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Original Article

In vitro evidence for differential involvement of CTGF, TGFβ, and PDGF-BB in mesangial response to injury

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Abstract

Background. Connective tissue growth factor (CTGF) is a profibrotic growth factor, which is upregulated in wound healing and renal fibrosis, including anti-Thy-1.1 nephritis. The kinetics of CTGF mRNA expression in anti-Thy-1.1 nephritis suggested that CTGF regulation might contribute to glomerular response to injury downstream of transforming growth factor-β (TGFβ). In anti-Thy-1.1 nephritis the initial damage is followed by mesangial repair and limited sclerosis, which involves mesangial cell (MC) activation (α-smooth-muscle actin (αSMA) expression), proliferation, migration, and extracellular matrix production. The present *in vitro* study addresses the possible role of CTGF in these different aspects of mesangial response to injury, and how CTGF activity might relate to effects of TGFβ and platelet-derived growth factor-BB (PDGF-BB).

Methods and Results. Immunostaining and ELISA showed that αSMA expression and transformation of MC into myofibroblast-like cells was induced by TGFβ, but not affected by PDGF-BB, CTGF, or neutralizing anti-CTGF antibodies. [^{3}H]thymidine incorporation and Ki67 staining demonstrated that, unlike PDGF-BB, neither CTGF nor TGFβ induced the proliferation of MC. In contrast, both CTGF and TGFβ induced MC migration, as evidenced by approximation of wound edges in scrape-wounded, non-proliferating rat MC monolayers. In addition, fibronectin expression was upregulated by both CTGF and TGFβ, as measured by dot-blot analysis. Anti-CTGF completely blocked the effect of added CTGF. Moreover, anti-CTGF significantly reduced TGFβ-induced increase in fibronectin.

Conclusion. It thus appears that CTGF is specifically involved in a subset of the adaptive changes of MC involved in mesangial repair and sclerosis, which makes it an interesting candidate target for future intervention strategies.

Keywords: CTGF; extracellular matrix; mesangial; migration; TGFβ; wound healing

Introduction

Connective tissue growth factor (CTGF) is a 38-kDa cysteine-rich protein and is a member of the CCN (CTGF/Fisp 12, Cyr 61/CEF-10, Nov) immediate early gene family of proteins. CTGF was originally cloned from human umbilical vein endothelial cells (HUVEC) and identified by its platelet-derived growth factor-like (PDGF) activity for normal rat kidney (NRK) fibroblasts [1]. CTGF expression is upregulated by transforming growth factor β (TGFβ), but not by PDGF in NRK, human foreskin fibroblasts, NIH3T3 cells, glomerular visceral epithelial cells (GVEC) and mesangial cells (MC) [2–6]. We have previously shown that renal CTGF expression is highly upregulated in a subset of human kidney diseases and that it may be a common factor in the development of renal fibrosis, as a downstream mediator of TGFβ [3]. CTGF is thought to be essential for the mediation of some of the actions of TGFβ [4,5,7]. Full length CTGF (38 kDa) has been shown to induce mitogenic activity for NIH 3T3 cells, whereas the 10-kDa form is mitogenic for Balb/c 3T3 cells and vascular smooth-muscle cells [1]. Furthermore, CTGF is involved in

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regulation of endothelial cell migration [8], matrix production by human foreskin fibroblasts and mesangial cells *in vitro* and in granulation tissue formation [9–11].

In experimental mesangial proliferative glomerulonephritis, induced by a single injection with anti-Thy-1.1 antibody, complement-mediated lysis of the mesangium can be largely restored in 2 weeks by repopulation of the mesangial area by the activation, proliferation and migration of extraglomerular mesangial cells, as well as by remodulation of the mesangial matrix, including transient increase of extracellular matrix proteins [6,12]. Both PDGF and TGFβ have been implicated as essential factors in the pathogenesis of anti-Thy-1.1 nephritis. We have observed in this model, that CTGF mRNA is highly upregulated in MC and GVEC, and that this precedes the increase in expression of α smooth-muscle actin (αSMA) by periglomerular and mesangial cells, which is indicative of activation and transformation into myofibroblast-like cells. *In vitro*, all TGFβ isoforms are equally capable of inducing transient CTGF mRNA upregulation in rat mesangial cells, and more sustained upregulation of CTGF mRNA in GVEC [6]. It is not known what aspects of the mesangial response to glomerular injury involve CTGF, and how this would relate to the role of TGFβ and PDGF-BB in anti-Thy-1.1 nephritis and other processes of tissue repair [13–16].

To study these questions, we compared the effects of CTGF with those of TGFβ and PDGF-BB on rat mesangial cells in an *in vitro* model for wound healing. For this, a scrape-wounding assay was developed using mesangial cell monolayers, in which αSMA expression, cell proliferation, and cell migration could be addressed. Expression of fibronectin was assessed in conditioned medium of cultured mesangial cells after stimulation with CTGF, TGFβ, or PDGF-BB.

CTGF, TGFβ1 and PDGF-BB may each contribute to different aspects of the mesangial response to injury.

Subjects and methods

Growth factors and antibodies

Recombinant human (rh) CTGF, pre-immune chicken IgY (pCIgY13) and neutralizing chicken anti-CTGF antibody (pIgY13) were provided by FibroGen Inc. (South San Francisco, USA). rhCTGF was generated using a baculovirus expression system and purified by heparin–Sepharose affinity chromatography as described previously [9]. Peak fractions containing rhCTGF were determined by immunoblotting and Coomassie staining of SDS–polyacrylamide gels. Neutralizing anti-CTGF antibody was raised in chicken by immunization with purified baculovirus-derived full-length rhCTGF protein as previously described [4], and was subsequently affinity purified through a rhCTGF–Sepharose column. rhTGFβ1, rhPDGF-BB, and neutralizing rabbit pan-specific TGFβ antibody (panTGFβ) were purchased from R&D Systems (Abingdon, United Kingdom).

Culture of rat mesangial cells

An established rat mesangial cell line [17] was used and cultured in complete medium consisting of DMEM (Gibco BRL, Breda, The Netherlands), supplemented with 20% fetal calf serum (Gibco BRL), 5 µg/ml insulin (Sigma, Zwijndrecht, The Netherlands), penicillin (Gibco BRL) and streptomycin (Gibco BRL) in a humidified 37°C, 5% CO_2, 95% air incubator.

Western blot analysis

Rat mesangial cells were cultured for 24 h with and without TGFβ1 (5 ng/ml) in the absence of fetal calf serum (i.e. under serum-free conditions), and conditioned medium was harvested to which protease inhibitors (PMSF 1 mmol/l, aprotinin 2 µg/ml, leupeptin 5 µg/ml, pepstatin 0.7 µg/ml (Roche, Almere, The Netherlands)) were added. Heparin-binding proteins were extracted from conditioned medium with heparin–Sepharose CL-6B beads (Amersham Pharmacia, Roosendaal, The Netherlands) for 2 h at 4°C. Bound proteins were eluted by boiling in 2× SDS sample buffer and resolved on a 8% SDS–polyacrylamide gel and subsequently transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Membranes were blocked in PBS/0.05% Tween 20/5% BSA and incubated with pIgY13 or control chicken Ab (pCIgY13) at 0.5 µg/ml in blocking buffer, followed by HRPO-conjugated rabbit anti-chicken Ab (Zymed, South San Francisco, USA) in blocking buffer. Immobilized antibodies were detected with the enhanced chemiluminescence system (ECL, NEN Lifesciences, Zaventem, Belgium) according to the manufacturer's instructions and exposure to X-Omat blue XB-1 films (Kodak, NEN Lifesciences).

Scrape wounding assay

Cells (1.5×10^5/ml) were plated in 12-well plates (Costar, Badhoevedorp, The Netherlands), in complete medium. After overnight culturing, cells were kept in serum free media for 24 h and two wounds per well were made by scraping the surface of the dish with a plastic pipette tip. Subsequently, plates were washed with Dulbecco's PBS (Gibco BRL) and DPBS was replaced by fresh serum free medium, to which various factors were added. In each experiment, wound closure was measured at several time points in triplicate wells for each condition, at six marked sites per well (i.e. $3\times6=18$ observations per time point/condition/experiment), by inverse light microscopy with a grid reticule in the eyepiece of the microscope. Wound closure was quantified as percentage of the starting distance of the wound edges. All experiments were repeated three times, on separate days for each condition. Data are presented as the mean ± standard deviation of three independent experiments, performed on separate days. For statistical evaluation, a repeated measurement test followed by a Duncan's *post hoc* test was applied.

Cell proliferation assay

[^{3}H]Thymidine incorporation was measured to assess DNA synthesis in mesangial cells. Cells were plated in 24-well (Costar) tissue culture plates at a concentration of 2.5×10^4 cells/well. Cells were serum-starved for 24 h, followed by a 48h incubation with growth factors before addition of

[^{3}H]thymidine (5 μCi/well) during the last 2 h of the assay. Cells were washed with Dulbecco's PBS (Gibco BRL) and fixed in methanol, followed by solubilization with 0.2 N NaOH and quantification of [^{3}H]thymidine in a liquid scintillation counter. Experiments were performed in triplicate and data are expressed as means ± standard error. For statistical evaluation, a LSD test with Bonferroni correction was applied.

Immunocytochemistry

Scrape-wounded mesangial cell monolayers were fixed in methanol and washed in PBS/0.05% Tween 20, followed by incubation with a monoclonal anti-αSMA antibody (Sigma, clone 1A4, Zwijndrecht, The Netherlands) or a monoclonal anti-Ki67 antibody (Dako, Glostrup, Denmark). Immobilized mouse antibodies were detected by a two-step immunoperoxidase technique using HRPO-conjugated rabbit anti-mouse immunoglobulin (Dako), followed by HRPO-conjugated swine anti-rabbit immunoglobulin (Dako). Enzyme activity of HRPO was detected using 3,3′-diaminobenzidine (Sigma). Specificity was checked by omission of the primary antibodies and the use of isotype-matched non-immune IgG as negative controls.

αSMA ELISA

Rat mesangial cells were plated at 5000 cells/well in a 96-well tissue culture plate (Costar). Cells were serum-starved for 24 h after reaching approximately a 70% confluence level, after which growth factors were added in fresh serum-free medium in triplicate wells. After another 24 or 48 h of incubation, cells were fixed in methanol at 4°C and non-specific protein binding sites were blocked for 1 h by PBS/1% BSA. Cells were incubated with a monoclonal anti-αSMA antibody in PBS/1% BSA followed by a 2-step detection with HRPO-conjugated rabbit anti-mouse IgG (Dako) and HRPO-conjugated swine anti-rabbit IgG (Dako). Finally, *o*-phenylenediamine dihydrochloride (Sigma) was added as substrate for HRPO. Absorbance was measured at 490 nm in an ELISA Titertek reader. Experiments were repeated at least 3 times per condition and evaluated by a LSD test with Bonferroni correction.

Fibronectin dot-blot analysis

Rat mesangial cells were cultured and subsequently serum-starved for 24 h after reaching a 70% confluence level. Conditioned medium of cells exposed for 24 h to various factors in the absence or presence of neutralizing antibodies were harvested and protease inhibitors (PMSF 1 mmol/l, aprotinin 2 μg/ml, leupeptin 5 μg/ml, pepstatin 0.7 μg/ml (Roche, Almere, The Netherlands)) were added. Conditioned media were blotted in dilution series onto a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) with a Biorad dot-blot apparatus (Biorad, Utrecht, The Netherlands). Blots were blocked with PBS/0.05% Tween 20/5% non-fat milk and incubated with a polyclonal rabbit anti-fibronectin antibody (Dako, Glostrup, Denmark), followed by a HRPO-conjugated swine anti-rabbit antibody (Dako). Immobilized antibodies were detected with an enhanced chemiluminescence detection system (NEN Lifesciences), followed by exposure to a X-Omat blue film (Kodak, NEN Lifesciences). Specificity was checked by omission of the primary antibody and use of isotype-matched IgG as negative control. Experiments were repeated at least three times per condition and statistically evaluated by a LSD test with Bonferroni correction.

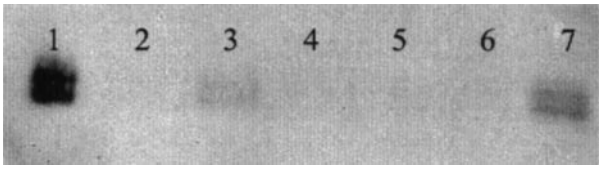

Fig. 1. Induction of CTGF protein expression by TGFβ1 in cultured rat mesangial cells. Cells were serum starved and stimulated with TGFβ1 (5 ng/ml) for 24 h. Heparin-binding proteins in the conditioned medium (CM) were precipitated using heparin–Sepharose (HS) CL 6B beads. Lane 1, rhCTGF (10 ng) was run as a standard; lane 2, control CM; lane 3, TGFβ1-stimulated CM; lane 4, supernatant of control HS-treated cultures; lane 5, supernatant of TGFβ1 HS-treated cultures; lane 6, SDS-eluted heparin-binding proteins from HS-treated control cultures; lane 7, SDS-eluted heparin binding proteins from HS-treated TGFβ1-stimulated cultures.

Results

TGFβ induction of CTGF protein in rat mesangial cells

Western blot analysis was performed on culture supernatants from mesangial cells that were serum-starved for 24 h and subsequently stimulated with TGFβ1 (5 ng/ml). As indicated in Figure 1, conditioned medium showed an increase in CTGF protein expression in TGFβ1-stimulated cultures compared to control cultures (lanes 2 and 3) of the same molecular weight (36–38 kDa) as the recombinant protein (lane 1). Supernatants of heparin–Sepharose treated conditioned medium did not show any reactivity with the anti-CTGF antibody (Ab) (lanes 4 and 5). In contrast, when heparin-binding proteins were resolved from heparin–Sepharose beads by boiling in SDS sample buffer, a strong CTGF protein signal was observed in TGFβ1-stimulated cultures (lanes 6 and 7).

Induction of morphological changes and αSMA expression by TGFβ1, but not by PDGF-BB or CTGF

Upregulation of αSMA *in vivo* is a marker for transformation of mesangial cells into myofibroblast-like cells [18]. In the anti-Thy-1.1 glomerulonephritis model, increased αSMA expression was observed after the elevation of CTGF mRNA [6]. Therefore we examined scrape-wounded cultures for changes in morphology and αSMA expression in the presence or absence of added growth factors. Phase-contrast microscopy revealed that scrape-wounded cultures exhibited a seemingly uniform monolayer of slightly elongated, flattened cells with multiple slender extensions. However, immunostaining with monoclonal anti-αSMA antibody revealed heterogeneity, with intense positive staining of a small minority of cells ($< 14\%$), which were scattered throughout the monolayer either as single cells or grouped in small clusters between the large majority of negative cells throughout the monolayer. These αSMA-positive cells appeared

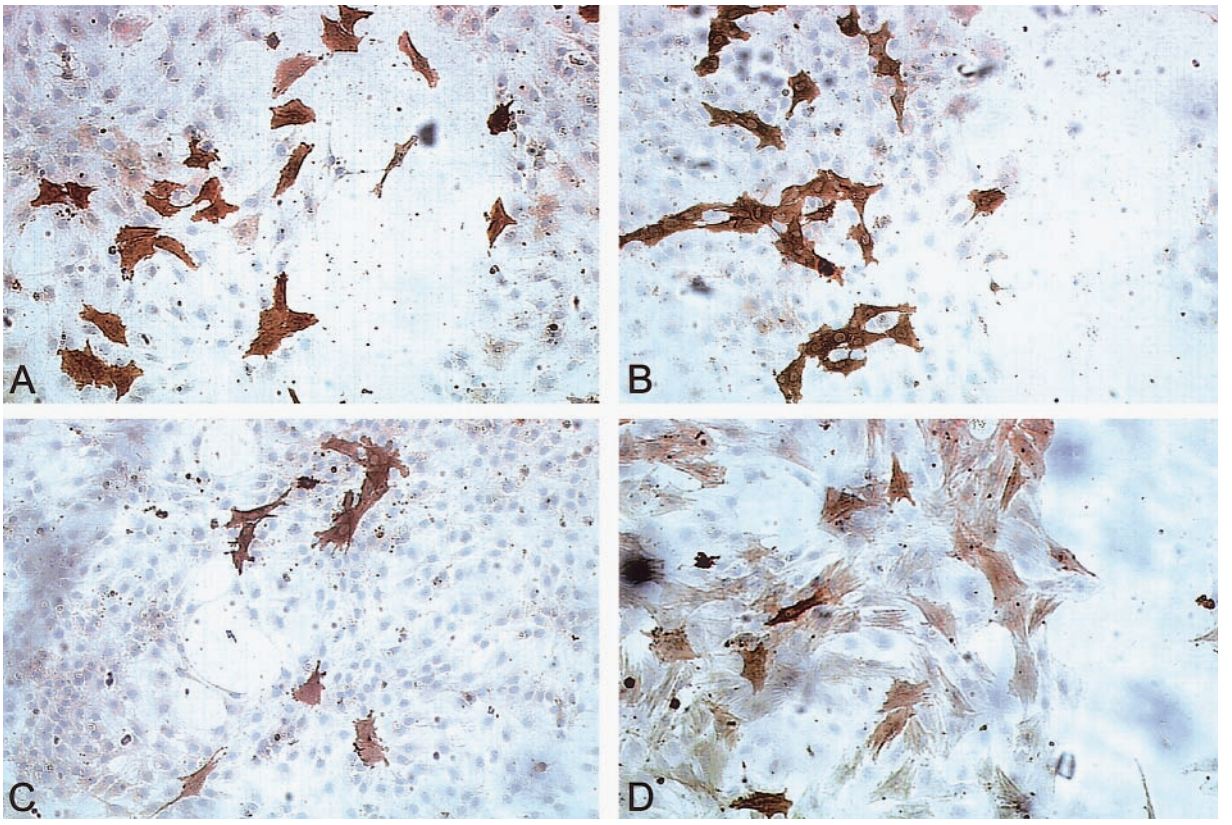

Fig. 2. Scraped rat mesangial cells were stained for αSMA. (**A**) Control, (**B**) rhCTGF (20 ng/ml) and (**C**) PDGF-BB (10 ng/ml) stimulated cultures showed positive staining of a minority of cells with a hypertrophic appearance scattered throughout the monolayer. (**D**) TGFβ1-stimulated cells (5 ng/ml) were more spindle-shaped and fibroblast-like and αSMA staining was positive throughout the monolayer.

hypertrophic and polygonal, and resembled myofibroblasts (Figure 2a). The morphology and the αSMA staining pattern of mesangial cells were not different in CTGF- or PDGF-BB-stimulated cultures, except that the latter stood out by the appearance of many mitotic figures (Figure 2b,c, 7). In contrast, cells in TGFβ1-stimulated cultures developed a uniformly spindle-shaped and fibroblast-like phenotype throughout the monolayer. Immunostaining of these cultures for αSMA was positive in over 95% of the cells throughout the monolayer (Figure 2d).

To measure αSMA expression, an ELISA was performed on methanol-fixed rat mesangial cell monolayers. As shown in Figure 3, 3.1- and 2.4-fold inductions of αSMA expression were observed after incubation with TGFβ1 for 24 and 48 h respectively. In contrast, αSMA expression in CTGF- and PDGF-BB-stimulated cultures did not significantly differ from control levels. αSMA expression induced by TGFβ1 was not influenced by addition of neutralizing anti-CTGF antibody, suggesting that TGFβ1-induced expression of αSMA was mediated by a CTGF-independent pathway.

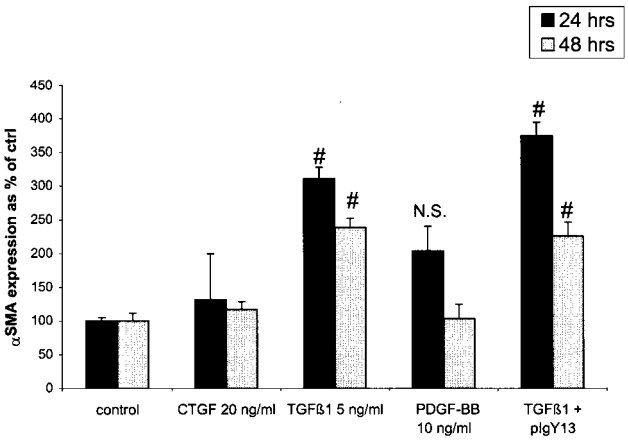

Fig. 3. αSMA expression was measured semi-quantitatively in an ELISA on mesangial cells, stimulated for 24 and 48 h with CTGF (20 ng/ml), TGFβ1 (5 ng/ml), PDGF-BB (10 ng/ml) or TGFβ1 (5 ng/ml) in the presence of anti-CTGF neutralizing antibody (pIgY13; 0.4 μg/ml). Significant increase in αSMA expression was observed only in TGFβ1-stimulated cultures ($P < 0.05$) after both 24 and 48 h of stimulation. Co-addition of CTGF neutralizing antibody to TGFβ1-stimulated cultures did not influence TGFβ1-induced αSMA expression ($P < 0.05$).

Induction of wound closure by CTGF

Wound closure in the absence of added growth factor occurred only very slowly, leaving a minimum of $88 \pm 15\%$ of the original width of the wound at 72 h after scraping. At 72 h after scraping, wound closure induced by rhCTGF at a concentration of 20 ng/ml was almost complete (Figure 4). rhCTGF enhanced

wound closure in a dose-dependent manner (Figure 5a). In the presence of neutralizing chicken anti-CTGF antibody (pIgY13) at 0.4 μg/ml no significant wound closure was measured as compared to controls. Addition of control chicken IgY (pCIgY13) (0.4 μg/ml) or neutralizing pan-specific TGFβ antibody (1 μg/ml) did not influence CTGF-induced wound healing (Figure 5b). Short term exposure (4 h) of scrape-wounded cells to rhCTGF did not result in significant wound closure (data not shown). So continuous presence of rhCTGF in the culture medium was required to induce wound healing of mesangial cell monolayers. Since previous studies have shown that heparin can significantly increase the proliferative response of NRK fibroblasts to CTGF, the influence of heparin on CTGF-induced wound closure was examined [9]. Heparin (10 μg/ml), added in the presence or absence of exogenous growth factors, did not alter the rate of wound closure induced by CTGF in our assays (data not shown).

Induction of wound closure by TGFβ1 and PDGF-BB

When PDGF-BB (10 ng/ml) or TGFβ1 (5 ng/ml) was added to cultures, significantly increased wound closure was measured after 48 and 72 h post scraping, leaving 24 ± 1.4 and $41 \pm 6.4\%$ respectively of the width of the wound. The kinetics of wound healing in TGFβ1-stimulated cultures were similar to those of CTGF-stimulated cultures. Addition of neutralizing anti-CTGF antibody to TGFβ1-stimulated cultures did not influence TGFβ1-induced wound healing, but neutralizing pan-specific TGFβ antibody completely blocked TGFβ1-induced wound closure (Figure 6a).

In PDGF-BB-stimulated cultures, a much quicker closure of the wounds was observed, as compared to cultures stimulated with CTGF or TGFβ1. Addition of neutralizing anti-CTGF antibody or neutralizing pan-specific TGFβ antibody did not influence wound closure of PDGF-BB-stimulated cultures (Figure 6b).

Induction of proliferation by PDGF-BB, but not by TGFβ1 or CTGF

To get an impression of the relative contribution of proliferation to wound closure, immunostaining was performed with a monoclonal anti-Ki67 antibody. Only the PDGF-BB-stimulated cultures, but not the CTGF- or TGFβ1-stimulated cells, displayed a high fraction of Ki67-positive cells. These were located mainly at the wound edges of the mesangial cell monolayer (Figure 7). In agreement with this, only PDGF-BB and not CTGF and TGFβ increased [^{3}H]thymidine incorporation (10 times that of control cultures, Figure 8). Addition of heparin (10 μg/ml)

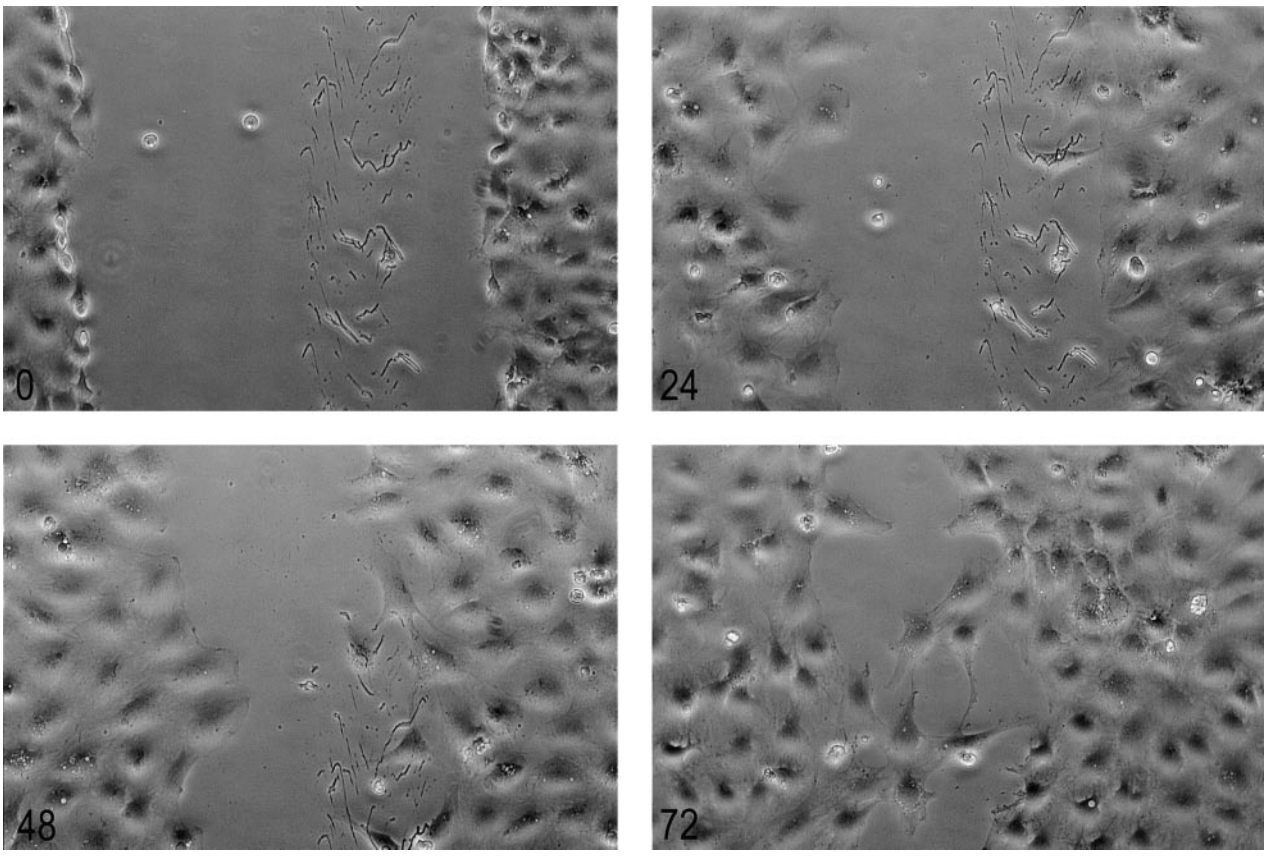

Fig. 4. Time-lapse photography of a rat mesangial cell monolayer, stimulated with rhCTGF (20 ng/ml), at 0, 24, 48 and 72 h after scraping. At 72 h after scraping $16 \pm 27.3\%$ of the original distance between the wound edges remained. Magnification $\times 200$.

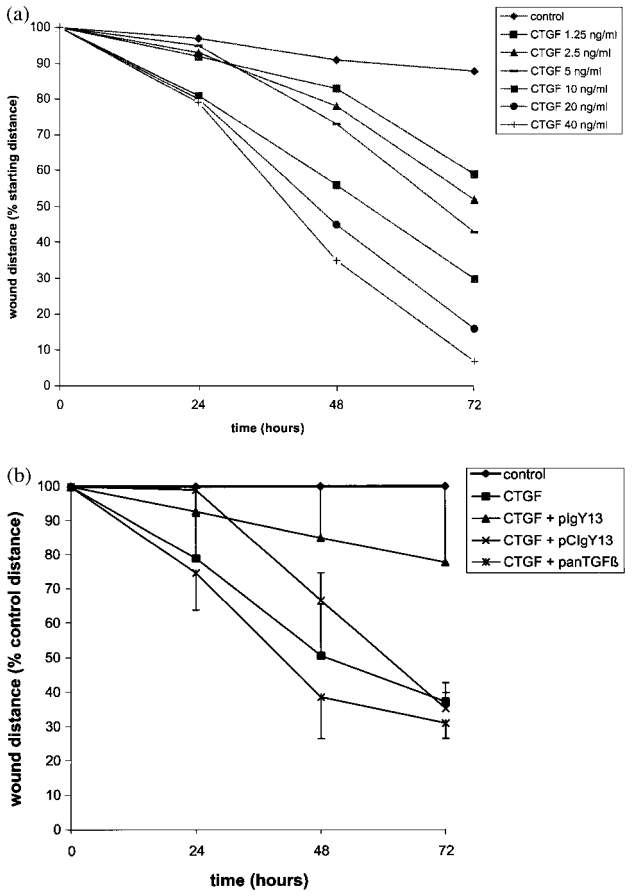

Fig. 5. (**a**) CTGF-induced wound closure of scraped rat mesangial cell monolayers in a dose-dependent manner. (**b**) CTGF-induced wound closure of scraped rat mesangial cell monolayers is not affected by neutralizing anti-TGFβ antibodies. Wound closure was measured as percentage of starting distance. For better comparison, data are presented after correction for 'spontaneous' wound closure as observed in control cultures. CTGF (20 ng/ml)-induced wound closure was completely blocked by addition of a chicken neutralizing anti-CTGF antibody (pIgY13; 0.4 μg/ml). Significant differences in wound closure were observed at 48 and 72 h after scraping ($P<0.05$; control *vs* CTGF; control *vs* CTGF + panTGFβ at 48 h. Control *vs* CTGF, control *vs* CTGF + pCIgY13, control *vs* CTGF + panTGFβ, CTGF *vs* CTGF + pIgY13, CTGF + pIgY13 *vs* CTGF + pCIgY13, CTGF + pIgY13 *vs* CTGF + panTGFβ at 72 h). Co-addition of control chicken IgY (pCIgY13; 0.4 μg/ml) did not affect CTGF- induced wound healing. Co-addition of a neutralizing pan-TGFβ antibody (1 μg/ml) did not influence CTGF-induced wound closure, suggesting that CTGF was capable of induction of wound healing in a TGFβ-independent manner.

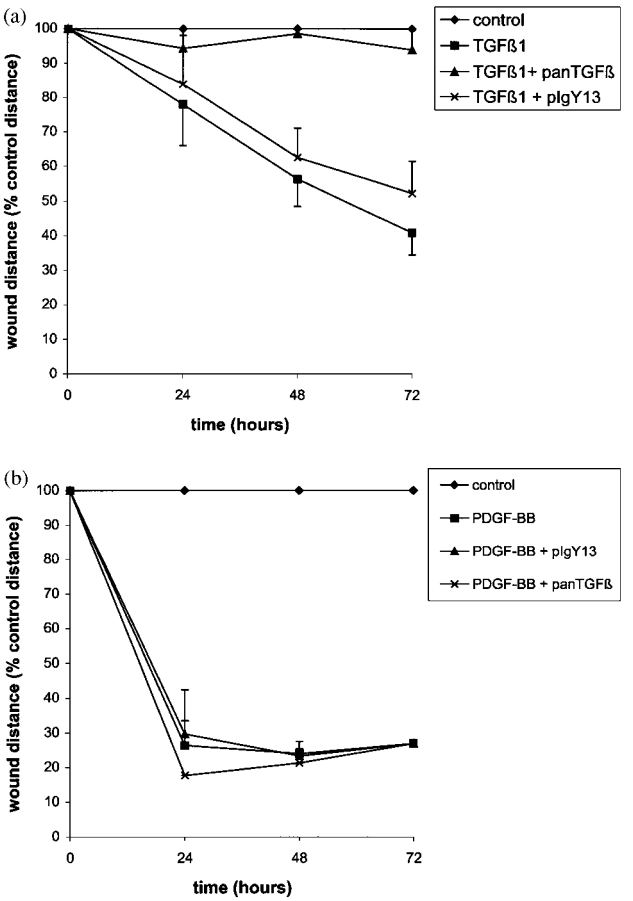

Fig. 6. (**a**) TGFβ-induced wound closure of scraped rat mesangial cell monolayers is independent of CTGF. Wound closure is presented as percentage of starting distance and corrected for background values of control cultures. TGFβ1 (5 ng/ml) induces significant wound closure of rat mesangial cell monolayers at 72 h after scraping ($P<0.05$; control *vs* TGFβ, control *vs* TGFβ + pIgY13, TGFβ *vs* TGFβ + panTGFβ). Effects of TGFβ1 on wound closure could be blocked completely by co-addition of neutralizing pan-specific TGFβ antibody (1 μg/ml) ($P<0.05$) but not by neutralizing anti-CTGF antibody (pIgY13; 0.4 μg/ml). (**b**) PDGF-BB-induced wound healing of scraped rat mesangial cell monolayers is independent of CTGF. Wound closure is presented as percentage of starting distance and corrected for background values of control cultures. PDGF-BB (10 ng/ml)-induced wound closure of mesangial cell monolayers is already significant 24 h after scraping ($P<0.05$; control *vs* PDGF-BB, control *vs* PDGF-BB + pIgY13, control *vs* PDGF-BB + panTGFβ). PDGF-BB effects were not affected by the addition of neutralizing anti-CTGF antibody (pIgY13; 0.4 μg/ml), nor by the addition of a neutralizing panTGFβ antibody (1 μg/ml).

in the presence or absence of added growth factors did not influence the proliferation rate of mesangial cells (data not shown).

Induction of fibronectin expression by CTGF and TGFβ

An important aspect of the mesangial response to injury is growth factor-induced expression of extracellular matrix proteins, which is essential for normal wound healing. A strong accumulation of fibronectin, among other matrix proteins, is typically seen in anti-Thy-1.1 nephritis. Therefore, we tested the ability of CTGF to influence fibronectin expression in mesangial cells and investigate the possible role of CTGF in the previously reported induction of fibronectin synthesis by TGFβ.

In conditioned medium of CTGF-stimulated cells a 4.3 ± 0.2-fold upregulation of fibronectin protein was measured compared to control cultures. This CTGF-induced fibronectin synthesis could be completely blocked by neutralizing anti-CTGF antibodies (pIgY13, 0.4 μg/ml) but not by pre-immune control chicken IgY (pCIgY13, 0.4 μg/ml). TGFβ-stimulated fibronectin expression was significantly reduced in the presence of neutralizing anti-CTGF antibodies (3.0 ± 0.5-fold, as compared to 7.8 ± 1.0-fold over

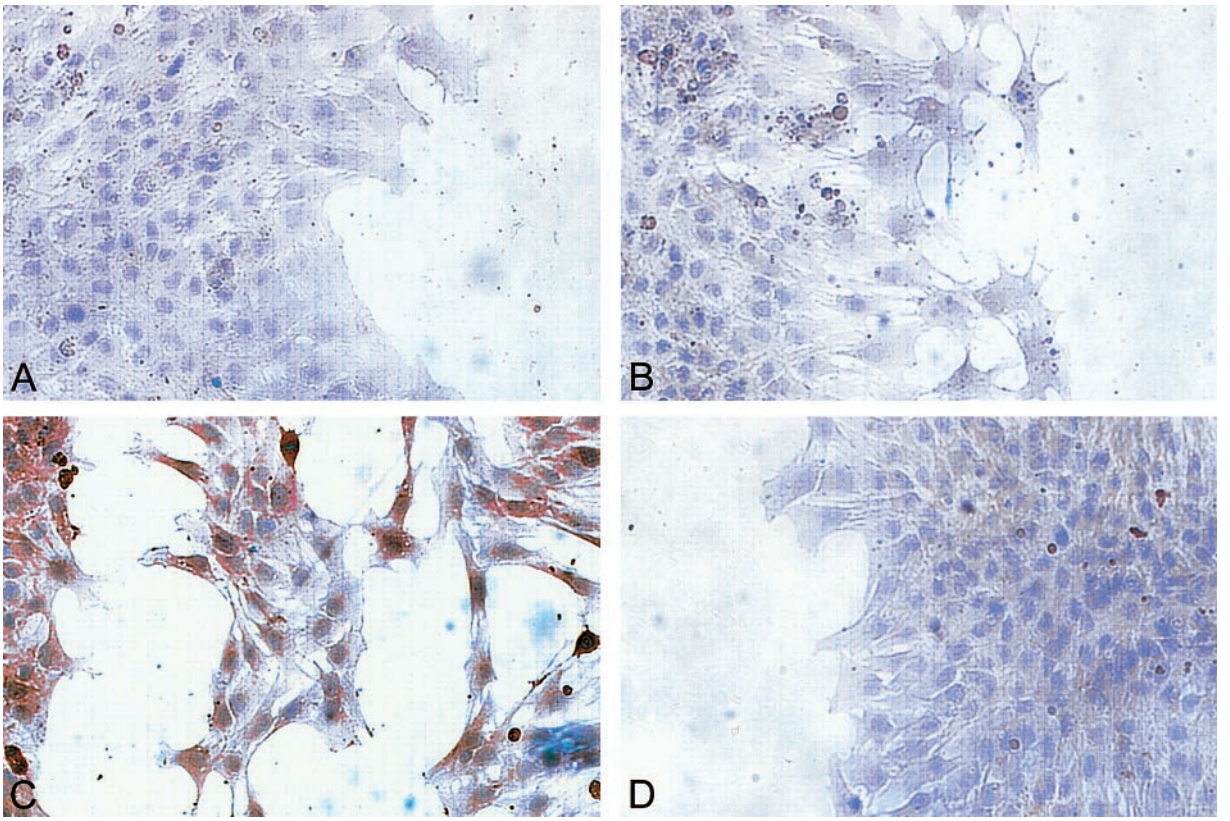

Fig. 7. (**A**) Control cells and cells exposed to (**B**) CTGF (20 ng/ml) or (**D**) TGFβ1 (5 ng/ml) did not express Ki67 antigen as judged by immunostaining. However, (**C**) a large fraction (about 58%) of mesangial cells stimulated with PDGF-BB (10 ng/ml) showed strong staining for Ki67.

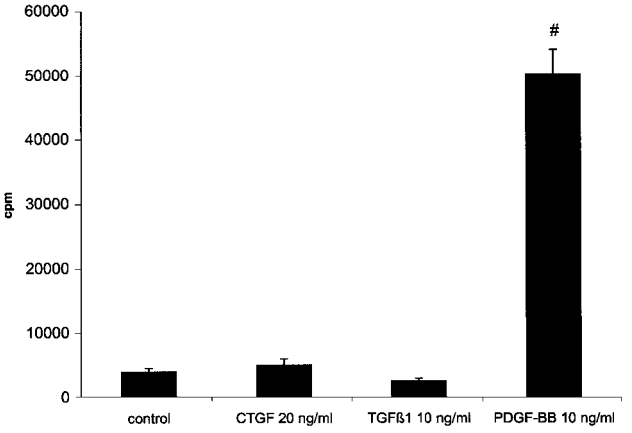

Fig. 8. No induction of [^{3}H]thymidine incorporation was observed in control, CTGF or TGFβ1-stimulated cultures. However, a 10-fold increase in proliferation was measured in PDGF-BB-stimulated cultures as compared to controls ($P < 0.05$).

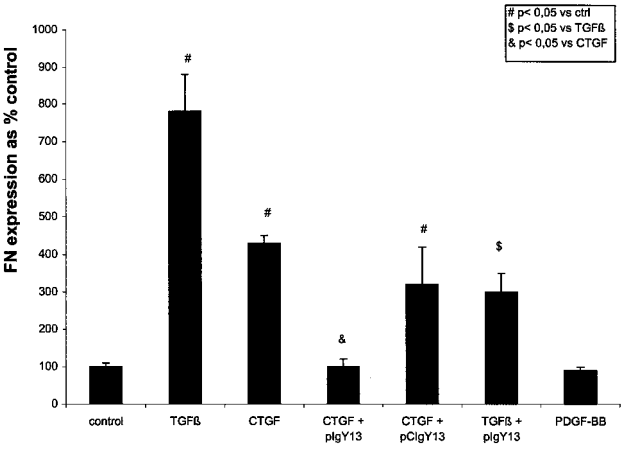

Fig. 9. Dot blot analysis of fibronectin expression in conditioned medium of rat mesangial cell cultures. Both rhCTGF (4.3 ± 0.2-fold 20 ng/ml) and TGFβ1 (7.8 ± 1-fold 5 ng/ml) induced expression of fibronectin. CTGF stimulated fibronectin expression was completely blocked by neutralizing anti-CTGF Ab (pIgY13, 0.4 µg/ml) but not by control IgY (pCIgY13, 0.4 µg/ml). TGFβ1 induced fibronectin expression was significantly blocked by anti-CTGF Ab. PDGF-BB (10 ng/ml) did not show induction of fibronectin protein expression.

baseline). PDGF-BB (0.9 ± 0.1) did not significantly change the basal fibronectin expression in this rat mesangial cell line (Figure 9).

Discussion

Tissue repair is a highly regulated process of obvious importance throughout life. To study tissue repair in response to glomerular injury, anti-Thy-1.1 nephritis has been used extensively as an *in vivo* model for mesangial proliferative glomerulonephritis.

Recently we observed early transient upregulation of CTGF mRNA in this model, which preceded the

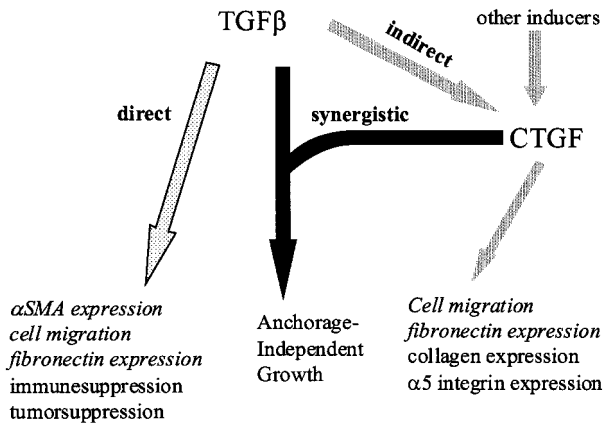

Fig. 10. Different pathways by which TGFβ mediates its action on cells, independent and dependent of CTGF. In *italic*: effects of TGFβ and CTGF observed in this study (modified from Kothapalli *et al.* 1997 [4]).

increase in αSMA [6]. As far as growth factor involvement in the anti-Thy-1.1 model is concerned, TGFβ and PDGF-BB have been implicated as key factors in the repair process. Effects of PDGF-BB include proliferation, chemotaxis, and extracellular matrix synthesis, and its expression is strongly correlated with the degree of glomerular injury in glomerulonephritis [13,15,16].

TGFβ1 is critically involved in wound healing and fibrosis, but also in tumour suppression and control of the immune response and inflammation [9,14,19,20]. Some of the effects of TGFβ1, such as anchorage-independent growth of NRK fibroblasts and collagen synthesis by these cells, have been demonstrated to be mediated *via* a CTGF-dependent pathway [4,5,7,11]. However, CTGF without active TGFβ was not able to induce anchorage-independent growth. The question remains concerning which other actions of TGFβ might be mediated *via* CTGF-dependent and -independent pathways (Figure 10).

Conversely, CTGF is known to have mitogenic effects on proliferation, migration, matrix production, angiogenesis, adherence and granulation tissue formation [1,4,5,8–10]. However, it has not been firmly established whether these actions of CTGF require TGFβ activity.

The present *in vitro* studies were performed to investigate whether the increased expression of CTGF in the healing phase of the anti-Thy-1.1 single injection model might contribute to the various aspects of mesangial repair and how this would relate to effects of TGFβ and PDGF-BB. Therefore we assessed the *in vitro* response of mesangial cells to these growth factors in terms of changes in morphology and αSMA expression, migration, proliferation, and fibronectin induction.

To assess the effect of CTGF, TGFβ, and PDGF-BB on activation of mesangial cells and on their transformation into myofibroblast-like cells, scrape-wounded cultures were stained for αSMA. The morphology and number of αSMA-positive cells and the level of αSMA expression were not affected by CTGF and PDGF-BB. In contrast, TGFβ1-stimulated cells were more spindle-shaped and arranged in typical fibroblast-like bundles. Moreover, αSMA expression was upregulated in all cells throughout the monolayer in these cultures and semi-quantitative analysis by an indirect ELISA for αSMA on mesangial cells showed up to a threefold increase of the αSMA expression level. Neutralizing anti-CTGF antibodies did not influence these effects of TGFβ on mesangial cells, which suggests that the effect of TGFβ on αSMA expression is not dependent on CTGF.

Migration and proliferation of mesangial cells, two other aspects of mesangial repair in anti-Thy-1.1 nephritis, were investigated by measuring the closure of wound margins by the scraped mesangial cell monolayers. We found that CTGF stimulates wound closure of mesangial cell monolayers in a dose-dependent manner. This effect of CTGF was completely blocked by the addition of neutralizing chicken anti-CTGF antibody but not by neutralizing pan-specific TGFβ antibody. From this it seems that CTGF-induced wound healing in this *in vitro* model is TGFβ-independent although we cannot exclude some role for autocrine TGFβ stimulation, which might have escaped TGFβ blocking antibodies in the culture medium. To stimulate significant wound closure, short-term exposure to CTGF was not sufficient. When scrape-wounded cultures were stimulated with TGFβ, similar kinetics of wound closure were observed as in the presence of CTGF. TGFβ-induced wound healing was completely blocked by a neutralizing pan-specific TGFβ antibody, but neutralizing anti-CTGF antibody had no detectable effect on TGFβ-induced wound healing. It thus appears that CTGF- and TGFβ-induced wound healing share various characteristics but are mutually independent.

To investigate the possible involvement of proliferation in CTGF- and TGFβ-induced wound closure, cells were evaluated for the expression of the Ki67 antigen and for [^{3}H]thymidine incorporation. Although CTGF is a mitogen for NRK fibroblasts and for vascular smooth-muscle cells [9,22] and while TGFβ is known to be mitogenic for some mesenchymal cells (e.g. NRK fibroblasts), neither CTGF nor TGFβ induced mesangial-cell proliferation in our assays. This suggests that mitogenic responses to CTGF and TGFβ may be cell-type dependent.

In PDGF-BB-stimulated cultures wound closure was more rapid than in cultures exposed to CTGF or TGFβ, complete at 24 h as compared to the 72 h needed in CTGF- and TGFβ1-stimulated cultures. PDGF-BB-induced wound closure was characterized by strongly increased proliferation, as shown by [^{3}H]thymidine incorporation and anti-Ki67 staining.

Since proliferation appeared not to be necessary for CTGF- and TGFβ-induced wound closure, it seemed more likely that wound healing was the result of hypertrophy and/or migration of mesangial cells. Based on data from earlier studies [22], that CTGF-induced migration of vascular endothelial cells depends on expression of $\alpha_{v\beta3}$ integrin, it might be

suggested that this could also be the case for mesangial cells. However, involvement of integrins in CTGF-stimulated mesangial cells has not been studied thus far.

The literature on a possible relationship between αSMA expression and proliferation, morphology or cellular hypertrophy seems contradictory. Stephenson *et al*. [23] reported a strong correlation of αSMA expression with hypertrophy and an inverse correlation with proliferation of mesangial cells *in vitro*, whereas Hugo *et al*. [12] showed in the *in vivo* anti-Thy-1.1 model that αSMA is expressed mainly in proliferating Ki67-positive mesangial cells. We observed no increase in αSMA expression in proliferative PDGF-BB-stimulated cultures, nor in non-proliferative CTGF-stimulated cultures. This observation indicates that *in vitro* neither the proliferation, nor the migration of rat mesangial cells requires the upregulation of αSMA. As far as the *in vivo* correlation of αSMA expression with proliferation and migration is concerned, this might reflect independent effects of regulation by multiple growth factors.

A further aspect of mesangial repair in anti-Thy-1.1 nephritis, upregulation of extracellular matrix production, was addressed by investigating the effects of CTGF and TGFβ on fibronectin synthesis. Both CTGF and TGFβ stimulated fibronectin expression by mesangial cells. Moreover, TGFβ-induced fibronectin expression was significantly diminished in the presence of neutralizing anti-CTGF antibodies. This suggests that, at least in part, TGFβ-induced fibronectin synthesis is regulated by a CTGF-dependent pathway. Whether this holds also true for regulation of expression of other extracellular matrix (ECM) components, and ECM-degrading enzymes, such as MMPs, remains to be elucidated. Although it has been reported [24], that PDGF-BB can induce fibronectin expression, we did not observe any effect on fibronectin expression by mesangial cells in our system. Differences in cell lines and culture conditions might be responsible for this apparent discrepancy.

In the complex process of wound repair, the levels of growth factors may determine the outcome of the repair process, i.e. hypertrophic scarring, *vs* true repair and restoration of functional tissue architecture. From *in vitro* as well as *in vivo* studies it is well known that TGFβ is critically involved in various aspects of the repair process, but TGFβ also plays a pivotal role as a suppressor of immune function and tumour growth. The fact that CTGF thus far seems to be involved more specifically in the tissue response to injury might make it a more attractive target for intervention strategies to prevent excess fibrosis, when CTGF is overexpressed.

The results of this study show that in the case of mesangial response to injury, TGFβ effects may occur in part indirectly, through the effects of TGFβ-induced CTGF expression on mesangial cells, and in part directly, through CTGF-independent pathways. It also has become apparent that the mesangial response to injury might also involve TGFβ-like effects induced by CTGF overexpression in response to other stimuli, and independent of TGFβ activity. However, it is still unclear whether increased expression of CTGF is primarily involved in temporary adaptation to the situation of acute damage and repair, or mainly in progressive scarring. Therefore the crucial question remains as to how therapeutic modulation of CTGF expression might affect the outcome of repair and scarring following glomerular injury. This will be investigated in the rat anti-Thy-1.1 model by analysis of histomorphological integrity and residual function after *in vivo* administration of neutralizing anti-CTGF antibodies during different phases of glomerulonephritis.

In conclusion, PDGF-BB, TGFβ, and CTGF all induce *in vitro* wound healing of rat mesangial cell monolayers. In the case of PDGF-BB, wound closure appears to be independent of CTGF and to be mainly due to increased proliferation. TGFβ-induced wound healing was in part independent of CTGF (migration and αSMA expression), and in part regulated *via* a CTGF-dependent pathway (fibronectin synthesis). CTGF effects (migration and fibronectin production) appeared to be TGFβ independent. These data add to the notion that mesangial response to injury involves the interaction of a number of growth factors with different regulatory and effector profiles. By manipulation of the balance between these factors it might become possible to influence the outcome of the repair process favourably, and to minimize (progressive) loss of function by excessive scarring. CTGF might be one of the more suitable targets for future intervention strategies.

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