Fibroblasts in Mechanically Stressed Collagen Lattices Assume a “Synthetic” Phenotype*

Received for publication, February 20, 2001, and in revised form, July 20, 2001
Published, JBC Papers in Press, July 23, 2001, DOI 10.1074/jbc.M101602200

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Fibroblasts are subjected to changes of the mechanical force balance during physiological as well as pathological situations, such as wound healing, development of hypertrophic scars, and fibrogenesis. However, the molecular response and the changes in fibroblast gene expression upon mechanical stimulation remain poorly understood. As an in vitro model, human dermal fibroblasts were cultured within a three-dimensional network of fibrillar collagen either under high (stressed) or low tension (relaxed). cDNA microarray technology in combination with Northern blot analysis led to identification of mechano-responsive genes coding for extracellular matrix proteins, fibrogenic growth factors, protease inhibitors, components of focal adhesions, and the cytoskeleton. Application of biaxial strain to fibroblasts cultured on flexible silicone membranes revealed that the type of strain as well as the properties of the substrate induced different patterns of gene regulation. The transcriptional profile of mechanically induced genes in collagen lattices suggests that mechanical stimuli lead to a “synthetic” fibroblast phenotype characterized by induction of connective tissue synthesis while simultaneously inhibiting matrix degradation.

Regulation of genes by mechanical forces has been studied extensively relating to the biology of vascular endothelial and smooth muscle cells or chondrocytes that are obviously subjected to high fluid shear or pressure load (1). In contrast, dermal fibroblasts are less well characterized in their response to mechanical load, despite the fact that skin in its physiological state is constantly exposed to stretching and bending forces. Moreover, healing of skin wounds represents a special situation in which fibroblasts develop tensile forces against the granulation tissue matrix in order to bring the wound margins together and to obtain fast wound closure. It has been postulated that during wound contraction mechanically stressed fibroblasts differentiate into the specialized myofibroblast phenotype, characterized by expression of α-smooth muscle actin and formation of prominent stress fibers and of fibronexus junctions (2–4). The scar that finally develops is itself a tissue under increased mechanical forces, at least as long as scar resolution is not complete. The specialized cases of abnormal scarring, e.g. keloids, represent a further situation to which mechanical forces are of relevance; such lesions have long been known to develop in regions of the body that are subjected to relatively higher mechanical forces than others. Furthermore, fibroblasts in fibrotic skin lesions are thought to be subject to considerable mechanical tension.

Thus, there are examples of physiological and disease conditions that strongly argue for the presence of mechanical forces acting upon skin fibroblasts. To analyze the effects of such forces, several experimental models have been designed that clearly demonstrated that fibroblasts respond to mechanical stress. In a classical experiment, it has been shown that fibroblasts develop forces against a malleable substrate, which was wrinkled by the cells (5). Further development of elastic membrane-based culture models has proven valuable because defined strains, e.g. static or stepwise or cyclic, can easily be applied. Such planar culture models have provided valuable insight into cellular responses to substrate deformation, e.g. induction of collagen and proteoglycan synthesis by vascular smooth muscle cells (6). However, for some cell types, including skin fibroblasts, cultures on such substrates does not sufficiently represent the three-dimensional network of ECM1 macromolecules with which these cells interact in vivo. Three-dimensional collagen lattices are more suitable culture models (7). To study the effects of mechanical stress on dermal fibroblasts, we compared retracting collagen lattices, in which collagen fibers are remodeled to a floating disc of one-tenth of the original diameter, and tension is very low and distributed isotropically to stressed lattices that cannot be retracted due to adherence of the collagen lattice to a nylon thread (or steel wire) placed at the inner perimeter of the dish (8). In the latter model, cells develop tension against the resisting matrix. By contrast, in the mechanically relaxed matrix of the retracting lattice, tension is self-generated as opposed to the externally applied stress generated by substrate deformation and is distributed anisotropically. The magnitude of force developed in similar restrained lattices has been measured and amounted to 0.25 g per million cells (9) or 40–60 dynes per million cells (10).

A prerequisite for force generation is the presence of serum (11, 12), TGF-β (13), or platelet-derived growth factor (14). The

* This work was supported in part by Deutsche Forschungsgemeinschaft Grant KI 556/11. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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1 The abbreviations used are: ECM, extracellular matrix; TGF, transforming growth factor; kb, kilobases/kilobase pairs; TRITC, tetramethylrhodamine B isothiocyanate; CTGF, connective tissue growth factor; VEGF, vascular endothelial growth factor; GAPDH, glyceraldehyde-hyde-3-phosphate dehydrogenase; PCLP, podocalyxin-like protein; µN, micronewtons.
active substance in serum is lysophosphatidic acid, which stimulates contraction via cAMP, activation of Rho with inhibition of myosin light chain phosphatase, and via Ca²⁺-dependent activation of myosin light chain kinase (15). Within collagen lattices, fibroblasts utilize αβ₁ integrin receptors to mediate cell-collagen contact (16, 17). αβ₁-mediated contraction leads to activation of protein kinase Cζ and NFκB (18). By their transmembrane nature, αβ₁ receptors physically connect external collagen fibers with the actin filament network and thereby transmit forces and information required for contraction into the cells (19). Cytoskeletal networks must be intact, as cytochalasin-mediated actin microfilament disruption (11) as well as absence of intermediate filaments (20) severely impair contraction.

Cytoskeletal structures were shown to change drastically depending upon mechanical load, i.e. fibroblasts in stressed matrices develop prominent α-smooth muscle actin-containing stress fibers (11) resembling myofibroblasts. Thus, mechanical forces influence the differentiation state of cells. In parallel, cellular morphology also changes; in relaxed lattices fibroblasts appear stellate with short processes, whereas in restrained lattices they are elongated, bipolar, and oriented along the lines of tension (8, 21). Cell proliferation is low in relaxed (7) and active in restrained lattices (8).

In view of these profound changes, an adaptation of gene expression profiles induced by mechanical stress is expected. As compared with relaxed lattices, overall protein synthesis is increased 4–6-fold in stressed collagen gels (8). In particular, the following genes were found induced in fibroblasts in stressed as compared with mechanically relaxed collagen gels: α1(1) and α2(1) collagen, α1(III) collagen, all three α-chains of type VI collagen, fibronectin and β-actin, whereas elastin was only slightly induced and MMP-1 was strongly repressed (8). These changes were regulated at a pretranslational level. Also using fibroblasts but a different experimental model, Chiquet and co-workers (1) demonstrated induction by stress of collagen XII and tenasin-C mRNA as well as protein. Based on comparative promoter analyses, the authors (22, 23) suggested a core sequence of GAGACC, which was initially detected to render a platelet-derived growth factor promoter in vascular endothelial cells responsive to shear stress, to be part of a stretch-responsive element.

The aim of this study was to obtain a comprehensive overview on changes in gene expression induced by tensile stress in fibroblasts cultured in restrained (termed “stressed” in the following) versus mechanically relaxed (termed “relaxed”) three-dimensional collagen lattices. To address this, we applied a combination of techniques to identify differentially expressed genes at the level of steady-state mRNA. For a number of sequences thus identified, differential expression was verified by Northern blot hybridization.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Fibroblast cultures were established by outgrowth from skin biopsies of healthy donors as described (24) in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (PAA Laboratories, Linz, Austria), glutamine (2 mmol/liter, Biochem, Berlin, Germany), penicillin (100 units/ml, Bioch), streptomycin (100 μg/ml, Bioch), and sodium ascorbate (50 μg/ml, Sigma) and were grown in the moist atmosphere of a CO₂ incubator (5% CO₂) at 37 °C. Cells were subcultured by trypsinization (0.1% trypsin, 0.02% EDTA, Biochrom) in phosphate-buffered saline and resuspended at a ratio of 1:2. Absence of mycoplasma was checked routinely by bisbenzimidazole fluorochrome (Hoechst 33258) staining.

**Preparation of Collagen Lattices and Force Measurement**—Acid-extracted, not pepsinized collagen I from newborn bovine skin was obtained from Prof. Charles Lapiere (Lieu, Belgium) and the Institut fur Biomedizinische Forschung (Leipzig, Germany). Collagen from rat tail tendons was from First Link (Brierley Hill, UK). Collagen was redissolved at 3 mg/ml in sterile 0.1% acetic acid.

Three-dimensional collagen lattices were prepared as described with minor modifications (8). Human dermal fibroblasts were seeded at 2 × 10⁵ cells/ml into a solution containing collagen 1 (0.3 mg collagen/ml) and grown for 20 h at 37 °C in 100 mm-diameter bacterial dishes. Cells were mechanically relaxed (term ‘relaxed’ will be used) were allowed to contract the gel matrix, whereas in stressed lattices (term ‘stressed’) contractile behavior was prevented by a braided nylon thread (0.5 mm diameter) placed at the inner perimeter of the dish (Fig. 1G). In parallel, monolayer cultures of 3 × 10⁶ fibroblasts were incubated for 20 h at 37 °C in 145-mm culture dishes.

The set up used to measure isometric forces was a modification of systems described previously (9, 10) with a force transducer (KG 7A with Bridge-Amplifier DUBAM 7C, Scientific Instruments GmbH, Heidelberg, Germany) and a fixed post connected to the lattice by two polyethylene bars (18 × 5 × 4 mm). The lattice (17 × 22 × 6 mm) was poured between the two attachment posts from a solution containing 2.5 × 10⁶ fibroblasts per ml and 1.75 mg/ml rat tail collagen in Dulbecco’s modified Eagle’s medium and 10% fetal calf serum. Following polymerization (5 min at 37 °C), the lattice was connected to the measuring device, and the set up was moved to a CO₂ incubator (37 °C, 5% CO₂). The force transducer output was digitized at 0.23 Hz (Analog to Digital Converter μMeter4 and software Nextview light from BMC Systeme GmbH, Maisach, Germany) and recorded using a personal computer.

**Culture on Flexible Membranes**—A mechanical strain device (FX-3000, Flexcell International Corp.) was used to apply radial and circumferential cellular strain to human dermal fibroblasts. Fibroblasts were cultured as monolayers at 80–90% confluency on collagen I-coated silicone membranes in 6-well plates (BioFlex Culture Plate, Flexcell Corp.) in the moist atmosphere of a CO₂ incubator (5% CO₂) at 37 °C. Six planar cylinders (25 mm diameter) served as loading posts for the culture plates which were centered beneath each 35-mm well. By application of vacuum, cyclic equibiaxial strain was applied to the fibroblast monolayer, and the membrane surface area was increased by 20%. The membrane either underwent static deformation (20% surface increase for 24 h) or it was deformed with a constant, sinusoidal frequency (0.1 Hz, 20% surface increase, 24 h). Before staining, culture medium was exchanged, and 3 ml of fresh Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum was added. Cells cultured in identical wells without externally applied tension were used as controls.

**RNA Isolation and Northern Blot Analysis**—Collagen lattices and cells from monolayer cultures were lysed and homogenized in Trizol reagent (Life Technologies, Inc.), and total RNA was prepared according to the manufacturer’s instructions. mRNA was isolated from total RNA employing Oligotex mRNA Kit (Qiagen, Hilden, Germany). For Northern blot analysis, 5–15 μg of total RNA were separated by electrophoresis in 1% agarose, 0.66 M formaldehyde gels and transferred to GeneScreen hybridization transfer membranes (PerkinElmer Life Sciences). After UV cross-linking (Stratalinker, Stratagene, La Jolla, CA) membranes were stained with 0.04% methylene blue. Probes were as follows: (a) human PCLP, 1.5-kb HindIII fragment; (b) human MMP-1, 2.8-kb PstI fragment of clone FN7 (26); (c) human αVI collagen, 1.8-kb fragment of clone P18 (27); (d) human CTGF, 1.1-kb EcoRI fragment; (e) human TGF-β1, 0.36-kb EcoRI/HindIII fragment; (f) human α1(III) collagen, 1.8-kb fragment of clone P18 (27); (g) human CTGF, 1.1-kb EcoRI fragment; (h) human TGF-β3, 0.15-kb EcoRI/HindIII fragment; (i) human decorin, 0.9-kb EcoRI/HindIII fragment of clone PG40 (30); (h) human α5 integrin, 3.6-kb EcoRI fragment of clone 2.72L G1; (i) human β₁ integrin, 1.3-kb EcoRI/HindIII fragment of clone P32 (32); (j) human GAPDH, 1.3-kb PstI fragment of cDNA (33); and (k) human PAI-2, 1.9-kb EcoRI fragment of pPAI-2.7 (34). The following cDNA probes were obtained from Incyte Genomics (Palo Alto, CA): (a) human PCLP, EcoRI fragment of plN1Y2297562; (m) human Fl14, EcoRI/NorI fragment of plN1Y1042815; (a) human Cdc-like kinase, EcoRI/NorI fragment of plN1Y2464289; (a) human SHIBP2, EcoRI/NorI fragment of plN1Y270638; and (p) human SOD2, EcoRI/NorI fragment of plN1Y635178.

All cDNA probes were radiolabeled by random priming (Amersham Pharmacia Biotech) and hybridized overnight at 42 °C either in 50% formamide, 5 × SSC, 0.1% SDS, 5 × Denhardt’s buffer, and 0.1 mg/ml denatured herring sperm DNA, or in ULTRAhyb solution (Ambion, Austin, TX, USA). Each filter was washed for 15 min in 2 × SSC, 0.1% SDS at room temperature 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 with a 24-base oligodeoxynucleotide complementary to 18S rRNA labeled with [³²P]ATP by terminal deoxynucleotidyltransferase (Life Technologies, Inc.).
Gene Regulation in Mechanically Stressed Fibroblasts

RESULTS

In order to achieve a comprehensive characterization of dermal fibroblasts subjected to tensile stress regarding induction or repression of genes, cells were cultured for 20 h in threedimensional collagen lattices. Parallel cultures were either allowed to contract freely, i.e. these cells were cultured under isotonic conditions and not subjected to significant tensile stress (Fig. 1F). Furthermore, this will be referred to as the "relaxed" system. The other set was cultured in the presence of self-generated tension in lattices bound by circular nylon threads (stressed system, Fig. 1G). The time point of analysis (20 h) was chosen because by this time the relaxed cultures had reached maximal contraction (determined in preliminary experiments, not shown) and were regarded to be in mechanical equilibrium. From both sets of cultures, mRNA was isolated and used as starting material for different comparative analyses including hybridization to a cDNA microchip array comprising ~7100 expressed sequences (known and anonymous cDNAs). This approach yielded a large number of potentially differentially expressed sequences. Induction or repression of individual sequences by mechanical force was verified by Northern blot hybridization.

Potential Tension-inducible Genes Identified by cDNA Microarray Analysis—As expected, the simultaneous hybridization of Cy3-labeled cDNA generated from stressed collagen lattices and a Cy5-labeled probe from the relaxed culture system to a cDNA microchip array yielded the most comprehensive information. Signals were rated according to "balanced differential expression," which takes into account labeling efficiency as well as hybridization background. Signal intensities are expressed as x-fold Cy3:Cy5 intensities (stressed:relaxed). Positive numbers indicate higher expression in stressed lattices, and negative numbers indicate x-fold higher expression in relaxed lattices. 2-Fold or higher differences were considered significant. In total, we detected 60 known genes induced in mechanically stressed fibroblasts, whereas transcription of 64 known genes was found to be increased in the mechanically relaxed system if 3-fold or higher induction of gene transcription was considered.

Table I shows a list of 57 genes with known functions that are all induced at least 2-fold in stressed fibroblasts. No anonymous sequences are listed here; a full list is available from the authors upon request. For clarity, these genes are grouped according to their involvement in specific cellular tasks or properties.

In keeping with increased fibroblast proliferation in mechanically stressed lattices demonstrated by thymidine incorpora-

![Fig. 1. Double labeling for F-actin and vinculin in fibroblasts in stressed and relaxed collagen lattices.](image)
tion (8), proliferation-associated and cyclin gene expression was induced (Table I, group A). Of the signal transducer/ion channel group (group B), Rho GTPase-activating protein and myosin light chain were induced, as would be expected from studies using specific inhibitors (15). Genes involved in nucleoside metabolism and in particular induction of RNA polymerase II, the enzyme responsible for mRNA synthesis, reflect a generally activated condition and are in agreement with induced cell proliferation and gene expression (group C).

| Differential expression | Transcript | GenBank™ accession numbers |
|-------------------------|------------|---------------------------|
| A. Cell-cycle regulators and proliferation-associated genes | | |
| 4.6 | Proliferation-associated protein 2G4 | U59435 |
| 3.3 | Human minichromosome maintenance (MCM2) | AA251518 |
| 3.0 | Human RAS-related nuclear protein (RAN) | AA305368 |
| 2.9 | RAN-binding protein 1 | AI354212 |
| 2.9 | Cyclin D3 | AI354297 |
| 2.6 | Proliferation-associated gene A (peroxiredoxin 1) | AA315886 |
| B. Intracellular modulators/ion channels/signal transducers | | |
| 6.3 | Human leukemia virus receptor 1 | L20859 |
| 4.5 | Tax interaction protein 1 (TIP1) | AA299422 |
| 4.3 | Chloride intracellular channel | N27723 |
| 3.7 | Annexin I | AA348570 |
| 3.6 | Calmodulin 1 (phosphorylase kinase Δ) | AI205289 |
| 3.4 | Rho GTPase activating protein 1 | AA443506 |
| 3.1 | Calbindin 2 | AI193531 |
| 3.0 | Myosin, light polypeptide 1 (skeletal fast) | M312211 |
| 3.0 | Myristoylated alanine-rich PKC substrate | AI350287 |
| 2.3 | She-transforming protein | X81148 |
| 2.3 | Annexin IV | D78152 |
| C. DNA synthesis/modification/transcription and nucleoside metabolism | | |
| 3.4 | DNA (cytosine-5)-methyltransferase 1 | AA296506 |
| 3.3 | AHNAK nuclear protein (desmoyokin) | M80899 |
| 3.1 | DNA-directed RNA polymerase II | N85585 |
| 3.0 | Replication protein A2 | AA130737 |
| 3.0 | Nucleoside phosphorylase | H64313 |
| D. Transcription factors and DNA-binding proteins | | |
| 7.6 | Inhibitor of DNA-binding 1 (Id1) | S78825 |
| 6.8 | Inhibitor of DNA-binding 3 (Id3) | AL021154 |
| 3.4 | Homeobox B2 | X16665 |
| 3.3 | Muscle segment homeo box 1 (MSX1) | AA46197 |
| 2.9 | Forkhead-related activator 4 (FREAC-4) | U50931 |
| E. Receptors and cell surface proteins | | |
| 15.9 | Podocalyxin-like protein (PCLP) | N80294 |
| 9.9 | FGF-inducible protein 14 (Fn14) | AI492143 |
| 4.0 | Endoglin | AA355269 |
| 3.1 | Bradykinin receptor B2 | AA490192 |
| 2.4 | Syndecan 1 | R22500 |
| 2.1 | Thrombomodulin | RT8072 |
| F. Cytokines, growth factors, and chemokines | | |
| 7.0 | Cyr61 | AA459762 |
| 6.2 | Vascular endothelial growth factor-C (VEGF-C) | H07991 |
| 3.2 | ENA-78 | U12709 |
| 2.8 | Hepatoma-derived growth factor (HDGF) | D16431 |
| 2.1 | Connective tissue growth factor (CTGF) | AA552384 |
| G. Protease inhibitors | | |
| 10.3 | Plasminogen activator inhibitor 2 (PAI-2) | H81869 |
| 3.8 | Protease inhibitor 8 (PI-8) | L40377 |
| 3.4 | Plasminogen activator inhibitor 1 (PAI-1) | AA296506 |
| 2.3 | Tissue inhibitor of metalloproteinase 3 (TIMP-3) | AA115348 |
| 2.1 | Tissue inhibitor of metalloproteinase 1 (TIMP-1) | AI199040 |
| H. Extracellular matrix proteins | | |
| 2.9 | Collagen α1(I) | AA456983 |
| 2.1 | Tenascin-C | AA134100 |
| I. Cytoskeletal components | | |
| 5.1 | α-Tubulin | AA390346 |
| 3.6 | α-Smooth muscle actin | X13839 |
| 3.0 | α-Centaurin | Z14978 |
| 2.8 | Tropomyosin 1α | AA093973 |
| 2.7 | Microtubule-associated protein | AI124707 |
| 2.6 | Filamin A (actin-binding protein 280) | AA852906 |
| J. Focal adhesion components | | |
| 5.3 | Leupaxin | A1289901 |
| 3.5 | Moesin | F08375 |
| 3.3 | Integrin-linked kinase | U40262 |
| 3.2 | Zyxin | X94991 |
| 2.7 | Vinculin | N87855 |

* Differential expression by >2.0-fold, among ~7100 cDNAs investigated.
Furthermore, mechanical tension altered gene expression of a variety of transmembrane proteins (group E). Interestingly, of all known genes found induced in this assay, the transmembrane protein human podocalyxin-like protein (PCLP), not yet described as expressed gene in fibroblasts, reached the highest score. PCLP was originally identified on podocytes in the kidney glomerulus as a ligand for L-selectin, with a proposed role in tethering and rolling of lymphocytes on endothelia (37).

Group F shows a list of growth factors significantly induced in mechanically stressed collagen lattices. The highest induction of transcription was detected for Cyr61 mRNA that encodes a secreted, cysteine-rich heparin-binding protein that shares striking structural homologies with the fibrogenic mediator connective tissue growth factor (CTGF) (38). CTGF itself was detected at significantly increased levels in stressed lattices, confirming results generated by independent experiments (cDNA filter array, CLONTECH, data not shown) and Northern blot analysis (Fig. 3).

Of note, vascular endothelial growth factor-C (VEGF-C), a member of the VEGF protein family, was induced in fibroblasts subjected to tensile stress.

Group G summarizes induced expression of different protease inhibitors, including PAI-2 with the second highest score achieved in this assay but also the functionally related PAI-1. The MMP-inhibitors, TIMP-1 and TIMP-3 were found slightly induced.

The groups H–J consist of ECM proteins and structural components of the cytoskeleton and focal adhesion sites. Agreeing with previous reports (8, 39), collagen α1(I) and tenasin-C were induced. The microarray hybridization also confirmed induced expression of α-smooth muscle actin (group I), the isoform specifically expressed by myofibroblasts (40).

In keeping with the concept that tension is transmitted through focal adhesions (41), induced expression of leupaxin, integrin-linked kinase, moesin, zyxin, and vinculin was observed (group J). Changes in focal adhesion and actin cytoskeleton architecture were confirmed by immunostaining. In stressed collagen matrices vinculin is localized in small adhesion complexes at the cell margins (Fig. 1, C and E). Mechanical tension led to development of distinct actin stress fibers traversing the entire cell body (Fig. 1, A and E, visualized by TRITC phalloidin staining). In contrast, lack of tension induced total reorganization of actin cytoskeleton and focal adhesion architecture (Fig. 1, B, D, and F). Fibroblasts appeared rounded (Fig. 1F), and vinculin was distributed diffusely in the cytoplasm or in the perinuclear space (Fig. 1D), whereas actin stress fibers disappeared (Fig. 1B).

Time Course of Expression of Selected Genes—To obtain insight into the mechanisms of gene induction in human dermal fibroblasts cultured in a different mechanical environment, and to confirm cDNA microchip hybridization results, we studied the response of various genes to self-generated tension in more detail (Figs. 2–6). These genes were selected for further characterization due to their relevance in connective tissue metabolism (Figs. 2–4) or high levels of differential expression demonstrated by microarray analysis (Figs. 5 and 6).

First, we investigated the time-dependent regulation of extracellular matrix molecules in stressed and relaxed collagen gels. Our aim was to identify mechanisms whereby expression of one gene was dependent on previous regulation of another.

Highest expression of α1(I) collagen was observed in monolayer cultures on plastic at 20 h, whereas expression in both collagen lattice types was lower (Fig. 2A). Tension-loaded gel cultures consistently produced stronger signals than relaxed gel cultures, confirming previous results (8, 42). Elevated expression was already detected at 4 h of lattice culture, and signal intensity did not change significantly over 20 h. α1(VI) mRNA was also detected at increased levels after 4 h; however, a clear peak in expression was evident at 12 h, which declined after 20 h to the level seen at 4 h (Fig. 2A). In contrast to α1(I) collagen mRNA, the highest levels of α1(VI) mRNA were observed in stressed gels, not in monolayer cultures. Expression of MMP-1 was low in monolayers at 20 h and lattices at 4 h, whereas the strongest induction was detected in relaxed lattices at 20 h, as indicated previously (19, 43). Decorin expression was equally low in monolayers at 20 h and early lattice
cultures but increased significantly and to the same extent in both gel systems over 20 h.

Fig. 3 depicts expression of TGF-\(\beta\)-1, -\(\beta\)-3, and CTGF. The isoforms \(\beta\)-1 and \(\beta\)-3 were both expressed at low levels in monolayers and induced by culture in collagen lattices (Fig. 3A). TGF-\(\beta\)-1 reached maximal expression levels at 12 h in collagen gels; at 20 h expression remained high only under tension, whereas it declined in the relaxed system. TGF-\(\beta\)-3 mRNA was increased in stressed lattices at 12 and 20 h, but peak levels were lower than those of TGF-\(\beta\)-1 (Fig. 3A). In contrast, CTGF transcript levels were lower in both types of lattices than in monolayer cultures with highest expression at 4 h and declining until 20 h (Fig. 3A). At all time points, CTGF levels in stressed cells were higher than in relaxed cells. Thus, CTGF expression in the stressed fibroblasts does not seem to depend on previous up-regulation of TGF-\(\beta\).

In accordance with previous reports (16), \(\alpha_5\) integrin mRNA was strongly induced in contracting lattices but only marginally elevated by tension in stressed lattices over 20 h. Expression of \(\beta_3\) integrin mRNA was low at 4 h in collagen lattices but increased at 12 h (Fig. 4). Expression levels thereafter remained unaltered in both stressed and relaxed cultures and were comparable to expression in monolayer cultures. Of note, the housekeeping gene GAPDH, which is often used as an unmodified internal standard for mRNA expression, showed strong induction at 20 h in relaxed collagen gels and can therefore not be recommended as control for quantification of RNA levels in these systems.

Stress-induced Gene Transcription Requires Tension or Combined Tension and ECM—Induction of PAI-2 and PLCP, which showed the highest levels of differential expression, was verified by comparing the expression levels in monolayer cultures on plastic and in stressed and relaxed lattices at 20 h by using fibroblasts isolated from four different healthy donors (Fig. 5). The same analysis was applied to confirm enhanced expression of fibroblast growth factor-inducible protein 14 (Fn14) which is expressed at elevated levels in heart, placenta, and pancreas (44). A large induction of gene transcription of these genes by tension was observed in all fibroblast strains tested. Interestingly, expression of Fn14 transcripts was of comparable intensity in monolayer cultures on plastic, whereas no signal was detected in relaxed gels. We concluded that tension as it is generated by culturing fibroblasts on the rigid, planar culture plastic substratum is sufficient to up-regulate transcription of genes like Fn14. This is contrasted by the induction of PAI-2 and PLCP which in addition to tension requires the presence of ECM.

This requirement for either tension or ECM under tension was also investigated for genes that were strongly induced in the mechanically relaxed cultures at 20 h, identified by cDNA microchip analysis (Fig. 6). Northern blot hybridization using RNA from four different donor strains confirmed enhanced expression of Cdc-like kinase, superoxide dismutase 2 (SOD2), and SH3-binding protein 5 (SH3BP5) in relaxed versus stressed cultures. The largest differential expression was seen in SH3BP5, where signals in stressed gels are barely detectable. SH3BP5 was originally identified as an intracellular protein binding to the SH3 domain of Bruton’s tyrosine kinase and is thought to be involved in regulation of B-cell apoptosis (45). Cdc-like kinase and SOD2 were expressed at high levels in both collagen lattice types with comparably higher levels in relaxed than in the stressed system. Interestingly, this regulation was identical for both transcripts of each gene. However, expression of Cdc-like kinase was restricted to lattice cultures, indicating that ECM is required for gene activation, whereas activation of SOD2 and SH3-binding protein 5 did not show this requirement. SOD2, which is involved in the detoxification of reactive oxygen species, displayed donor-dependent variation of mRNA expression levels.

Quantitative Assessment of Force Development and Regulation of Gene Expression—To correlate regulation of gene expression with increasing force development, PAI-2 was selected as a representative marker gene, and mRNA levels were assessed at 500, 800, and 900 \(\mu\)N. For this purpose, a uniaxial collagen lattice was connected to a force transducer. Fig. 7A depicts the isometric force developed by fibroblasts in a colla-
The collagen lattice having the following dimensions: 17 mm long (x) × 22 mm wide (y) × 6 mm high (z), i.e. the x-directed force developed while the total length of the lattice remained constant. A linear increase was observed over 3–4 h, which slowly progressed into a plateau at 13 h. With this setting, the maximal force developed amounted to 950–1000 N. PAI-2 mRNA levels in fibroblasts from two different donors (Fig. 7B) showed an increase at 800 N by 1.5- and 2.6-fold in comparison to levels at 500 N (Fig. 7C). A further increase in the force to 900 N did not result in higher transcript levels, suggesting that cells had adapted to the strain.

Substrate Properties and Type of Mechanical Strain Influence Gene Regulation—To determine the specific influence of fibroblast-generated tension on the transcription of α1(I) collagen and MMP-1 in three-dimensional lattices as opposed to passive stretching of fibroblasts on a deformable substrate, cells were seeded onto collagen I-coated membranes and subjected to either steady (Fig. 8, lane 2) or cyclic (Fig. 8, lane 3) stretch. Steady strain was maximal for the apparatus used and produced a 20% increase in area for 24 h. Cyclic strain also produced an increase in area by 20%, but the membrane was stretched with a frequency of 0.1 Hz (6 cycles per minute, in a sinusoidal wave). In contrast to the three-dimensional collagen gels, expression of both genes was further augmented by deformation. The level of α1(I) collagen mRNA even exceeded the transcript levels of the non-stretched control (Fig. 8, lane 1). Cyclic stretching of the silicone membrane at low frequencies produced a significant increase of the MMP-1 mRNA level, whereas expression in fibroblasts cultured on plastic and in stressed lattices at 20 h remained low (Fig. 8, lanes 1–3). These
findings are interesting because significant induction of MMP-1 transcription has been described previously (43) in mechanically relaxed collagen lattices.

DISCUSSION

The data presented here indicate that fibroblasts cultured in an environment of three-dimensional fibrillar collagen in the presence of mechanical stress adapt by inducing as well as repressing transcription of a variety of genes and by reorganizing intracellular structures.

Although a number of genes have been described previously (8, 39, 46) that are differentially regulated by the application of mechanical stress to dermal fibroblasts, all these studies lack a comprehensive description of a mechanically stressed fibroblast “phenotype.” To obtain a transcriptional profile of dermal fibroblasts cultured with or without self-generated tension...
against a three-dimensional collagen environment, we employed a cDNA microchip array for the simultaneous analysis of a large number of genes. cDNA array techniques have recently become a powerful tool to explore gene expression, and this method was used to explore differential regulation of serum-responsive genes in fibroblasts (47). Additionally, we combined this analysis with detailed Northern blot hybridization of selected transcripts, and we demonstrated, by directly measuring the forces generated by the cells, a close correlation during the forces generated by the cells, a close correlation between force development and gene expression. The magnitude of force measured was in good agreement with the data published by Brown et al. (10).

Analysis of differential gene expression confirmed that application of tension leads to an increase of transcripts encoding proteins involved in cellular proliferation. In this context it is interesting that transcripts coding for the transcription factors Id1 and Id3 are highly induced. These proteins belong to a family of helix-loop-helix proteins that in general function as negative regulators of cell growth and negative regulators of cell differentiation in many cell types analyzed (36). It is tempting to speculate that Id1, known to inhibit skeletal muscle differentiation via MyoD, may play a role in myofibroblast determination.

Furthermore, from the variety of known genes that we found activated in this setting, we conclude that mechanically stressed fibroblasts are activated to a “synthetic” phenotype characterized by connective tissue synthesis in concert with induction of various protease inhibitors, while simultaneously down-regulating production of proteases and inflammatory mediators. Confirming previous results, we observed induction of ECM synthesis, namely of collagen α1(I) and tenascin-C as well as down-regulation of MMP-1 transcription in the strained system as compared with the freely contracting gel. In parallel, transcription of protease inhibitors such as PAI-1, PAI-2, and PAI-8 and inhibitors of matrix metalloproteinases, TIMP-1 and TIMP-3, was significantly induced upon mechanical stimulation. As inhibitors of plasminogen activators, PAI-1 and PAI-2 can either function as direct inhibitors of matrix degradation or indirectly by inhibiting the proteolytic activation of matrix metalloproteinases (48). In the context of mechanical stimulation and tissue remodeling, induction of PAI-1 transcription was recently demonstrated in vascular smooth muscle cells exposed to homogeneous biaxial strain on flexible membranes (49), whereas the important role of PAI-2 protein in different stages of tissue regeneration after injury has been described (50). Increased levels of these inhibitors could disturb the subtle balance between matrix synthesis and degradation, leading to accumulation of extracellular matrix.

A variety of tension-inducible growth factors, e.g., VEGF-C, has been identified. Interestingly, mechanosensitive regulation of another member of this protein family, VEGF, has been reported in stressed mesangial and vascular smooth muscle cells (49, 51). VEGF-C was reported to stimulate selectively the growth of lymphatic vessels by binding to VEGFR-3 and, at very low efficiency, growth of blood vessels (52). Given that lymphatic endothelial cells are induced to proliferate in response to pressure, e.g., in edema, and the close contact of lymphatic vessels to ECM structures (53), fibroblasts can play an important role in controlling lymphatic cell proliferation through VEGF-C/VEGFR-3 interaction. These regulatory pathways can be influenced by mechanical pressure, allowing an adaptation of lymphatic vessel formation to biological requirements.

The concept of a “matrix-synthesizing” fibroblast is strongly supported by the identification of a variety of tension-inducible growth factors that are known to stimulate matrix deposition in processes like wound healing, scarring, and fibrotic diseases (54). Beside induction of TGF-β1 and -β3, we observed significant mechanical inducibility of transcripts coding for CTGF and Cyr61, both of which belong to the CCN protein family (CTGF, Cyr61/Cef10, Nephroblastoma overexpressed gene). Enhanced CTGF gene expression has been demonstrated during fibrogenesis and abnormal scar formation (55, 56), whereas the structurally related Cyr61 regulates cell adhesion, migration, as well as proliferation in fibroblasts and endothelial cells (57). Interestingly, as demonstrated by time course expression analysis, CTGF expression is independent of high TGF-β levels and induction appears to be directly by mechanical stress. This is in contrast to reports suggesting that TGF-β regulates CTGF gene expression by autocrine or paracrine mechanisms (55).

Coordinate induction by tension raises the interesting question whether these genes share common regulatory elements responding to a mechanical stimulus or if they are regulated by local growth factor release. In addition to a TGF-β-responsive element, the CTGF promoter contains an element homologous to a core sequence known as stretch-responsive element (GAGACC) that may be involved in a direct regulation of CTGF expression by the application of mechanical stress (22, 58). This element is highly conserved among different species and present in the promoters of many tension-inducible genes, e.g., tenascin-C and collagen XII gene (1). Data base comparison of the promoter regions of selected tension-responsive genes yielded a positive correlation between the presence of GAGACC-like motifs in the promoters of α1(I) collagen, CTGF, PAI-2, β-actin, and fibronectin and tension inducibility of gene expression. However, the MMP-1 gene which is not induced upon self-generated tension also contains the core sequence motif, suggesting that a more complex element must be involved in this response.

Changes in transcripts associated with focal contacts or cy-
toskeletal structure were identified by the microarray assay and matched the immunostaining results. Formation of stress fibers only in fibroblasts under tension had been described previously (4). In the relaxed lattices, actin filaments were still detectable; however, no clear structure can be depicted with the exception of filamentous bundles that seem to connect adjacent cells. Clearly defined vinculin-positive focal adhesion sites in stressed cells (Ref. 4 and this report) contrast with the staining in relaxed fibroblasts, which appears diffuse and cytosolic. This picture is reminiscent of a study showing diffuse staining and non-characteristic architecture of focal contacts in fibroblasts cultured on polymeric versus monomeric collagen (59). Thus cells recognize and adapt their morphology and intracellular structures not only to the individual matrix molecule (60) but differentiate between physical properties as well as supramolecular structure of the particular matrix.

Our Northern hybridization results demonstrate that the type of mechanical stress and the properties of the in vitro model seemed to crucially affect gene regulation. We identified two groups of mechanically regulated genes. For induction of some genes, such as collagen α1(I) and the fibroblast growth factor-inducible protein Fn14, a prestressed substrate is sufficient with no requirement for a (three-dimensional) extracellular matrix, whereas transcription of genes like PAI-2 and the glycoprotein PCLP requires a stressed collagenous environment. In contrast, passive stretching of fibroblasts on a flexible gel system produced different cellular responses, Fibroblasts reacted - (depending on the nature of the substrate) differently to the technique used (subtractive hybridization), which also in- 

Our microarray analysis and Northern blot studies have revealed 57 tension-inducible known genes and even more, if anonymous sequences are included. Our findings are in accordance to a recent report (49) on the stress response by vascular smooth muscle cells. This study presented a transcriptional profile analyzing 5000 genes of vascular smooth muscle cells seeded onto deformable silicone membranes. Only three transcripts were found to be induced significantly, i.e. cyclooxygenase-1, tenascin-C, and PAI-1. Interestingly, two of these transcripts were also found increased in our study involving a different cell type and a different stress-eliciting system.

In addition, a recent study on the activation of genes in fibroblasts by UV irradiation also showed only four genes, three of which are of known function, including TIMP-3 (61). The low number of genes detected in this study may result from the technique used (subtractive hybridization), which also in our hands produced only few differentially regulated genes. Interestingly, a number of tension-induced genes (PAI-1, PAI-2, CTFG, Id3, and TIMP-3) were also detected by cDNA microarray screening for serum-inducible genes in fibroblasts (47), suggesting that these genes belong to the expression profile of activated fibroblasts. Since further genes, in particular some related to inflammation, e.g. IL-6, ICAM-1, COX-2, were induced by serum but down-regulated by tension, we conclude that, depending on the external factors, there are different phenotypes of activated fibroblasts. Their role in vivo during mechanical stimulation of tissues or inflammation remains to be elucidated.

The transcriptional profile presented here may still underestimate the number of mechanically regulated genes in stressed fibroblasts. The total number of genes analyzed here represents ~10% of expressed human sequences, and our results reflect only changes in steady-state mRNA levels, whereas the regulation at the levels of translation and protein modification remains to be investigated.

Acknowledgments—We thank Charles Lapiere and Betty Nussgens (Liège, Belgium) for generously supplying purified collagen and for continued stimulating discussion, Gabriele Pfitzer (Cologne) for generous support with the force-measuring equipment, and Monique Aumailley (Cologne) for helpful discussion of intracellular structures.

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