# Activation of Rat Liver Perisinusoidal Lipocytes by Transforming Growth Factors Derived from Myofibroblastlike Cells

## A Potential Mechanism of Self Perpetuation in Liver Fibrogenesis

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## Abstract

**Rat liver perisinusoidal lipocytes (PL) cultured on uncoated plastic transform spontaneously within 6–10 d to myofibroblastlike cells (MFBlC). Parallel to the transformation the TGFα- and TGFβ1-mRNA expression increased and was highest in MFBlC. Competitive radioligand binding assays demonstrated that in contrast to untransformed PL the MFBlC synthesize and secrete transforming growth factor (TGF)-α (15 fmol/cell per 24 h) and predominantly the latent form of TGFβ1 (0.2 fmol/cell per 24 h). Medium conditioned by MFBlC (MFBcM) significantly stimulated PL proliferation with little effect on PL proteoglycan synthesis. By transient acidification of the MFBcM, known to activate the latent form of TGFβ1, the stimulatory effect on PL proteoglycan synthesis was enhanced and furthermore PL transformation (measured by expression of iso-α smooth muscle actin and loss of retinylpalmitate) was accelerated. Preincubation of this medium with neutralizing antibodies to TGFβ resulted in (*a*) the growth inhibitory effect was converted to a growth stimulation and (*b*) the stimulatory effect on proteoglycan synthesis was abolished. In summary our data indicate that progressive activation of PL on plastic (transformation to MFBlC) leads to an enhanced expression of the TGFα- and TGFβ1-mRNAs and secretion of the corresponding proteins. Medium conditioned by MFBlC stimulates proliferation, transformation, and PG synthesis of untransformed PL. These mechanisms are suggested to be relevant in self perpetuation of liver fibrogenesis. (*J. Clin. Invest.* 1992. 89:19–27.) Key words: lipocytes • proliferation • transformation • proteoglycans • transforming growth factors**

## Introduction

In experimental (1, 2) and in human alcoholic liver injury (3) perisinusoidal lipocytes (PL)[1] (fat storing cells, Ito cells, stellate cells) have the ability to proliferate strongly and transform via transitional cells (1) into myofibroblastlike cells (MFBlC) (3) showing dispersed nuclear chromatin (4); hypertrophy of rough endoplasmic reticulum (5, 6); reduction of number and size of fat droplets; decrease of retinoids; and long cytoplasmic extensions (5–7). PL cultured on uncoated plastic materials lose their differentiated phenotype and transform within 1–2 wk to highly proliferative and activated MFBlC (6, 7). PL and to an even higher amount, their transformed counterpart produce significant quantities of connective tissue components (6–9) and interestingly, a pattern of proteoglycan (PG) and collagen similar to that found in fibrotic extracellular matrix (for review see references 7 and 9).

Studies from others and our laboratory have shown recently that secretions of activated Kupffer cells and platelets stimulate proliferation of PL in culture (10–13) and also the synthesis of collagens (13), proteoglycans (14), and hyaluronan (15) by these cells. Potential candidates of the Kupffer cell and platelet-derived fibrogenic activity seem to be transforming growth factor α/epidermal growth factor (TGFα/EGF) and TGFβ (7, 16–18). It was shown recently that (*a*) TGFβ stimulates collagen- (16, 19) and proteoglycan synthesis in PL (20), (*b*) in acute $CCl_4$ induced liver injury the level of TGFβ-mRNA rises (21), (*c*) PL in culture express the TGFβ gene (22) and furthermore (*d*) TGFβ gene expression is significantly enhanced during active fibrogenesis associated with liver disease in man (23). These results indicate a central role of TGFβ in hepatic fibrogenesis. Furthermore, it was shown that TGFβ1 inhibited PL proliferation in a dose-dependent manner (19, 24), but significantly stimulated chondroitin sulfate and hyaluronate synthesis in these cells (untransformed PL) (20).

The current study described cell–cell interactions via soluble mediators between MFBlC and untransformed PL. Our results indicate that MFBlC synthesize and secrete soluble mediators and stimulate thereby PL proliferation (TGFα), transformation (TGFβ), and proteoglycan synthesis (TGFβ) in a paracrine way.

## Methods

### *Materials*

Collagenase H (clostridiopeptidase A, EC 3.4.24.3), trypsin (EC 3.4.21.4), and iso-α smooth muscle actin antibodies and FCS were from Boehringer Mannheim, GmbH, Mannheim, Germany. TGFβ1 (source human platelets) and TGFα (synthetic rat) were purchased from ICN Biochemicals, Cleveland, OH. [$^{125}$I]EGF (100 μCi/μg) was from Amersham Buchler, Braunschweig, Germany. [$^{35}$S]Sulfate (364.4–505.4 mCi/mmol) was from New England Nuclear, Boston, MA. [6-$^{3}$H]Thymidine (15 Ci/mmol) was from New England Nuclear-DuPont (Dreieich, Germany). [$^{125}$I]TGFβ1 (1 μCi/ng) was from Paesel & Lorei, Frankfurt, Germany. Nycodenz (analytical grade) was from Nyegaard and Co. AS, Oslo, Norway. Calf thymus DNA (type I) and

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1. *Abbreviations used in this paper:* MFBcM, myofibroblastlike cell conditioned medium; MFBlC, myofibroblastlike cells (transformed perisinusoidal lipocytes); PG, proteoglycans; PL, perisinusoidal lipocytes; TGFα, transforming growth factor alpha; TGFβ1, transforming growth factor β1.

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pronase were from Sigma Chemical Co., Munich, Germany. Protein A-purified neutralizing antibodies against porcine and human TGFβ1 were supplied by R&D Systems, Minneapolis, MN. [$^{32}$P]dCTP (3,000 Ci/mmol), [$^{32}$P]ddATP (5,000 Ci/mmol) and the Hybond N blotting membrane were from Amersham/Buchler (Dreieich, Germany). The TGFα-cDNA probe (sp 65C17N3) was kindly provided by Dr. R. Derynck (Genentech, Inc., South San Francisco, CA), and the TGFβ1-cDNA probe (pTGFβ33) by Dr. P. Kondaiah, National Institutes of Health, Bethesda, MD (25). Antibodies against vimentin, desmin, and bromodeoxyuridine were from Dakopatts, Hamburg, Germany.

## *Methods*

### PL ISOLATION AND CULTURE

Detailed procedures of the isolation and culture of PL from rats have been published previously by this laboratory (10, 12, 14, 15, 26). The purity of the PL preparations attained > 80% (nonvital hepatocytes, Kupffer cells, and endothelial cells represented the remaining 20%) as assessed by light microscopy using the presence of perinuclear lipid droplets and the typical cell shape as a marker. Viability assessed by trypan blue exclusion was > 80%. With the first (~ 8 h after seeding) and second (~ 20 h after seeding) medium change most of the contaminating cells (nonvital hepatocytes and endothelial cells) were removed, and the PL monolayers were essentially (> 97%) free of impurities. With the third medium change (~ 44 h after seeding) FCS in DME was reduced to 0.5 or 2% and aliquots of the conditioned media were added. Control and treated cultures received the same amount of FCS. 24 h after the first stimulation media were added again during a further medium change whereby cultures were labeled with radionuclide.

### CULTURE OF MYOFIBROBLASTLIKE CELLS

Confluent PL in primary culture (6–7 d after seeding) were trypsinized in the presence of EDTA (0.05% wt/wt trypsin, 0.2% wt/wt EDTA) and seeded in DME containing 10% FCS.

### INDIRECT IMMUNOPEROXIDASE (IMMUNOFLUORESCENCE) STAINING

Cultures were washed twice with ice-cold PBS-Dulbecco, then fixed using alcohol/acetic acid 95:5 (vol/vol). After three washes with PBS-Dulbecco unspecific binding was blocked with 1:2 diluted calf serum (30 min, 22°C). The cells were next reacted with anti-iso-α smooth muscle actin (1:50) in PBS containing 1% BSA for 1 h at 22°C on a rotating platform. After three washes in PBS cells were reacted with peroxidase-conjugated anti-mouse IgG (1:50) in PBS containing 1% BSA or alternatively with fluorescein isothiocyanate-conjugated anti-mouse IgG (1:50) in PBS containing 1% BSA. After three washes in PBS the FITC treated cells were viewed under a microscope with an epifluorescence attachment. Cells treated with peroxidase-conjugated anti-mouse IgG were stained using 3,3'-diaminobenzidine tetrahydrochloride as substrate and hydrogen peroxide 0.001%, nickel chloride, and cobalt chloride in distilled water as intensifier. Thereafter, cells were washed with distilled water and then viewed under a microscope equipped with an optical grid and phase contrast. In cultures serving as negative control the first antibody was omitted.

### PRODUCTION OF CONDITIONED MEDIA

After washing the near confluent monolayers with DME, fresh DME was conditioned for 24 h in the absence of FCS by subconfluent-confluent PL in primary culture (3, 6, and 10 d after seeding) and MFBlC (secondary culture after 1–3 passages). Conditioned media were removed sterilely, centrifuged (450 *g*, 7 min, 4°C) to remove cell debris and finally clarified by passing through a 0.45-μm filter (Millex-GS; Millipore Corp., Bedford, MA). These media were used for all subsequent studies.

One half of the appropriate medium was dialyzed for 48 h against 100 vol distilled water at 4°C in tubings with a molecular cut-off 3.5 kD, lyophilized, and resolved in DME or binding buffer. The other half of the medium was acidified (pH 2.0) with 5 M HCl for 30 min at 4°C and neutralized with 5 M NaOH prior dialysis. In some experiments the MFBcM was dialyzed against DME without lyophilization. Before the medium was added to the cultures it was sterilized by passing through a 0.22-μm pore size filter (Millex-GS; Millipore Corp.).

### DETERMINATION OF CELL PROLIFERATION

*DNA measurement.* DNA content of the cultures was measured fluorometrically using calf thymus DNA as a standard (27).

*[$^3$H]Thymidine incorporation.* Thymidine incorporation was measured as previously described (10, 12, 24) by TCA precipitation of cells that had been treated for 24 h with [$^3$H]thymidine (0.5 μCi/ml medium) beginning on the third day after seeding.

*Bromodeoxyuridine incorporation.* PL and MFBlC were seeded with a density of $10^4$ cells/cm$^2$ in DME with 10% FCS in six well plates. 24 h after seeding cells were washed twice with DME and incubated for 48 h in DME alone or with 0.5, 2, and 10% FCS and for 24 h with bromodeoxyuridine ($5 \times 10^{-5}$ M final concentration). At the end of the culture period monolayers were washed twice with ice-cold PBS (pH 7.4) and then fixed with ice-cold ethanol/acetic acid 95:5 (vol/vol). Thereafter, the cells were incubated for 1 h at 21°C with antibromodeoxyuridine immunoglobulins (dilution 1/20 with PBS) and desoxyribonuclease (5 IU/ml) for DNA denaturation. After washing three times with PBS (pH 7.4) cells were incubated with peroxidase-conjugated rabbit anti-mouse immunoglobulins (dilution 1/50) for 2 h at 21°C. Thereafter cells were washed again three times with PBS and nuclei which had taken up bromodeoxyuridine were stained with DAB as substrate and hydrogen peroxide 0.001%, nickel chloride, and cobalt chloride in water as intensifier. Cells were counted using an Olympus phase contrast microscope equiped with a 1-mm$^2$ optical grid.

*Cell numeration.* Cells were counted after trypsination in a standard hemocytometer or alternatively using an Olympus phase contrast microscope equipped with a 1-mm$^2$ optical grid. With the second procedure cells were counted after fixation and indirect immunoperoxidase staining.

### DETERMINATION OF THE SYNTHESIS OF TOTAL MEDIUM PROTEOGLYCANS

The synthesis of sulfated proteoglycans (PG) was determined by the incorporation of [$^{35}$S]sulfate (20 μCi/ml medium) during a labeling period of 24 h (details described elsewhere [12, 14, 20, 26]). Proteoglycan synthesis was expressed as radioactivity on the basis of DNA.

### NEUTRALIZATION OF TGFβ ACTIVITY

To bind and neutralize active TGFβ1 anti-TGFβ IgG (2.5–50 μg/ml medium) was added to transiently acidified MFBcM and incubated for 1 h at 21°C on a rotating platform. In a control experiment anti-TGFβ IgG was incubated with pure TGFβ1.

### MOLECULAR HYBRIDIZATION AND BLOTTING TECHNIQUES

Total RNA from PL and MFBlC was isolated as described (28). For slot blot analysis, 20 μg RNA was blotted on Hybond N membranes using a Millipore slot blot chamber. The measurement of ribosomal S6 gene expression was used as internal standard. The 0.8-kb probe for TGFβ1 was obtained by Sac I-Pvu II digestion of the pTGFβ33 plasmid, subsequent separation by gel electrophoresis, elution of the 0.8-kb fragment and labeling with ($^{32}$P)-dCTP. For hybridization of TGFα the 1.4 kb Eco RI fragment of sp 65C17N3 was used and labeled as described above. Hybridization was carried out in a buffer containing 50% formamide and Denhardts solution at 42°C. Because TGFα-cDNA was a human probe, washing conditions were low stringent. The blots were exposed for 4–6 d at −80°C to Hyperfilm MP (Amersham Buchler, Braunschweig, Germany) using an intensifying screen.

### COMPETITIVE RADIOLIGAND ASSAY TO QUANTITATE EGF/TGFα and TGFβ

Subconfluent MFBlC in six-well culture plates and skin fibroblasts in six-well culture plates were washed with binding buffer (128 mmol NaCl, 5 mmol KCl, 1.2 mmol $CaCl_2$, 1.2 mmol $MgSO_4$, 50 mmol Hepes, pH 7.5, and 5 mg/ml BSA) and incubated for ~ 30 min at 37°C

in binding buffer. Receptor binding was performed in 0.5-ml binding buffer per well. After equilibration for 10 min at 20°C appropriate concentrations of [$^{125}$I]TGF$\beta$1 or [$^{125}$I]EGF, and either unlabeled peptide or dialyzed 10-fold concentrated conditioned media, were added. Incubations proceeded for 3 h at 20°C on a shaking platform and were terminated by aspiration of the binding buffer and washing the monolayers three times with ice-cold binding buffer.

Bound radioactivity was liberated with 1% Triton X-100 in PBS and measured in a gamma-counter. Nonspecific binding was determined at a 200-fold excess of cold ligand and substracted from total binding. Nonspecific binding of [$^{125}$I]TGF$\beta$1 never exceeded 10%, of [$^{125}$I]EGF 5% of total binding. All binding experiments were done in duplicate and repeated at least three times.

CELLULAR RETINYL-PALMITATE CONTENT

All the following steps were performed in the dark. PL cultures in six-well culture plates were washed twice in the dark with PBS. After washing, the retinoids were extracted with methanol/ethylacetate 1:1 (vol/vol) (1 ml per culture well, 2 h at 4°C). After centrifugation (10 min at 600 *g*) the transparent extraction fluid was used for HPLC. Retinyl-palmitate was determined in 100-$\mu$l aliquots by reversed phase HPLC using a RPC18 analytical column (Waters Associates, Milford, MA), developed at 1.3 ml/min for 30 min with methanol/ethylacetate/water 510:140:25 (vol/vol/vol) and fluorescence detection (Ex. 338 nm, Em. 425 nm). The retinyl-palmitate concentration was determined by comparing the peak area of the cell extracts with external standards of retinyl-palmitate in methanol/ethylacetate.

STATISTICAL ANALYSIS

Data are presented as mean±SD. Differences between groups were tested using variance analysis (Scheffé multiple range test). An $\alpha$-level of $P < 0.05$ was considered statistically significant.

## Results

*Characterization of the PL in culture during transformation to MFBlC.* The PL which were isolated from rat liver and seeded on uncoated culture wells had a purity of > 97% after the second medium change. The purity had been assessed by light-, fluorescence-, and electron microscopy, respectively. The relative amount of Kupffer cells which was assessed additionally by staining for endogenous peroxidase and by phagocytosis of latex beads was < 1% and decreased further during culture. Within the first 3 d after seeding the cells (untransformed PL) appeared homogenous and showed a characteristic morphology with lipid droplets surrounding the nucleus (Fig. 1 *a*). During the next several days the cells transdifferentiated spontaneously into MFBlC. This transdifferentiation was associated with a cell spreading, the development of long cytoplasmic extensions, a loss of fat droplets, and the appearance of one or two nucleoli (Fig. 1 *b*). Furthermore the desmin and iso-$\alpha$ smooth muscle actin expression (Fig. 1 *c*) increased. Transmission electron microscopy indicated that the size of the fat droplets de-

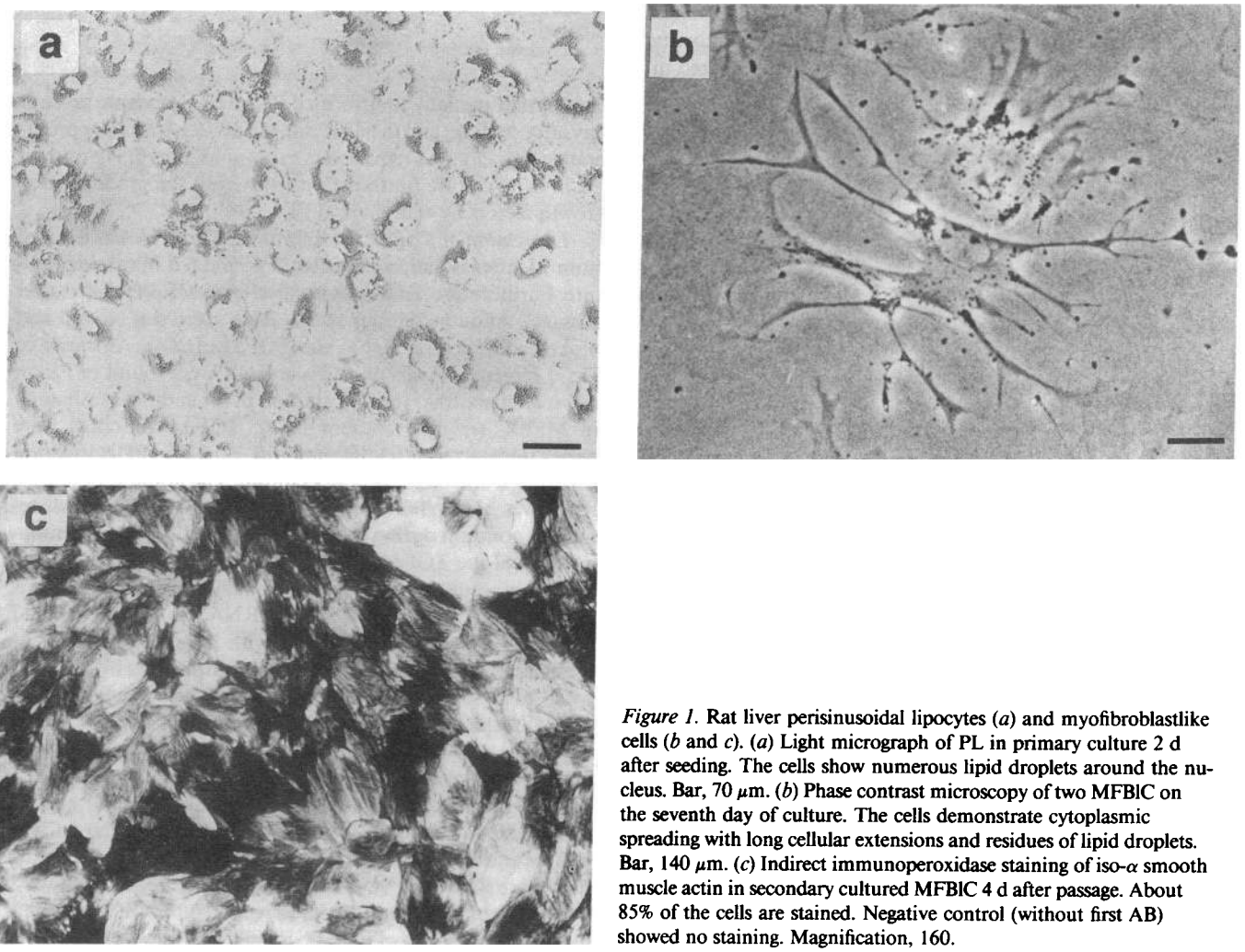


*Figure 1.* Rat liver perisinusoidal lipocytes (*a*) and myofibroblastlike cells (*b* and *c*). (*a*) Light micrograph of PL in primary culture 2 d after seeding. The cells show numerous lipid droplets around the nucleus. Bar, 70 $\mu$m. (*b*) Phase contrast microscopy of two MFBlC on the seventh day of culture. The cells demonstrate cytoplasmic spreading with long cellular extensions and residues of lipid droplets. Bar, 140 $\mu$m. (*c*) Indirect immunoperoxidase staining of iso-$\alpha$ smooth muscle actin in secondary cultured MFBlC 4 d after passage. About 85% of the cells are stained. Negative control (without first AB) showed no staining. Magnification, 160.

*Table I. Effect of FCS on PL and MFBlC Proliferation*

| | DME 0 FCS | DME with 0.5% FCS | DME with 2% FCS | DME with 10% FCS |
|---|---|---|---|---|
| PL (3 d) | | | | |
| (% BrdUrd +) | 0.1±0.1 | 2.0±1.0 | 13±4.8 | 46±6.2 |
| PL (7 d) | | | | |
| (% BrdUrd +) | 10±1.6 | 23±4.0 | 38±5.3 | 58±7.7 |
| MFBIC | | | | |
| (% BrdUrd +) | 21±4.6 | 29±8.7 | 44±7.6 | 60±15.0 |

Rat liver PL and MFBIC were seeded in DME with 10% FCS (density, $10^4$ cells/cm$^2$) on 6er plates. In PL (3 d) and MFBlC fetal calf serum was reduced at the first day after seeding to the indicated concentrations (0, 0.5, 2, and 10% respectively); in PL (7 d) fetal calf serum was reduced at the fifth day after seeding. 1 d after the reduction of the FCS serum cells were labeled without a further medium change for 24 h with $5*10^{-5}$ M bromodeoxyuridine (BrdUrd). Thereafter cultures were stopped by fixation using alcohol/acetic acid (95:5, vol/vol). Cell proliferation was measured by indirect immunoperoxidase staining of nuclei which had incorporated BrdUrd using BrdUrd-mAb and peroxidase-conjugated anti-mouse IgG (both diluted with PBS 1/50). Cells were counted using a phase contrast microscope equipped with an optical grid. 10 10-mm$^2$ squares/culture were counted. Data represent mean±SD of cells which had incorporated BrdUrd (% BrdUrd +).

creased during transformation and that the MFBlC developed both, a hypertrophy of rough endoplasmic reticulum and a hypodense dispersed nuclear chromatin as signs of highly active protein synthesis. Observations which are summarized in

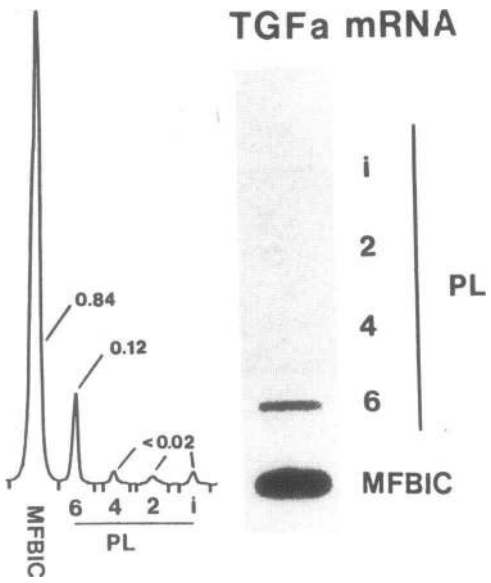


*Figure 2.* Expression of TGFα-mRNA in perisinusoidal lipocytes and myofibroblastlike cells. RNA was prepared from PL after isolation (*i*) and after 2, 4, and 6 d in primary culture and from MFBlC (6 d after passage). Aliquots (20 μg) of the isolated RNA were slot blotted and probed with a human TGFα-cDNA. The relative amount of TGFα-mRNA was quantitated using a laser densitometer (LKB 2202 Utroscan). The amount of blotted RNA was controlled by the mRNA of the S6-gene (see Fig. 3). Northern hybridization resulted in a single band with the size of 4.6–4.8 kb (data not shown). (*PL,* perisinusoidal lipocytes; *i,* RNA from PL after isolation; 2, 4, 6, RNA isolated from PL after 2, 4 and 6 d in primary culture; *MFBlC,* myofibroblastlike cells).

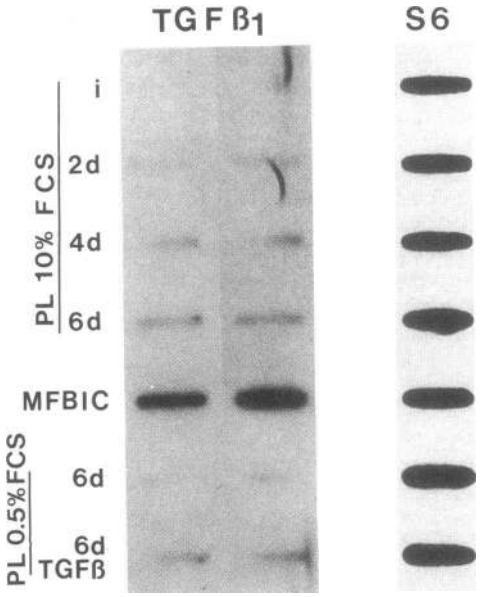


*Figure 3.* Expression of TGFβ1-mRNA in perisinusoidal lipocytes and myofibroblastlike cells. RNA was prepared from PL and MFBlC grown in the presence of 10% FCS (according to Fig. 2) or from PL grown for 6 d in the presence of 0.5% FCS with and without 1 ng/ml TGFβ1. The amount of blotted RNA (20 μg) was controlled by the mRNA of the S6-gene. Northern blot analyses resulted in a single band slightly below the 28 S ribosomal RNA (data not shown). (*PL,* perisinusoidal lipocyte; *MFBlC,* myofibroblastlike cell; *FCS,* fetal calf serum).

Table I that partially transformed PL (primary culture seventh day after seeding) and in particular MFBlC have a higher proliferative capacity and require less exogenous growth factor than PL in early culture (untransformed) suggest the production of growth factors by transformed PL.

*Expression of TGFα- and TGFβ-mRNA.* RNA was isolated from PL after isolation and after 2, 4, and 6 d in primary culture. Furthermore, RNA was isolated from MFBlC (3–6 d after passage). Aliquots (20 μg) of this RNA were slot blotted and probed with specific TGFα- and TGFβ1-cDNAs. As shown in Fig. 2 increased levels of TGFα-mRNA were found in PL on day 6 after seeding (relative amount 0.12) and predominantly in MFBlC (relative amount 0.84). TGFα transcripts were nearly undetectable (relative amount < 0.02) in PL at isolation and after 2 and 4 d in primary culture. The amount of blotted RNA was controlled by the RNA of the S6-gene which remained constant during transformation (Fig. 3). Fig. 3 demonstrates that the TGFβ1 transcripts were first detected in PL 2 days after seeding and increased gradually in primary culture. The highest expression of TGFβ1-mRNA was found in MFBlC (Fig. 3). If PL were treated for 6 d with 1 ng/ml TGFβ1 in the presence of 0.5% FCS the TGFβ1 transcripts were higher compared to unstimulated cells (Fig. 3). This result indicates that TGFβ1 induced its own mRNA expression in PL.

*TGFα and TGFβ quantification.* The TGFα and TGFβ1 protein concentration in media conditioned by PL and in MFBcM was determined by competitive radioligand binding assays and calculated by comparison of dilution curves with that of standard curves. Representative binding curves for TGFα are shown in Fig. 4, for TGFβ1 in Fig. 5. Competition of [$^{125}$I]EGF binding was achieved using rat TGFα (standard curve), competition of [$^{125}$I]TGFβ binding was achieved using

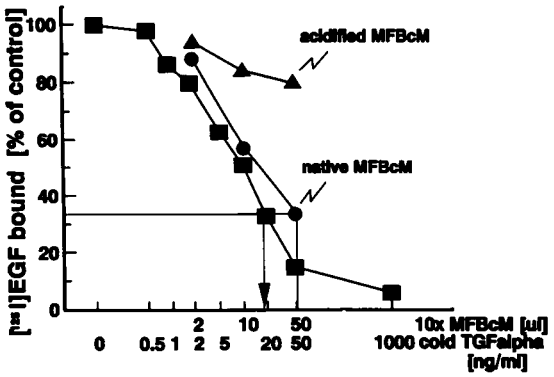


*Figure 4.* Measurement of TGF$\alpha$ protein concentration in MFBcM by a competitive radioligand binding assay. The media were conditioned for 24 h by MFBlC in the absence of FCS. Prior receptor binding aliquots of the media were acidified (pH 2) with 5 M HCl for 30 min and neutralized with 5 M NaOH prior dialysis against binding buffer. TGF$\alpha$ concentration was determined by competition of various volume equivalents of 10-fold concentrated MFBcM with [$^{125}$I]EGF binding to confluent skin fibroblasts (● — ● native MFBcM, ▲ — ▲ acidified MFBcM). Recombinant rat TGF$\alpha$ was used as cold ligand (■ — ■). The native MFBcM displayed a higher competition compared to the acidified MFBcM. In this representative recording competition of 50 $\mu$l 10-fold concentrated native MFBcM diluted with 450 $\mu$l binding buffer was equivalent 18 ng cold TGF$\alpha$. (*MFBcM*, medium conditioned by myofibroblastlike cells; *TGF$\alpha$*, transforming growth factor $\alpha$; *EGF*, epidermal growth factor).

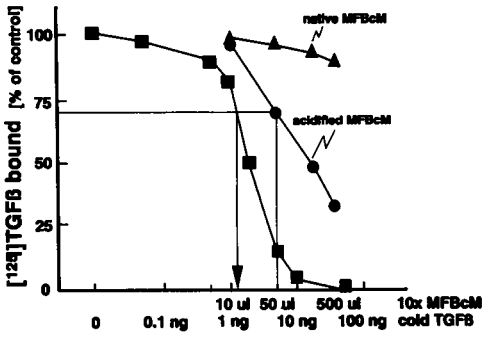


*Figure 5.* Measurement of TGF$\beta$ protein concentration in native and transiently acidified MFBcM by competitive radioligand binding assay. Media were conditioned and treated as described in Fig. 4. TGF$\beta$ concentration was determined by competition of 50 $\mu$l of 10-fold concentrated MFBcM with [$^{125}$I]TGF$\beta$1 binding to confluent skin fibroblasts (▲ — ▲ native MFBcM, ● — ● acidified MFBcM). Purified TGF$\beta$ from human platelets was used as standard (■ — ■). The acidified MFBcM displayed a higher competition compared to the native MFBcM. Interestingly, the slope of the acidified MFBcM was not exactly parallel to that of the standard, suggesting some nonspecific binding of [$^{125}$I]TGF$\beta$1 in the presence of high concentrations of MFBcM. For calculation (Table II) the competition of [$^{125}$I]TGF$\beta$1 binding by 50 $\mu$l 10-fold concentrated media was used. In this representative recording competition of 50 $\mu$l 10-fold concentrated aMFBcM diluted with 450 $\mu$l binding buffer was equivalent 1.2 ng cold TGF$\beta$1. (*MFBcM*, medium conditioned by myofibroblastlike cells; *TGF$\beta$1*, transforming growth factor $\beta$1).

human TGF$\beta$1. In previous experiments, incubation time and temperature were optimized (data not shown). Based on these data, binding was performed at 20°C for 3 h. Under these conditions, confluent monolayers of skin fibroblasts ($3 \times 10^4$ cells/cm$^2$) incubated with 20 fmol (0.46 ng) [$^{125}$I]TGF$\beta$/0.5 ml for 3 h at 20°C bind 4–6% of the input radioactivity.

By sequential 24-h medium collections from PL in primary culture during 10 d it was shown that TGF$\beta$ synthesis starts in parallel to transformation at day 5–6 after seeding, increased with culture time (transformation) and was highest in media conditioned by MFBlC (Table II). However, native MFBcM caused little [$^{125}$I]TGF$\beta$ competition, acidified MFBcM (dialyzed against binding buffer) resulted in high competition (Fig. 5). Based on the data of three different binding studies the native MFBcM was calculated to contain 0.15 ng/ml (5.7 pM) active TGF$\beta$1, whereas acidified MFBcM was contained 1.6±0.2 ng/ml (61±7.7 pM) active TGF$\beta$1. Furthermore we calculated that a single MFBlC produced ~ 0.2 fmol TGF$\beta$1 within 24 h. The fact that latent TGF$\beta$ does not bind to its receptor (data not shown) suggests that most of the TGF$\beta$ in the native MFBcM is in the inactive form. By transient acidification the latent form of TGF$\beta$1 was converted to the active (mature) form. In contrast to TGF$\beta$1 active TGF$\alpha$ was higher in native media. Native MFBcM was calculated to contain 22.5±6.6 ng/ml (4±1.2 nM) TGF$\alpha$ ($n$ = 3) and a single MFBlC was calculated to produce ~ 15 fmol TGF$\alpha$/24 h. In media conditioned by PL TGF$\alpha$ was not detected (Table II).

*Effect of conditioned media on PL proliferation.* Media conditioned by PL between the second and fifth day after seeding or by MFBlC after the first to third passage were tested on PL for their mitogenic activity. PL conditioned medium (PLcM) neither stimulated nor suppressed PL growth measured by DNA, cell count, and [$^3$H]thymidine incorporation (data not shown). In contrast MFBcM stimulated PL proliferation (Fig. 6). 10% of the MFBcM (0.02 ml 10-fold concentrated MFBcM added to 1.98 ml DME with 0.5% FCS) stimulated [$^3$H]-thymidine incorporation in primary cultured PL to 2.03-fold of control (Fig. 6) and DNA content to 1.60-fold of control. However, a higher dose of MFBcM inhibited PL proliferation ([$^3$H]thymidine incorporation at 30% MFBcM 1.3-fold and at

*Table II. TGF$\alpha$ and TGF$\beta$1 Production Rate*

| | PL 3rd d | PL 6th d | TC 10th d | MFBLC secondary culture |
|---|---|---|---|---|
| TGF$\alpha$ (fmol/cell/d) | n.d. | n.d. | 8 | 15 |
| TGF$\beta$1 (fmol/cell/d) | n.d. | 0.02 | 0.13 | 0.20 |

Media were conditioned for 24 h in the absence of FCS by rat liver PL (third and sixth day after seeding), partially transformed PL (TC: 10th day after seeding) and MFBLC (secondary culture, completely transformed). After removing the conditioned media cells were trypsinized and counted in a standard hemocytometer. TGF$\alpha$ was measured in native, TFG$\beta$1 in transiently acdified media by competitive radioligand binding assay (see Methods). In native media conditioned by PL 3 and 6 d after seeding TGF$\beta$1 was not detectable. In native media conditioned by TC and MFBLC TGF$\beta$1 was about one-tenth of the concentration obtained with transiently acidified media. Data points represent the mean TFG$\alpha$ and TFG$\beta$1 production rate per cell, which was calculated on the basis of TGFs concentration in conditioned media and cell count (at least two different cultures). (PL, perisinusoidal lipocyte; TC, transitional cell; MFBLC, myofibroblast-like cell; TGF$\alpha$, transforming growth factor $\alpha$; TFG$\beta$1, transforming growth factor $\beta$1; n.d., not detectable).

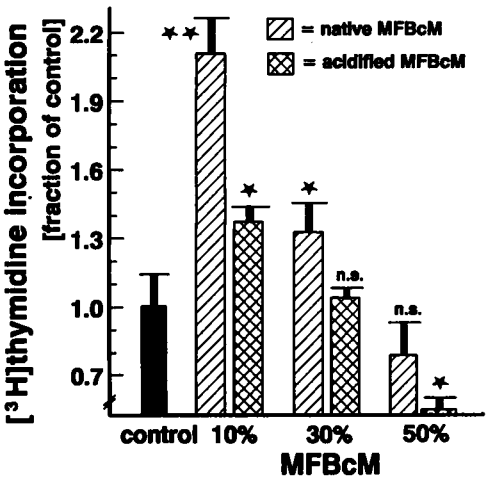


*Figure 6.* Effect of native and transiently acidified MFBcM on PL proliferation. Media were conditioned for 24 h by MFBlC in the absence of FCS. One-half of the MFBcM was acidified (pH 2) with 5 M HCl for 30 min and thereafter neutralized with 5 M NaOH to pH 7.4. Before conditioned media were added, both the acidified and native media were dialyzed against DME. Control media (DME with 2% FCS) were not dialyzed. Each culture (control and media stimulated) received DME with 2% FCS. The media were added to PL in primary culture in the appropriate concentrations (10, 30, and 50%) on the second and third day after seeding. Cells were labeled with 0.5 $\mu$Ci [$^3$H]thymidine/ml medium for 24 h between the third and fourth culture day. Mean values + SD of three independent experiments, each with $n = 4$. Statistically significant differences between control and treated cultures are indicated by stars (*$P < 0.05$; **$P < 0.01$; *MFBcM,* medium conditioned by myofibroblastlike cells).

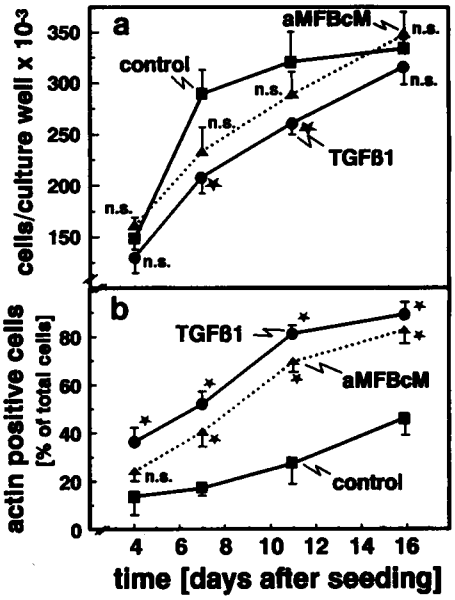


*Figure 7.* Effects of TGF$\beta$1 and transiently acidified medium conditioned by myofibroblastlike cells on perisinusoidal lipocyte growth (cell numeration) and expression of iso-$\alpha$ smooth muscle actin. Seeding density of PL was $0.08 \times 10^5$ cells/cm$^2$. The control as well as the treated cells were seeded and cultured in DME with 10% FCS. Beginning on the second day after seeding one-third of the cultures received 1 ng/ml TGF$\beta$1 (*circles*), one-third received 40 $\mu$l transiently acidified 10-fold concentrated MFBcM (final concentration of MFBcM 40%) (*triangles*) and one-third received only 10% FCS (*squares*). On day 4, 7, 11, and 16 after seeding part of the cultures were stopped by fixation using alcohol/acetic acid 95/5 (vol/vol); the remainder was stimulated again with TGF$\beta$1 or aMFBcM. After immunoperoxidase staining for iso-$\alpha$ smooth muscle actin each culture well was examined at least 10 times in different squares of 1 mm$^2$ each. (*a*) PL growth was determined by cell numeration using an Olympus phase contrast microscope equiped with a 1-mm$^2$ optic grid. All cells visible by phase contrast microscopy (positive and negative stained) were counted. The data points represent the average of counts on triplicate cultures (mean±SD) and the results were expressed as the number of cells/culture well. (*b*) Transformation was assessed by expression of iso-$\alpha$ smooth muscle actin. After fixation the cells were stained using anti-iso-$\alpha$ smooth muscle actin, peroxidase conjugated anti-mouse IgG and DAB. The data points represent the average of iso-$\alpha$ smooth muscle actin positive cells as a percentage of the total cell number (mean±SD). (*aMFBcM,* transiently acidified medium conditioned by myofibroblast-like cells; *TGF$\beta$1,* transforming growth factor $\beta$1; *n.s.,* not significant; *$P < 0.05$ significant difference between control and treated cultures).

50% MFBcM 0.73-fold of control) suggesting that, beside mitogens, potent growth inhibitors also must be present in MFBcM. Transient acidification of the MFBcM resulted in a depressed [$^3$H]thymidine incorporation (10% MFBcM, 2.03-fold vs. 1.4-fold; 50% MFBcM, 0.8-fold vs. 0.5-fold).

We have shown previously that TGF$\beta$1 acts as a potent growth inhibitor of the EGF/TGF$\alpha$ stimulated PL proliferation (24). Interestingly, the dose response curve of native MFBcM is parallel to that of pure TGF$\beta$1 in the presence of 20-ng/ml EGF or TGF$\alpha$.

*Effect of conditioned media on PL transformation.* Light and phase contrast microscopy of PL treated with acidified MFBcM suggested a stimulated PL transformation (flat cell shape, faster reduction of the number, and the size of fat droplets). As the expression of the cytoskeletal protein iso-$\alpha$ smooth muscle actin, and the reduction of cellular retinyl-palmitate in PL are the best indicators for objective measurement of transformation we determined these two parameters at different time intervals after seeding. As shown in Fig. 7 *b* 20% acidified MFBcM in DME with 10% FCS stimulated the expression of iso-$\alpha$ smooth muscle actin. Compared to the control, the difference was most pronounced 11 d after seeding. At that time interval, 70% of the PL treated with acidified MFBcM were iso-$\alpha$ smooth muscle actin positive; in the control cultures only 27% of the cells were iso-$\alpha$ smooth muscle actin positive. The total cell number was not significantly effected by 20% acidified MFBcM (Fig. 7 *a*). Furthermore, as demonstrated in Fig. 8, transiently acidified MFBcM added to PL in primary culture stimulated the loss of cellular retinyl-palmitate. The difference between the cultures treated with a MFBcM and the control was significant after 6 d in culture.

*Effect of conditioned media on fat storing cell PG synthesis.* To detect potential fibrogenic mediators stimulating PG synthesis in conditioned media these media were added in appropriate concentrations (10, 30, 50%) to PL in primary culture grown in DME with reduced (0.5%) FCS. Native and transiently acidified PLcM did not influence PG synthesis, whereas MFBcM stimulated PG synthesis measured as [$^{35}$S]sulfate incorporation based on DNA (Fig. 9). Maximal stimulation of PG synthesis per single cell was seen with native MFBcM at dilution 1/2 (1.45-fold of control), and with acidified MFBcM at dilution 1/10 (2.2-fold of control), respectively (Fig. 9).

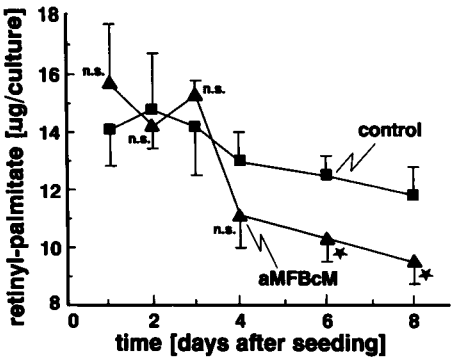


*Figure 8.* Effect of transiently acidified MFBcM on PL transformation (loss of retinyl-palmitate). The control as well as the treated cells were seeded and cultured in DME with 10% FCS. Beginning on the first day after seeding one-half of the cultures received transiently acidified MFBcM (final concentration of MFBcM, 40%) (*triangles*) and the other half received only 10% FCS (*squares*). On day 1, 2, 3, 4, 6, and 8 after seeding part of the cultures were stopped by extraction of retinoids with methanol/ethylacetate. Retinyl-palmitate was determined by HPLC using fluorescence detection. (*aMFBcM*, transiently acidified medium conditioned for 24 h by myofibroblastlike cells; *n.s.*, not significant; *significant difference ($P < 0.05$) between control and treated cultures).

*Identification of TGFs as fibrogenic mediators produced by MFBlC.* The activation of the growth inhibitory and PG synthesis stimulatory effect by transient acidification of the MFBcM (whereby TGFβ1 is activated) suggested that TGFβ1 is responsible for these effects. To verify this hypothesis MFBcM were preincubated with TGFβ neutralizing antibodies before addition to PL. To demonstrate the neutralizing potency of anti-TGFβ IgG 2 ng/ml pure human TGFβ1 was incubated for 1 h at 22°C with increasing doses of anti-TGFβ (2.5–50 µg/ml) before adding to PL monolayers. The growth inhibitory activity and PG synthesis stimulating activity of these 2 ng/ml TGFβ1 could be neutralized completely by 12.5 µg/ml anti-TGFβ (data not shown). As outlined in Fig. 10 *a* the PG synthesis stimulating activity of 20 and 50% acidified MFBcM (2.1-fold and 1.6-fold of control) was neutralized by preincubation of the medium with 12.5 µg/ml anti-TGFβ1. Furthermore, the growth inhibitory activity of 40% transiently acidified MFBcM was abolished by preincubation with 2.5 µg/ml anti-TGFβ and even converted to a growth stimulation by higher concentrations of anti-TGFβ (Fig. 10 *b*). These results not only serve to further confirm the presence of TGFβ in MFBcM, but also provide evidence about the presence of one or more mitogens in MFBcM.

## Discussion

Using cell culture systems, it has previously been shown that Kupffer cells stimulate via soluble mediators rat liver PL proliferation (10, 11, 13) and extracellular matrix synthesis by these cells (13–15, 17). Similar results, i.e., an enhanced PL proliferation and PG synthesis were obtained using platelet lysate as stimulus (12). Concerning the nature of the macrophage- and platelet-derived factor(s) stimulating PL EGF/TGFα was shown to stimulate proliferation (24), whereas TGFβ1 inhibited PL proliferation (19, 24) but significantly stimulated PL

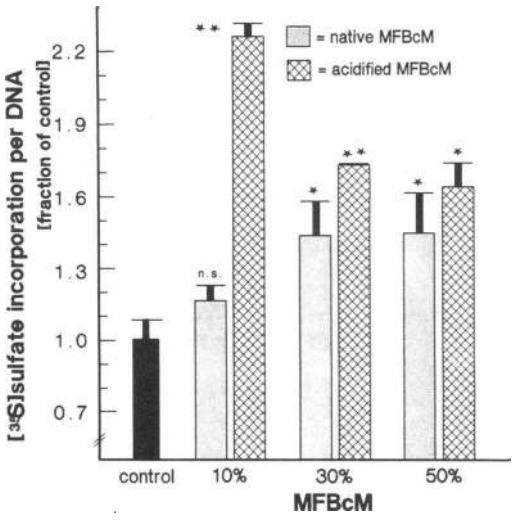


*Figure 9.* Effects of native and transiently acidified media conditioned by myofibroblastlike cells on perisinusoidal lipocyte proteoglycan synthesis. Conditioned media were treated as described in Fig. 6 and added to PL monolayers in the appropriate concentrations at the third and fourth day after seeding. Control and treated cultures received DME with 0.5% FCS. Between the fourth and fifth culture day cells were exposed for 24 h to 20 µCi [$^{35}$S]sulfate/ml medium. Proteoglycan synthesis was determined by liquid scintillation counting after binding of the proteoglycans to DEAE-sephacel. Values are expressed as mean±SD of three cultures on the basis of DNA. (*MFBcM*, medium conditioned for 24 h by myofibroblastlike cells; *n.s.*, not significant; *$P < 0.05$, **$P < 0.01$, significant difference between control and treated cultures).

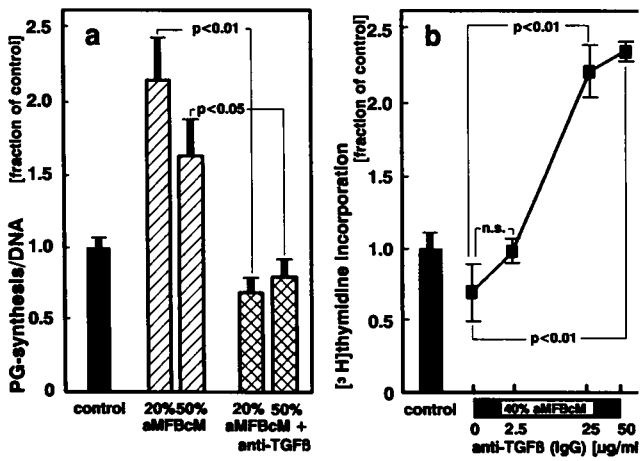


*Figure 10.* Effect of neutralizing anti-TGFβ1 IgG on MFBlC induced stimulation of PG synthesis (*a*) and inhibition of PL proliferation (*b*). Media were conditioned and acidified as described in Fig. 6. Anti-TGFβ IgG (2.5, 12.5, 25, and 50 µg/ml) was added to the media and incubated for 1 h at 21°C on a rotating platform. Thereafter, these media were added to PL monolayers in the appropriate concentrations at the third and fourth day after seeding. (*a*) The effect 12.5 µg ml anti-TGFβ1 on stimulated PG synthesis is shown. Control and treated cultures received DME with 0.5% FCS. Cells were exposed for 24 h to 20 µCi [$^{35}$S]sulfate/ml medium and proteoglycans were measured as described in Fig. 9. Mean values + SD of a representativ experiment with $n = 4$. (*b*) Control and treated cultures received DMI with 2% FCS. Cells were labeled with 0.5 µCi [$^{3}$H]thymidine/ml medium for 24 h between the fourth and fifth culture day. Mean values + SD of a representative experiment with $n = 4$.

collagen- (16, 19) and PG synthesis (20). In combination, both TGFs seem to be potent fibrogenic mediators enhancing predominantly chondroitin sulfate and hyaluronan synthesis by PL (7, 20). Very recently, TGFβ was identified as the prominent mediator elaborated by Kupffer cells stimulating PL collagen (17) and PG synthesis (18). Conclusions drawn from these results suggest that in liver injury infiltrating platelets and activated Kupffer cells elaborate transforming growth factors, acting as fibrogenic mediators initiating in a paracrine way not only PL proliferation and transformation but also the synthesis of PG, hyaluronan, and collagen (for review see references 7 and 9).

If PL are cultured on uncoated plastic materials they lose their normal phenotype (4) and transform within 1–2 wk to highly active MFBlC showing reduced number and size of fat droplets; hypertrophy of rough ER; long cytoplasmic extensions; and pronounced positive staining for iso-α smooth muscle actin (6, 7, 29). The current study demonstrates that these transformed cells (MFBlC) have the capacity to produce soluble mediators stimulating thereby PL proliferation, transformation, and enhanced PG synthesis. Medium conditioned by MFBlC stimulated PL proliferation with little effect on PG synthesis. Transient acidification of this medium, whereby latent TGFβ is activated (30–32), inhibited PL proliferation but enhanced PG synthesis. These results suggest that TGFβ (mainly in the inactive form) together with mitogenic stimuli are present in MFBcM. By competitive radioligand binding assays, TGFβ was detectable in PLcM in increasing amounts beginning from day 6 after seeding and particularly in MFBcM (secondary culture). By a further experiment, evidence has been obtained for relevant TGFβ-like activity in MFBcM. The biological activity of the acidified MFBlC conditioned media (inhibition of PL proliferation and stimulation of PG synthesis) could be neutralized by TGFβ antibodies.

Because MFBlC produce the latent form of TGFβ, activation is required before receptor binding. In general, latent TGFβ may be activated by extreme, unphysiological pH (pH $< 2$ or $> 12$) (30–32), by several proteases (31), and by glycosidases (33). The nature and magnitude of the TGFβ mediated PL stimulation may be regulated by the TGFβ production rate; the balance of activation/inactivation; and the expression of TGFβ receptors on cell surface. Because essentially all cell types of the liver seem to have receptors for TGFβ the TGFβ activating mechanism is considered to be the major regulatory step. However, the mechanism of TGFβ activation in PL cultures and in injured liver remains to be established. Recently obtained results showing that alpha2-macroglobulin is secreted by PL in increasing amounts beginning with the fifth day after seeding (34) and data indicating that alpha2-macroglobulin may bind with and inactivate TGFβ (35, 36), suggest a potential role of alpha2-macroglobulin as a scavenger for active TGFβ at sites of liver injury. Interestingly, heparin, which is related in its structure to the PG heparan sulfate predominantly produced by parenchymal cells (37), may liberate active TGFβ from its complex with alpha2-macroglobulin (38). The pathobiochemical relevance of this mechanism is unclear. Furthermore, binding of TGFβ to fibronectin (39) and subsequent dissociation on acidic conditions at sites of tissue injury and inflammation may be relevant in the development of liver fibrogenesis.

The result that PL proliferation is stimulated by MFBcM preincubated with neutralizing anti-TGFβ IgG suggests the synthesis of one or more mitogens in addition to TGFβ by MFBlC. The radioligand binding assay indicates that significant amounts of EGF or TGFα must be present in MFBcM. Because the EGF cell surface receptor binds both growth factors with similar affinity (40), the radioligand binding assay is unable to differentiate between EGF and TGFα. However, the expression of TGFα-mRNA in MFBlC suggests that at least one factor with mitogenic activity is TGFα. Preliminary data suggest that insulinlike growth factor 1 (somatomedin C) may be a second candidate of the MFBlC-derived mitogenic activity (41, 42). Platelet-derived growth factor (PDGF-BB and PDGF-AB) a further potent mitogen for PL was not detected in cellfree supernatants of passaged PL (43).

In conclusion, our data together with previously reported results (7, 10–15, 17, 44) suggest that in culture on plain plastic and even more important in liver injury a cascade of stimulatory mechanisms act on PL. In early primary culture and in the early stages of tissue response to liver injury the release of TGFβ1 from Kupffer cells (17, 18) and platelets (12) results in an augmented transformation of PL to MFBlC. Thereafter, activated PL (MFBlC) itself produces fibrogenic mediators and thereby stimulates proliferation (TGFα), transformation (TGFβ) and proteoglycan synthesis (TGFβ) of untransformed PL. These findings also imply that autocrine stimulation of transformed PL (MFBlC) may occur in vivo via secretion of these cytokines. However, further in vivo and in vitro studies should confirm this hypothesis.

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