with mechanically stressed fibroblasts; approx. 7100 cDNA sequences were investigated.

cellular functions. Table 1 lists 44 selected transcripts, which were significantly induced in relaxed fibroblasts. Results indicated fundamental reprogramming of the transcriptional profile in a

relaxed three-dimensional environment. Group A (Table 1) summarizes gene products that are involved in regulation of cell cycle and proliferation. The highest levels of induction were found for Cdc (cell-cycle division)-like kinase (8.0-fold induced in relaxed compared with mechanically stressed fibroblasts), and B-cell translocation gene (7.4-fold) that belongs to a family of cell cycle regulators with antiproliferative effects [35]. Gene products such as the nuclear receptor ROR α 1 [RAR (retinoic acid receptor)-related orphan receptor α 1] (Group A; 4.3-fold) and the basic helix-loop-helix protein DEC1 (differentiation of embryo chondrocytes; Group C) were shown to regulate cellular differentiation in various cell types. Group D summarizes intracellular signal modulators induced in relaxed fibroblasts. Of these, the nuclear protein tyrosine phosphatase type IVa (15.5-fold), which is expressed in proliferating, as well as in terminally differentiated, cells and tissues [36], reveals the highest differential expression coefficient. In the group of receptors and cell-surface proteins (Group E), induction of several cell-surface molecules involved in activation of fibroblasts (especially in the context of inflammation) was detected. Among them are endothelin receptor type B (3.9-fold), integrin α 1 and ICAM-1 (intercellular adhesion molecule-1; 2.3-fold). Additionally, we identified a group of cytokines and growth factors possibly involved in regulating the specific cellular response to mechanical alterations of the ECM (Group F). The highest differential expression in this group was detected for IL-6 (14.2-fold), confirming our previous results [37], which demonstrated induction of IL-6 in fibroblasts in relaxed collagen lattices as compared with monolayer cultures. Stanniocalcin (12.2-fold) has been described as a calcium-regulated hormone, which, in mammalian cells, functions as auto- or para-crine factor to inhibit intracellular uptake of calcium while simultaneously stimulating the uptake of phosphate ions [38]. In addition, we observed induction of inhibin β A (7.2-fold), a member of the TGF (transforming growth factor)- β superfamily of proteins, and induction of KGF (4.0-fold). The IGF (insulin-like growth factor)-binding proteins IGFBP-3 and -5 (3.6-fold and 2.9-fold respectively) have been described as modulators of IGF [39], but also of IGF-independent functions, e.g. of anti-proliferative effects and promotion of apoptotic events [40,41]. Group G gives an overview of various gene products involved in cellular stress response and regulation of apoptosis. Induction of pro-, as well as anti-, apoptotic factors was detected. The transcript with the highest differential expression in the whole screening procedure was the pro-inflammatory mediator COX-2 (20.0-fold). Groups H and I comprise molecules involved in ECM regulation, such as proteases and the collagen-modifying enzyme lysyl hydroxylase (5.2-fold).

Verification of differential gene expression

To confirm the cDNA microarray results by an alternative experimental approach, gene expression of selected transcripts with either high differential expression ratios or relevant to connective tissue regulation was verified by Northern analysis of RNA isolated from different donor strains (Figure 1). In general, Northern blot analysis confirmed the regulation tendencies resulting from the microarray hybridization. In independent experiments, fibroblasts were grown as monolayers, in stressed or in relaxed collagen lattices and mRNA was isolated after 20 h of culture. It became obvious that the three-dimensional organization and physical properties of the substrate played a crucial role in the regulation of transcript levels. Strong induction of COX-2 mRNA levels was detected in relaxed fibroblasts, whereas there was no significant expression in mechanically stressed or in monolayer fibroblasts. Using fibroblast strains derived from dif-

Figure 1 Verification of differential gene expression

Human dermal fibroblasts were cultured in stressed (+) or relaxed (-) collagen gels or on plastic tissue-culture dishes (M) for 20 h. Expression levels for COX-2, IL-6, FAP, STC (stanniocalcin), KGF and LH2 (lysyl hydroxylase-2) were assessed by Northern blot analysis as described in the Experimental section. RNA loading per lane for each probe was visualized by hybridization with 18 S-specific probes.

ferent donors, induction of COX-2 mRNA in relaxed lattices was verified; however, COX-2 induction varied between 7- and 19-fold, depending on the particular fibroblast strain. Transcripts encoding stanniocalcin, IL-6 and KGF revealed a similar expression pattern with strongest induction in relaxed fibroblasts. In contrast, FAP and lysyl hydroxylase-2, two ECM-modifying gene products, also showed enhanced transcription in relaxed lattices, but a basal level of gene expression was detected in mechanically stressed and monolayer fibroblasts.

COX-2 is expressed in stressed and relaxed fibroblasts in a time-dependent and tightly controlled manner

COX-2 has been described as an inducible pro-inflammatory enzyme catalysing the initial steps in prostaglandin biosynthesis [42]. In quiescent fibroblasts, COX-2 activity is not detectable and its expression is associated with cell activation in the context of inflammation. To obtain mechanistic insight into COX-2 regulation in relaxed fibroblasts, we investigated the time-course of mRNA in stressed and relaxed collagen gels (Figure 2). Significant induction of COX-2 in relaxed lattices started at 12 h, and mRNA levels increased during the time-course of the experiment, resulting in elevated COX-2 levels at 24 h (Figure 2A), as predicted by the array analysis. Detailed investigation of COX-2 levels between 12 and 72 h (Figure 2C) revealed that mRNA levels remained at consistently high levels in relaxed fibroblasts, whereas almost no expression of the transcript was detected in mechanically stressed cells. Surprisingly, at earlier time points (0.5–8 h after gel casting), Northern blot analysis revealed induction of COX-2 in both systems with 2- and 3.5-fold enhanced signal intensity at 2 and 4 h in stressed fibroblasts as compared with the freely contracting cells respectively (Figure 2B). At later time points, COX-2 expression was restricted to the relaxed phenotype with the highest

Figure 2 Biphasic induction of COX-2 by tension at early and by lack of tension at later time points

Human dermal fibroblasts were cultured in stressed (+) or relaxed (–) collagen gels for 4, 8, 12 and 24 h (A). In parallel, cells were grown on plastic tissue-culture dishes (M) for 24 h. Hybridization of RNA with a COX-2-specific probe revealed induction at 4 h in stressed, but a much stronger induction by lack of tension at 12 and 24 h. Therefore early (0.5–8 h) and later (12–72 h) time points were investigated in more detail (B and C respectively). RNA loading per lane was visualized by hybridization with an 18 S-specific probe.

Figure 3 Biphasic induction of IL-1 β by tension at early and by lack of tension at later time points

Human dermal fibroblasts were cultured in stressed (+) or relaxed (–) collagen gels for 4, 8, 12 and 24 h (A). In parallel, cells were grown on plastic tissue-culture dishes (M) for 24 h. Hybridization of RNA with an IL-1 β -specific probe revealed induction at 4, 8 and 12 h in stressed, but induction by lack of tension at 24 h (A). Therefore early (0.5–8 h) and later (12–72 h) time points were investigated in more detail (B and C respectively). Donor-dependent variations in signal intensity at early time points were observed, but high expression in stressed conditions at early and higher expression after 24 h in relaxed conditions were consistently seen with all donors. RNA loading per lane was visualized by hybridization with an 18 S-specific probe.

differential expression factor detected in the whole screening procedure. These results indicated that COX-2, which has been described as an immediate-early gene product in response to cellular stimulation (e.g. alteration of force balance), is tightly regulated in a biphasic, time-dependent manner.

Induction of COX-2 and IL-6 in relaxed fibroblasts is mediated by an IL-1-dependent mechanism

The strong and specific regulation of COX-2 gene expression by the fibroblast–ECM interaction raises the question of whether COX-2 gene expression is governed directly by changes in cell–matrix interactions or by soluble-factor-mediated mechanisms leading to the activation of the COX-2 gene promoter. In the context of fibroblast responses to inflammation, we were interested to investigate if a hierarchy exists of so-called pro-inflammatory mediators and which mechanisms underlie the induction. IL-1 (especially its β -isoform) and TNF (tumour necrosis factor)- α are potent inducers of inflammation that have been described as stimulators of COX-2 transcription in fibroblasts [43]. Since neither DNA array nor Northern blot analysis of stressed, relaxed and monolayer fibroblasts indicated any significant expression of TNF- α mRNA (results not shown), we concentrated on the elucidation of the role of IL-1. The microchip hybridization failed to detect differential expression of IL-1 α (see the supplementary material at <http://www.BiochemJ.org/bj/379/bj3790351add.htm>), and IL-1 β was not contained in the array. Lambert et al. [26] identified an autocrine IL-1 loop in fibroblasts grown in relaxed collagen gels for 48 h. Analysis of time-dependent IL-1 β expression in three-dimensional lattices (Figure 3A) revealed an ex-

pression pattern bearing striking similarities with COX-2 regulation: at the beginning of gel contraction (4–12 h after onset of the experiment), we detected significant induction of IL-1 β mRNA in mechanically stressed fibroblasts with maximal expression between 4 and 8 h, whereas at 24 h, IL-1 β mRNA was expressed exclusively in the relaxed lattices. In monolayer cells, IL-1 β mRNA was barely detectable. Figure 3(B) illustrates transcript levels at early time points: IL-1 β transcription is induced in both collagen-lattice models, with specific signals being detected at 1 h after onset of the experiment. At 2 h, IL-1 β signal intensity was slightly weaker in relaxed than in stressed fibroblasts. Although expression in relaxed fibroblasts almost vanished at early time points, IL-1 β levels in mechanically stressed fibroblasts reached a maximum between 4 and 8 h. A completely different picture emerged at later time points: similar to COX-2 expression pattern, IL-1 β mRNA was detected exclusively in relaxed fibroblasts with continuously elevated signal intensities up to 72 h (Figure 3C). Signal intensity varied slightly in a donor-dependent fashion, as seen by the variation between 4 and 8 h in Figures 3(A) and 3(B), but the tendency of induction in relaxed cultures up to approx. 12 h and later induction in stressed cultures was persistent in all different donor cell strains tested.

Furthermore, the temporal expression of IL-6 was studied. This pro-inflammatory cytokine is endogenously induced in fibroblasts seeded into contracting collagen lattices as compared with monolayer cells and upon exogenous stimulation with IL-1 [37]. The time-course of IL-6 mRNA expression showed enhanced transcript levels in relaxed fibroblasts at 12 and 24 h, but no significant expression was found in mechanically stressed cells (Figure 4). The similar expression patterns of IL-1 β , COX-2 and the specific

Figure 4 Induction of IL-6 by a relaxed collagen lattice

Human dermal fibroblasts were cultured in stressed (+) or relaxed (–) collagen gels for 4, 8, 12 and 24 h. In parallel, cells were grown on plastic tissue-culture dishes (M) for 24 h. Hybridization of RNA with an IL-6-specific probe revealed induction at 12 and 24 h only in relaxed lattices. RNA loading per lane was visualized by hybridization with an 18 S-specific probe.

Figure 5 Inhibition of induction by IL1ra

Human dermal fibroblasts were cultured in relaxed collagen gels for 20 h in the absence (0) or presence of IL-1ra at the indicated concentrations. Induction was abrogated in a dose-dependent fashion for IL-1 β , COX-2 and IL-6, whereas induced transcription of MMP-1 and KGF was not sensitive. RNA loading per lane was visualized by Methylene Blue staining of the membrane after RNA transfer.

induction of IL-6 in relaxed fibroblasts led us to a detailed mechanistic investigation. We blocked autocrine IL-1 action by adding IL-1ra to freely contracting collagen gels at the start of the experiment (Figure 5). Northern blot analysis of RNA isolated from relaxed fibroblasts after 24 h revealed that blocking of IL-1 receptors inhibited IL-1 activity partially and led to a significant reduction of IL-1 β and COX-2 mRNA levels in a dose-dependent manner (Figures 5A and 5B). At a concentration of 1 μ g/ml IL-1ra, IL-1 and COX-2 signal intensities were reduced by up to 70% as compared with the untreated controls. A concentration of 0.01 μ g/ml IL-1ra was sufficient to down-regulate IL-6 mRNA expression by 85% (Figure 5B). In contrast, expression of KGF, which was shown to be induced by IL-1 in many different cell types and culture systems (e.g. keratinocyte–fibroblast interactions), was not affected by the addition of IL-1ra. In accordance with data by Lambert et al. [26], MMP-1 levels remained unchanged in the presence of increasing IL-1ra concentrations.

Induction of IL-1 expression, which often characterizes activated cell phenotypes, seems to be a key mediator regulating gene expression in relaxed fibroblasts, leading to autocrine regulatory loops that in turn modulate the expression of downstream inflammatory players such as COX-2 and IL-6.

No changes in COX-2 transcript levels were observed after treatment of cultures with the COX-inhibitors aspirin (inhibits COX-1 and COX-2), ibuprofen (also inhibits both) and NS 398 (a specific COX-2-inhibitor) (results not shown).

DISCUSSION

The aim of the present study was the characterization of fibroblast gene-expression profiles in response to changes of their extracellular environment, with special emphasis on the mechanical resistance of the ECM. To analyse these interactions with the ECM in detail, we chose collagen lattices as a model system to compare fibroblasts developing self-generated tension against a fixed matrix with fibroblasts in relaxed lattices that undergo the transition from proliferating to a quiescent state during gel contraction. In collagen gel culture, fibroblasts acquire tissue-like characteristics not typically observed in monolayer cultures. Concentrating mainly on fibroblasts in mechanically relaxed lattices, i.e. without tensile stimulation, transcriptional profiling indicated fundamental phenotypic changes and reprogramming of the gene-expression profile. Analysis of approx. 7100 transcripts (this number corresponds to approx. 20–25% of expressed transcripts in human cells) yielded a large number of transcripts with specialized functions. These results led to the identification of new regulatory mechanisms with potential implications for the role of fibroblasts in inflammatory diseases.

Three striking biological responses occurring in relaxed fibroblast cultures have been described previously. These are reduced cellular proliferation and induction of apoptotic events [24,25,44], induction of matrix-degrading proteases [16] and down-regulation of collagen synthesis [45]. Fibroblasts grown in relaxed gel cultures display a marked decline in cellular DNA synthesis [13]. Cells become arrested in G₀, and cell regression begins [46,47]. Some of the transcripts identified in our screening are involved in cell-cycle regulation and are responsible for anti-proliferative effects in many different cell types, but have not been described in fibroblasts so far. For example, Cdc-like protein kinase belongs to the Cdc2/CDC28 gene family and reveals striking sequence similarity to various serine/threonine kinases. Its yeast counterpart has been described as a cell-cycle regulator [48]. Expression of the B-cell translocation gene has been described in myoblasts where it exerts anti-proliferative effects and is induced by PGE₂ (prostaglandin E₂) [35,49]. ROR α 1 is yet another transcript expressed in myoblasts during conversion from the proliferative into the post-mitotic state. This orphan nuclear receptor has no known ligand in the classical sense [50]. In relaxed lattices, anti-proliferative effects are paralleled by apoptotic events [44]. Mechanical unloading of fibroblasts that goes along with disruption of the ERK (extracellular-signal-regulated kinase) signalling pathway is thought to induce cellular quiescence [51]. These findings support the hypothesis that cytoskeletal tension and cellular force balance are critical factors for anchorage-dependent cell growth [24,52]. Transcripts regulating apoptosis and/or mediating anti-proliferative effects (such as IGFBP-3 and -5) could contribute to these phenomena.

Previous reports had demonstrated induced synthesis and/or activation in relaxed collagen lattices of matrix-degrading enzymes, e.g. MMP-1 [13,45], MMP-2 [20], MMP-13 [53] and MT1-MMP [54]. Although most of these were not on the array, FAP α was detected, and is expressed as integral membrane serine protease on activated fibroblasts during healing of skin wounds or during tumour growth [55,56]. Interestingly, expression of other serine proteases, such as members of the plasminogen activator system (plasmin or urokinase plasminogen activator), did not change. This indicates selective regulation of different proteases

(e.g. MMPs or serine proteases) by fibroblasts adapting to re-organized matrices.

The screen did not add much further information to the already well-documented regulation of matrix proteins in relaxed collagen lattices. The fibrillar collagens I and III [16,57] and collagen XII [58] are down-regulated, while the microfibrillar collagen VI remains unaltered [59]. Furthermore, tenascin-C [60] and elastin [13] are down-regulated in relaxed collagen lattices, whereas decorin [11], fibronectin [57,58] and fibulin-1 (see the supplementary material at <http://www.BiochemJ.org/bj/379/bj3790351add.htm>) are not affected by changes in force balance.

Possibly the most important aspect revealed by the cDNA microarray analysis was co-ordinated induction of inflammatory mediators, some of which also belong to the group of genes induced upon general cell stress. These included different types of molecules: the adhesion molecule ICAM-1, a glycoprotein generally involved in cell–cell adhesion, cytoskeletal organization and intracellular signal transduction, is significantly up-regulated at the mRNA level in relaxed fibroblasts. Its involvement in inflammation is mirrored by ICAM-1-deficient mice, which develop severe defects in response to inflammatory stimuli [61]. In the group of cytokines, we detected remarkable induction of KGF and IL-6. Both factors can be involved in the regulation of inflammation during connective-tissue regeneration and repair, and are potential targets of IL-1 action [37,62]. However, the most striking finding was the strongly enhanced production of COX-2 with the highest differential expression coefficient in the entire screen. COX-2 encodes a key enzyme in prostaglandin synthesis, e.g. at sites of inflammation, and it is the major isoform found in inflammatory cells. COX-2 gene expression is induced by numerous pro-inflammatory, mitogenic and oncogenic factors in many cell types, including fibroblasts (reviewed in [42]). Its expression supplies important defence mechanisms against environmental insults, and overexpression occurs during the course of inflammation, tissue injury and tumorigenesis. Mechano-sensitive induction of COX-2 mRNA was also observed in osteocytes subjected to tensile forces [63]. Interestingly, induction occurred in a biphasic fashion, with a second peak due to relaxation. Furthermore, disruption of cytoskeletal networks by microtubule and actin-filament-disrupting reagents induced COX-2 levels [64]. In these examples, COX-2 was regarded as an immediate-early gene product induced within a very short time period after stimulation, however no correlation with IL-1 was provided. In the present paper, we show that in relaxed lattices at 20 h, an autocrine IL-1 β loop contributes to enhanced COX-2 and IL-6 transcript levels. IL-1 β thus was found to be a central player in the regulation of inflammatory responses in our model system. However, classical IL-1-responsive genes, such as MMP-1 [26] and KGF, were not affected by blocking IL-1 function.

Strong up-regulation of inflammatory mediators in concert with growth inhibition, apoptosis and matrix degradation characterizes the modulation of fibroblast gene expression in contracting collagen lattices. Most interestingly, the data show that an inflammatory phenotype can be induced by the surrounding type I collagen and probably altered physical properties of the matrix. Kheradmand et al. [65] proposed that cell-shape changes are critical factors in transcriptional regulation of inflammatory mediators such as IL-1 α . In their fibroblast model, altered cell shape leads to rearrangement of cytoarchitecture and modulation of gene expression via integrin-mediated mechanisms. In our model, changes in cell shape could also be responsible for the altered transcriptional profile observed. Culture of fibroblasts in collagen lattices is a unique model system that illustrates the versatility of fibroblasts and demonstrates that fibroblasts are indeed able to actively contribute to inflammation. These findings extend the

current view on the role of fibroblasts involved in tissue regulation and stimulate investigations of these properties *in vivo* where the complex interplay between cell–matrix and cell–cell interactions orchestrates adaptation to physiological as well as pathological conditions.

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