The role of cytokines TGF-β1 and FGF-1 in the expression of characteristic markers of rat liver myofibroblasts cultured in three-dimensional collagen gel

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Short title: Liver myofibroblasts in collagen gel
Summary

Rat liver myofibroblasts (MFB) are the key cells involved in the deposition of extracellular matrix in fibrotic liver. They were isolated by repeated passaging of non-parenchymal cell fraction and cultured in 3-dimensional (3D) collagen gel mimicking tissue. The transfer of MFB from plastic dishes to collagen resulted in the change in their shape from large and spread to slender with long extensions. The expression of transforming growth factor-β1 (TGF-β1) and of MFB markers, α-smooth muscle actin (α-SMA) and cellular fibronectin (EDA-FN), on protein level was significantly decreased in collagen gel. The gel did not change the expression of metalloproteinase MMP-2 but activated the proenzyme. The experiments with inhibitors of metabolic pathways showed that EDA-FN and α-SMA were differently regulated. The expression of EDA-FN required functional TGF-β1 receptors and was also dependent on the activity of protein kinases MEK1 and MEK2. α-SMA expression was primarily determined by the 3D environment. Fibroblast growth factor-1 (FGF-1) in combination with heparin decreased the expression of α-SMA and increased the expression of EDA-FN in the cells on plastic. The cellular environment may influence the cells per se and may modify the action of other agents.

Key words

Liver myofibroblasts · α-smooth muscle actin · cellular fibronectin · matrix metalloproteinase-2 · TGF-β1 · FGF-1 · protein kinases
Introduction

Myofibroblasts (MFB) are the main cellular type responsible for tissue repair. They are involved in wound contraction, remodelling of provisional wound matrix and forming of the scar. They are contractile cells due to the presence of α-smooth muscle actin (α-SMA) containing stress fibres. They synthesize extracellular matrix (ECM) components and produce enzymes involved in tissue degradation and remodelling (Desmouliere et al. 2005). Various fibroblastic cells may acquire myofibroblastic phenotype. The main source of MFB in the liver is hepatic stellate cells (HSC) and portal fibroblasts. MFB are not present in normal liver but they appear in liver fibrosis (Iwaisako et al. 2012). They can be isolated from the nonparenchymal liver cell fraction released from the tissue by enzymatic treatment (Knittel et al. 1999, Ogawa et al. 2007, Jiroutová et al. 2013). MFB differentiation observed in vivo can be reproduced by culturing nonparenchymal liver cells on plastic dishes (Friedman et al. 1989, Guyot et al. 2006).

Cells in a tissue are surrounded with extracellular matrix (ECM), which is a network containing proteins, glycoproteins, proteoglycans and glycosaminoglycans. ECM provides both chemical and mechanical signals to cells. Chemical signals may originate in the chemical structure of ECM components or may be provided by cytokines and growth factors stored in the ECM and released from it under certain circumstances (Kanta 2015). The cells cultured in three-dimensional (3D) environment acquire tissue-like phenotype and behave differently than the cells in conventional tissue culture on stiff plastic dishes, as far as their morphology, differentiation, migration, and proliferation is concerned (Plant et al. 2009). The most abundant ECM component in fibrotic tissue is type I collagen (Aumailley and Gayrand 1998, Ramadori et al. 1998). 3D collagen matrices are often used as a model to study cell behaviour in the tissue-like environment (Grinnell and Petroll 2010).
Transforming growth factor-β1 (TGF-β1) is a cytokine thought to have a key role in liver fibrogenesis (Gressner et al. 2002). It is produced by endothelial cells, lymphocytes, macrophages and platelets (Branton and Kopp 1999). Autoinduction of TGF-β1 may occur in fibroblasts (Kelley et al. 1993, Wells et al. 2004). Nonparenchymal liver cells express this cytokines in CCl₄-damaged liver (Date et al. 1998). TGF-β1 forms a complex with receptors I and II and type I receptor phosphorylates signal transducers Smad2 and Smad3. The Smads enter the cell nucleus and regulate the transcription of specific genes (Inagaki and Okazaki 2007). Smad activity may be further modified by MAP kinases (ERK 1/2) that phosphorylate Smad3 (Leask and Abraham 2004).

TGF-β1 induces the expression of α-SMA, a marker of MFB differentiation, in fibroblastic cells of various origin (Malmström et al. 2004, Meyer-ter-Vehn et al. 2006, Hinz 2007). However, the induction is regulated by substrate stiffness; cell activation increases as the support becomes stiffer (Arora et al. 1999, Li et al. 2007).

TGF-β1 stimulates cellular fibronectin synthesis and its incorporation into ECM (Ignotz and Massagué 1986, Ramadori et al. 1992). EDA type of cellular fibronectin (FN) is involved in fibroblast differentiation and is essential for fibrosis development (Muro et al. 2008, Kohan et al. 2010). EDA-FN deposition precedes α-SMA expression during granulation tissue evolution in vivo (Serini et al. 1998).

Matrix metalloproteinase-2 (MMP-2) is expressed during liver fibrogenesis by α-SMA containing cells. It is secreted as a proenzyme and activated by a membrane-type MMP (Préaux et al. 1999). The influence of TGF-β1 on MMP-2 expression and activity varies from tissue to tissue. It is greatly enhanced in human ligament fibroblasts (Wang et al. 2011), weekly increased in rat tendon fibroblasts (Cui et al. 2011) and suppressed in rat lung fibroblasts (Ye et al. 2011).
Fibroblast growth factor-1 (FGF-1), also called acidic FGF (aFGF), is a member of the family of fibroblast growth factors. These cytokines control cell proliferation, migration and differentiation in various organs through different signal pathways (Boilly et al. 2000). FGF form a ternary receptor complex with tyrosine kinase and heparan sulfate glycosaminoglycan (McKeehan and Kan 1994). Heparin modulates FGF activity in this way (Turnbull and Gallagher 1993).

FGF-1 suppresses α1(I) collagen expression and induces MMP-1 in cultured fibroblasts (Becerril et al. 1999) and decreases α-SMA expression (Maltseva et al. 2001). It antagonizes the effect of TGF-β1 on α-SMA expression in fibroblasts (Ramos et al. 2006). FGF-1 actions can be characterized as the induction of the antifibrogenic phenotype. On the other hand, liver fibrosis is decreased in mice deficient in FGF-1 and -2 and chronically exposed to carbon tetrachloride treatment (Yu et al. 2003). However, the effects of FGF-1 on liver myofibroblasts have not yet been studied.

The aim of this study was to compare the expression of MFB markers, α-SMA, cellular fibronectin and MMP-2, in rat liver MFB embedded in 3D collagen gel with the expression in the cells cultured on standard plastic dishes. The role of TGF-β1 pathway was studied using SB431542, the inhibitor of TGF-β type I receptor. The inhibitor U0126 was used to study the role of MAP kinases. The effect of FGF-1 and heparin was tested in the cells cultured both in type I collagen gel and on plastic. TGF-β1 and other cytokines are candidates for the treatment of cirrhosis and their testing should therefore include conditions resembling 3D structure of tissues.
Methods

Animals

Male Sprague-Dawley rats (Anlab, Prague, Czech Republic) were fed commercial pelleted diet ad libitum and were maintained in an air-conditioned room at 22 °C. They were used at the age of 5 months when they weighed 450-550 g. The experiments were approved by the Committee for Work with Small Laboratory Animals of the Faculty of Medicine in Hradec Králové and by the Ministry of Education, Youth and Sports, File No. 40904/2011-30.

Cell isolation and culture

The rats were anesthetized with a mixture of ketamine (Narkamon, Bioveta, Ivanovice, Czech Republic) and xylazine (Xylased, Bioveta). The liver was perfused in situ through the portal vein with Ca^{2+}-free Hanks balanced salt solution (Gibco Life Technologies, Prague) for 10 min., followed by 100 ml of 0.2% pronase E (w/v) (Roche, Prague) solution and by 200 ml of 0.01% collagenase B (Serva, Heidelberg, Germany). The enzymes were dissolved in complete HBSS. Perfusion solutions were warmed in a water bath to maintain the temperature of the liver at 37°C. The cell suspension was incubated in 0.001% DNase (Roche) for 30 min. HSC fraction of liver cells was isolated by centrifugation on Optiprep (Axis-Shield, Oslo, Norway) gradient (Jiroutova et al. 2007). The cells were cultured in Dulbecco’s modified Eagle medium (DMEM; Sigma, Prague) containing 10% fetal bovine serum (FBS; PAA, Cölbe, Germany), 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma). MFB were obtained after 4 passages of the culture. They were cultured for 24 h before the tested agents were added.

FGF-1 was obtained from Peprotech (Rocky Hill, USA), heparin from porcine intestinal mucosa was obtained from Sigma. SB431542 (Sigma) was dissolved in dimethylsulfoxide
(DMSO; Sigma) and used at final concentration of 100 nmol/ml. The stock solution of U0126 (Cell Signaling, Danvers, USA) was in DMSO, final concentration was 30 nmol/ml.

**Collagen gels**

A modified procedure of Elsdale and Bard (1972) was used to prepare collagen. Rat tail tendons were cut to small pieces, extracted with 0.15 M NaCl overnight and then with 0.25 M acetic acid for 48 hrs with continuous stirring. The suspension was dialyzed against three exchanges of 0.02 M acetic acid and centrifuged at 16,000 g for 40 min. All these manipulations were done at 4°C. Collagen concentration was adjusted to 1.33 mg/ml. Filter-sterilized collagen solution was mixed with 4 x DMEM in the ratio 3:1 (v/v). The pH of the mixture was neutral judging by the colour of the indicator present in DMEM. The cells (230 thousands in 40 µl of DMEM) were introduced into 2 ml of the mixture in Petri 35 mm dishes. The gels were allowed to polymerize in the CO₂ incubator for 1 hour at 37°C and then they were overlaid with the same volume of DMEM containing 20% FBS and antibiotics. The resulting concentration of FBS was 10%. The same numbers of cells were plated on plastic dishes without collagen

**RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR)**

The cells on plastic were scraped in PBS, centrifuged and dissolved in guanidine thiocyanate (GTC) containing mercaptoethanol (Jiroutova et al. 2007). The gels were centrifuged at 12,000 g for 4 minutes and dissolved in saturated GTC containing mercaptoethanol. Total cellular RNA was extracted according to Chomczynski and Sacchi (1987) and purified using RNeasy Mini Kit (Qiagen, Hilde, Germany). RNA was reverse transcribed using cDNA Reverse Transcription Kit (Applied Biosystems Life Technologies, Prague) and quantified
with TaqMan Gene Expression Assays (Applied Biosystems). The results were normalized to 18S RNA expression.

**Western blot analysis**

The cells were scraped from the plastic, suspended in phosphate buffered saline (PBS) containing 4 mM EDTA and washed 3fold with this solution. Collagen gel was dissolved by Clostridium histolyticum collagenase NB 4G proved grade (Serva, Heidelberg, Germany). The cells were washed 3fold with EDTA-containing PBS. The proteins were extracted with cell lysis buffer (Cell Signaling). Protein content was determined with bicinchoninic acid (Sigma). Ten µg protein were applied on Novex NuPAGE 4-12% Bis-Tris gel (Invitrogen Life Technologies, Prague) under nonreducing conditions. The proteins were transferred to 0.2 µm Hybond nitrocellulose membrane (GE Healthcare, München, Germany). Staining with Ponceau S was used as a loading control. The membranes were incubated with antibodies to α-SMA (1A4, Sigma), cellular fibronectin (IST-9; Santa Cruz Biotechnology, Heildelberg, Germany) or MMP-2 (H-76, Santa Cruz), TGF-β1 (V, Santa Cruz), Smad2/3 (D7G7, Cell Signaling), Phospho-Smad2 (Ser465/467)/Smad3 (Ser423/425) (D27F4, Cell Signaling) at 4°C overnight. The secondary antibodies were from Santa Cruz. Detection was done with Western Blotting Luminol Reagent (Santa Cruz). The blots were scanned, quantified by program QUANTITY ONE 4.6 and normalized to the respective controls.

**Immunocytochemistry**

The cells were cultured for 24 h. FGF-1, heparin or SB431542 were then added and the cells were harvested 48 h later. Cells on collagen gel were fixed by paraformaldehyde and permeabilized with 0.1% Triton X-100. The unspecific binding was blocked with 1% FBS
for 30 min. The cells were incubated with the antibody to α-SMA (1A4, Sigma) at 4°C overnight. Polyclonal Goat Anti-Mouse Immunoglobulins/HRP (Dako) as a secondary antibody was applied in dark for 1 h. The nuclei were stained with DAPI. The slides were mounted in Vectashield mounting medium for fluorescence H-1000 (Vector, Peterborough, United Kingdom) and examined by fluorescence microscopy.

**Zymography**

The activity of MMPs was measured in culture media. The cells were cultured on collagen gels for 24 h, released by trypsin treatment and plated on plastic for 24 hours. The cells cultured on plastic were trypsinized and replated. Culture medium was harvested and protein concentration was adjusted to 1 mg/ml. Ten microliter samples were electrophoresed in 8% SDS-polyacrylamide gel containing 0.1% gelatin. The gel was washed with 2.5% Triton X-100 twice for 30 min and subsequently incubated for 16 h at 37°C in 50 mmol/l Tris-HCl buffer pH 7.4 containing 15 mmol/l sodium chloride and 10 mmol/l calcium chloride. The gel was stained with Coomassie brilliant blue and destained.

**ELISA determination**

Levels of extracellular TGF-β1 was determined in culture media using commercial ELISA kits for TGF-β1 (Invitrogen Life Technologies, Prague) according to the manufacturer’s instructions. The cells were cultured on plastic and on gel for 24 h before the tested agents (FGF-1, heparin, SB431542 or U0126) were added. The cultivation intervals used are given in Figures and Tables. The minimum detectable dose of TGF-β1 is less than 15.6 pg/ml. The total amount of TGF-β1 was normalized to the number of cells and media volume.
**Statistical analysis**

Two-tailed Student’s t-test was used to evaluate the statistical significance of the results. The significance level was $p = 0.05$. $n$ in the legends indicates the number of independent experiments (MFB from different animals).

**Results**

The 4fold passaged MFB spread on plastic surface (Fig. 1A). However, when they were embedded in collagen gel, they assumed a slender shape with long extensions and aggregated (Fig. 1C). The addition of FGF-1 and heparin did not have any effect on cell morphology (Fig. 1B, D). The expression of the proteins characteristic of MFB was evaluated by Western analysis. When the cells were harvested 72 h after transfer to collagen gel, the level of TGF-β1 was reduced to 16% in the 3D environment when compared to parallel cultures on plastic. The level of α-SMA decreased to 32% and the level of EDA-FN to 39%. The expression of MMP-2 did not change significantly (Fig. 1E, F).

The concentration of FGF-1 used in experiments with cells *in vitro* varies from 1 to 80 ng/ml. Heparin that increases the effect of FGF-1 is often used in concentrations of 5 to 100 μg/ml (Maltseva *et al*. 2001, Becerril *et al*. 1999). We tested the effects of FGF-1 alone in concentrations ranging from 4 ng to 25 ng/ml of culture medium containing 0.5% FBS without heparin. MFB were plated on plastic dishes (Fig. 2A) or embedded in collagen gel (Fig. 2B). Changes in the mRNA expression of four genes characteristic of MFB, TGF-β1, EDA-FN, α-SMA and MMP-2, were found at the lowest tested concentration, especially in the cells cultured on plastic. Untreated cells were used as control. The highest tested concentration produced results that differed most from the control but the results varied greatly and were not statistically significant.
The concentration of 4 ng FGF-1/ml was used in further experiments. To increase the effect of FGF-1, heparin was added at concentration 10 µg/ml. Culture medium contained 10% FBS. The influence of FGF-1 and heparin on the cells on plastic or in collagen during 96 hour experiments is shown in Fig. 3. The drugs were added to the cultures 24 h after plating. Each result was normalized to the untreated control harvested at the respective time interval and the asterisks indicate statistical significance of the results compared to this control. To show the difference in the protein expression between plastic and collagen gel, the level of the expression on plastic was considered 100%. FGF-1 plus heparin increased the expression of EDA-FN protein in the first 72 h in the cells on plastic but decreased the expression of α-SMA. The expression of EDA-FN and α-SMA in the cells embedded in collagen was insignificantly decreased at certain time intervals. TGF-β1 protein was not detectable by Western blotting at some time intervals (not shown) and therefore ELISA determination of the extracellular TGF-β1 concentration was used. The concentration of TGF-β1 secreted into the medium by the cells in collagen gel was significantly lower than that secreted by the cells on plastic. TGF-β1 levels were not much affected by the combination of FGF-1 and heparin (Fig. 3, Table 1). MMP-2 protein expression was not influenced by FGF-1 with heparin (Fig. 3). However, the activity of MMP-2 in the cells cultured on plastic was inhibited by drug combination (Fig. 4J).

SB 431542 inhibits the function of TGF-β type I receptor (Inman et al. 2002). In our experiments, it inhibited phosphorylation of Smad3 and Smad2, both in the cells on plastic and in collagen (Fig. 4K, L) and prevented Smad2 phosphorylation induced by FGF-1 and heparin. SB 431542 significantly decreased the expression of EDA-FN in the cells both on plastic and in collagen gel. α-SMA expression was much less affected. MMP-2 was not significantly
influenced (Fig. 5A, B). The effect of the inhibitor on the expression of α-SMA in collagen-embedded cells is illustrated in Fig. 4C, D. SB 431542 partially inhibited the secretion of TGF-β1 into the culture medium both in cells on plastic and in collagen gel, in the presence or absence of FGF-1 and heparin (Fig. 5C).

U0126 is an inhibitor of mitogen-activated protein kinases MEK1 and MEK2 (Favata et al. 1998). It suppressed the expression of EDA-FN in the cells in collagen gel. Its effect on α-SMA was not statistically significant. The cells cultured on plastic responded to the inhibitor by a slight decrease in α-SMA and EDA-FN expression. MMP-2 expression was not significantly affected by U0126 either in 3D environment or on plastic (Fig. 5D, E). The secretion of TGF-β1 into the medium was slightly inhibited (Fig. 5F).

Discussion

Rat MFB obtained by 4 passages of nonparenchymal fraction of liver cells spread well on plastic dishes. However, when they were embedded in collagen gel, they acquired a slender shape with long extensions. DNA synthesis and collagen expression are much higher in the cells cultured on stiff plastic than in the cells embedded in collagen gel attached to the walls of the culture dish (Lambert et al. 1992). Pathological stiffening of the tissue enhances fibrosis, while physiological levels of the tissue stiffness act as a brake on collagen synthesis. Exogenous TGF-β1 or matrix stiffening can overcome this physiological brake (Liu et al. 2010). The changes in cell proliferation and synthetic activities are accompanied with changes in cell morphology (Kanta 2015).

TGF-β1, α-SMA, EDA-FN and MMP-2 proteins were all expressed in our MFB. The concentration of immunoreactive TGF-β1 in the culture medium of the cells embedded
in collagen was significantly lower than that in the medium of cells cultured on plastic. The decrease in the key profibrotic cytokine TGF-β1 expression in the cells growing in collagen after transfer from plastic is in agreement with the above theory. Mouse mammary epithelial cells transferred from plastic to EHS matrix give similar results (Streuli et al. 1993). The opposite was found with fibroblasts isolated from human skin biopsies (Varedi et al. 2000). The cause may reside in the tissue from which the cells were isolated and in the physiological state of the cells. The behaviour of normal dermal cells resembles that of the fibroblasts cultured in collagen matrix detached from the walls of the dish and floating in the medium. These cells have very low proliferative capacity and ability to synthesize collagen (Grinnell 1994).

The inhibitor SB 431542 that blocks the activity of TGF-β receptor type I (Inman et al. 2002) partially decreased the concentration of extracellular TGF-β1 in the medium of MFB on plastic or in collagen gel. TGF-β1 is known to autoregulate its own production (Kim et al. 1990) and this result may reflect this ability. TGF-β1 secretion into the medium was also slightly decreased by U0126, the inhibitor of protein kinases MEK1 and MEK2.

The EDA-FN protein levels was significantly lower in the cells cultured in collagen gel than in the cells on plastic and it was further decreased by SB 431542. This compound also inhibited phorphorylation of Smad3 and Smad2. The low level of TGF-β1 may be the cause of decreased expression of EDA-FN. EDA-FN expression by MFB in collagen gel was strongly dependent on the function of MEK1 and MEK2 kinases, especially in the cells in collagen gel.

TGF-β1 induces the formation of granulation tissue in vivo with abundant α-SMA containing MFB (Desmouliere et al. 1993) and increases α-SMA expression in MFB embedded in collagen lattices in vitro. Fibronectin deposition precedes the expression of α-SMA by fibroblasts in granulation tissue in vivo and after TGF-β1 stimulation in vitro (Serini
et al. 1998). The expressions of α-SMA and fibronectin fibrils are induced by TGF-β1 but while the α-SMA appearance is reversible, that of fibronectin is not (Vaughan et al. 2000). The changes in EDA-FN level in our experiments did not parallel those in α-SMA. The expression of α-SMA was more influenced by the environment and differently dependent on signal pathways. The decrease in α-SMA expression in collagen matrix was a dominant effect, while the blockade of TGF-β1 receptor had a modifying influence. The expression of EDA-FN was much more sensitive to U0126, the inhibitor of MEK kinases (Favata et al. 1998), than the expression of α-SMA.

MMP-2 expression in MFB was not significantly influenced by culturing the cells in 3D collagen gel. TGF-β1 is known to regulate metalloproteinase expression in many cells. It induces MMP-2 gene transcription and activity in cultured human ligament fibroblasts (Xie et al. 2013) but suppresses MMP-2 expression by rat dermal fibroblasts (Howard et al. 2012). The inhibitor of TGF-β1 receptor function did not influence the expression of MMP-2 in our MFB. However, collagen gel induced activation of the proMMP-2, in agreement with the findings in human dermal fibroblasts (Seltzer et al. 1994) and in HSC (Théret et al. 1999).

FGF-1 with heparin induces antifibrogenic phenotype in human lung fibroblasts, decreases α-SMA expression, reduces transcription of type I collagen and induces collagenase transcription (Becerril et al. 1999, Ramos et al. 2006). The combination of these drugs inhibits α-SMA expression in rabbit corneal MFB (Maltseva et al. 2001). FGF-1 at the concentration of 20 ng/ml with 100 µg/ml heparin reverses the changes in morphology of human epithelial-like cells (Ramos et al. 2010). Cell morphology was not changed in our experiments where lower drug concentrations were used. MFB cultured on plastic responded to FGF-1 added with heparin by a decrease in α-SMA protein and an increase in EDA-FN. The response of the cells in gel was weaker but in the same direction in the case of EDA-FN and α-SMA.
The activity of MMP-2 determined by zymography was inhibited by the drugs in the cells cultured on plastic. The expected antimyofibroblastic effect of FGF-1 was small in MFB cultured in the gel.

The culture of rat liver MFB in 3D collagen gel modified the expression of the key profibrotic cytokine TGF-β1 and the expression of myofibroblastic markers EDA-FN and α-SMA to a different extent. The expression of MMP-2 did not change but the proenzyme was activated. EDA-FN expression was dependent on TGF-β1 and influenced by MAP kinases, while the expression of α-SMA was primarily determined by the environment. The combination of FGF-1 with heparin had larger effects on MFB cultured on plastic than on those in collagen gel. When compared with the standard culture of MFB on plastic, individual variables were selectively influenced by the 3D collagen environment that simulates conditions in tissues. MFB markers were regulated differently by various signalling pathways.

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Fig. 1. Morphology of MFB. The cells were cultured for 72 h on plastic (A, B) or in collagen gel (C, D). FGF-1 plus heparin was added after the first 24 h (B, D). Magnification 100x. Protein expression in MFB cultured for 72 h on plastic or in collagen gel. Protein extracts of the cells were subjected to Western analysis. The blots were scanned. The results are shown as percentage of the expression on plastic (E). Means ± S.E.M. (n = 5), *statistically significant results (p≤0.05). Examples of Western blots (1 of 5 independent determinations) (F).
Fig. 2. mRNA expression in MFB cultured on plastic (A) or in collagen gel (B). FBS concentration was 0.5%, heparin was not present, FGF-1 was added at the indicated concentrations 24 h after plating the cells and the cells were harvested after consecutive 16 h. The results are expressed as percentage of the untreated control. Means ± S.E.M. (n = 3), *statistically significant results (p≤0.05).
Fig. 3. Protein expression in MFB cultured on plastic (dark columns) or in collagen gel (light columns). FBS concentration was 10%. FGF-1 plus heparin were added 24 h after cell plating and the cells were harvested later at the indicated time intervals. The asterisks indicate statistical significance of the results compared to the untreated control at the respective time interval. To show the difference in the protein expression between plastic and collagen gel, the level of the expression on plastic was considered 100%. Means ± S.E.M. (n = 3), *statistically significant results (p≤0.05).
Fig. 4. Immunocytochemistry of MFB. The cells were cultured on collagen gel for 24 h. FGF-1 plus heparin (B, C) or SB 431542 (C, D) were then added and the cells were cultured for another 48 h. Control (A). MFB were stained with an antibody against α-SMA. FBS concentration was 10%. Magnification 600x. Zymography of culture media. MFB were cultured on plastic (E, I, J) or on collagen gel (F, G, H). FGF-1 plus heparin were added to some cultures (H, J). The expression of Smad2 and Smad3 and their phosphorylated forms. MFB were cultured on plastic (K) or in collagen gel (L) for 24 h. The specified drugs were then added and the cells were harvested 48 h later. Means ± S.E.M. (n = 3), *statistically significant results compared to untreated control (p≤0.05).
Fig. 5. Protein expression in MFB cultured on plastic (A) or in collagen gel (B) in the presence of the inhibitor SB 431542. All drugs were added 24 h after plating and the cells were harvested 48 h later. FBS concentration was 10%. TGF-β1 concentration in the medium was determined by ELISA (C). Protein expression in MFB cultured on plastic (D) or in collagen gel (E) in the presence of the inhibitor U0126. The drugs were added 24 h after plating the cells and the cells were harvested 48 h later. TGF-β1 concentration in the medium was determined
by ELISA (F). Means ± S.E.M. (n = 3), *statistically significant results compared to untreated control (p≤0.05).

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<th>Time (hours)</th>
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<tr>
<td>FGF-1 (4 ng/ml)</td>
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<tr>
<td>Heparin (10 μg/ml)</td>
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<td>Plastic</td>
<td>124 ± 14</td>
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<td>147 ± 13</td>
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<td>Collagen gel</td>
<td>35 ± 5∗</td>
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**Table 1.** TGF-β1 content (pg/ml) determined by ELISA in the medium of MFB cultured on plastic or in collagen gel. FBS concentration was 10%. The samples were taken at the indicated time intervals after cell plating. The cells in collagen gel were compared to those on plastic. Means ± S.E.M. (n = 3), *statistically significant results (p≤0.05).