Interleukin 4 Co-Stimulates the PDGF-BB- and bFGF-Mediated Proliferation of Mesangial Cells and Myofibroblasts

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Key Words

Interleukin 4 · Endothelial cells · Myofibroblasts · Mesangial cells · Tubular epithelial cells · Proliferation · Growth factors

Abstract

Background: Although many mediators involved in the pathogenesis of fibrosis are known, its precise mechanism is still unknown. In vitro experiments may contribute to the recognition of cellular changes which also take place during fibrosis. **Methods:** Renal tubular epithelial cells (EPC), mesangial cells (MC) and glomerular endothelial cells (GEDC) as well as endothelial cells (EDC) and myofibroblasts (MF) from cattle were isolated to measure the proliferation and protein synthesis in the presence of individual and combined cytokines/growth factors in cell cultures. **Results:** Cytokines stimulating or permitting the proliferation of myofibroblast-like cells (MFLC) (MC and MF), caused damage of endothelial cells (EDC, GEDC), whereas EPC were stable. The proliferation of MFLC was strongly stimulated by PDGF-BB and bFGF and elevated more than twofold in the presence of interleukin 4 (IL-4), but IL-4 alone had no effect. Furthermore,

the proliferation of transdifferentiated endothelial cells (TEC), obtained by incubation of EDC with $\text{TNF}\alpha$ and bFGF, was stimulated with both PDGF-BB/IL-4 and bFGF/IL-4 in the same way and proved to be stable with respect to $\text{TNF}\alpha$. **Conclusion:** Interleukin 4 co-stimulates the PDGF-BB- and bFGF-mediated proliferation of MC, MF, and TEC. $\text{TNF}\alpha$ does not inhibit the proliferation of extracellular matrix-synthesizing cells, but has an inhibitory or even toxic effect on EDC and GEDC. It may be concluded that cytokines released in inflamed renal tissue influence tubulointerstitial cells in different ways, resulting in progressive tissue damage and fibrosis in which the EDC would be the most sensitive cells. Thus, we speculate that microvascular injury in these areas leads to ischemia and malnutrition of tubular EPC and may be responsible for ongoing tubular damage and resulting renal interstitial fibrosis.

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Introduction

Renal interstitial fibrosis (RIF) and glomerulosclerosis are characterised by an accumulation of extracellular matrix (ECM) after a primary injury, and subsequent inflammation [1–3]. If the primary damage exceeds a certain limit, inflammation persists on a diminished level.

Cytokines and growth factors act on cellular functions and either determine the healing or progression of tissue

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0028-2766/02/0924-0868\$18.50/0

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damage resulting in fibrosis and organ failure. In fibrotic diseases, the affected tissue is unable to regulate the inflammatory process due to the persistence of inflammatory signals [4]. Although there is a growing list of inflammatory mediators [2, 5–7], little is currently known about the manner in which they initiate and sustain the disease. Furthermore, previous results obtained from animal models of RIF indicate that the same cytokines are involved in acute injury and progressive fibrosis [3, 5, 8].

In animal models of RIF, the focal progression of fibrosis has been proved [9, 10]. In damaged renal interstitial areas, resident cells (tubular epithelial cells, microvascular endothelial cells, ECM synthesizing myofibroblast-like cells) and invading inflammatory cells (mainly macrophages/monocytes, T-lymphocytes) are involved in cytokine secretion. High local cytokine concentrations may be the cause of disturbed functions of resident cells and possibly of their transdifferentiation [11–13]. The progression of fibrosis is cytokine-dependent and not the result of irreversibly changed autonomous ECM-producing cells as shown from the finding that tubular epithelial cell damage is essential for progressive fibrosis [14].

It has been shown that the action of cytokine combinations differs from the sum of actions of individual cytokines [15, 16]. Because the reaction of cells from different species to the same cytokine or growth factor may be considerably different, a comparison of cellular reactions should be performed using different cells from the same species. So far, such experiments have not been carried out, because the isolation and cultivation of the renal cells (endothelial cells (EDC), tubular epithelial cells (EPC), and mesangial cells/myofibroblasts (MC/MF)) obtained from humans and laboratory animals would be complicated. However, the easy isolation and handling of bovine EDC and tubular EPC will allow such an experiment. Using these cells, we compared the action of cytokines regarding cellular proliferation, protein synthesis and secretion. The results can help to decide which of these mediators or which combinations are essential for progressive fibrosis.

Materials and Methods

Sources, Abbreviations

Human growth factors and cytokines were purchased from Sigma (Deisenhofen, Germany), TGF β 1 and IL-4 were from NatuTec (Frankfurt, Germany). Cell culture medium and supplements were obtained from Biochrom (Berlin, Germany) and other substances were from Sigma.

The following abbreviations were used: transforming growth factor- β 1 (TGF β 1), platelet-derived growth factor-BB (PDGF-BB), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), tumor necrosis factor- α (TNF α), interleukin 1 β (IL-1 β), and interleukin 4 (IL-4). The designation of bovine cells was abbreviated as follows: renal tubular epithelial cells, endothelial cells from the lung artery (EDC), glomerular endothelial cells (GEDC), MC, MF, endothelial cell derived myofibroblast-like cells (TEC).

Cell Cultures

For the isolation of bovine cells, kidneys and lung arteries of adult cattles were obtained from a local slaughterhouse (Jena, Germany). Glomeruli were isolated by the sieving method. Renal cortex was minced and passed through serial sieves (tissue grinder kit; Sigma, Deisenhofen, Germany). For separation glomeruli passing through a 140 μ m sieve, being hold back on a 104- μ m sieve. A pure glomerular fraction was obtained by repeated sedimentation. The glomeruli were incubated in the presence of collagenase (Worthington, Freehold, N.J., USA; 216 IU/mg, 0.6 mg/ml) for 15 min at 37 °C. After washing twice with PBS, glomerular remnants consisting of MC and GEDC were suspended at a density of 1,000 remnants/ml in DMEM-Ham's F-12 [1:1] (D/H) supplemented with 10% FCS, penicillin/streptomycin, and *L*-glutamine solution (1:100, Biochrom).

For the isolation of GEDC, the suspension was given onto gelatin-coated culture dishes and incubated at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air. The medium was changed after 3–5 days, and nonadherent remnants were separated. After outgrowth of endothelial cells was visible in addition to MC, the cell cultures were trypsinized (0.025% trypsin, 0.5 mM EDTA) for a short time at room temperature to mainly detach the GEDC. The washed cells were plated onto gelatin-coated dishes at low density. GDEC fractions were isolated from semiconfluent cultures using cloning cylinders. The studies were performed using GEDC after more than 6 passages.

Bovine MC were obtained from glomerular remnants plated onto culture dishes (without gelatin) at a density of about 200 remnants/cm² in 20% FCS-containing medium. Under these conditions, mainly cultures of preferentially MC were obtained within 2–3 weeks. The culture medium after the first trypsinization was supplemented with 10% FCS. MC cultures were used for experiments after more than 8 passages.

Bovine tubular epithelial cells (EPC) were isolated from kidney cortex using tissue pieces (approximately 1–3 mm³). 10–15 fragments were placed onto the growth surface of 20 cm² culture dishes, which had been previously coated with collagen isolated from rat tails. The use of coated culture dishes is not essential but makes the detachment of cells, employing trypsin, easier. Outgrowths were obtained within some days. The cells were isolated by trypsinization and cultured in D/H medium without FCS but supplemented with insulin/transferrin/sodium selenite (ITS, $\times$ 100; Biochrom). Further growth stimulation with EGF (10 ng/ml) is only slightly effective. For experiments, cell cultures of passages 5–10 were used.

Bovine MF were obtained from lung arteries. Pieces of 2–4 mm³ were cut from the tunica media of the vessels and plated onto culture dishes (20 cm²). The pieces were moistened with FCS. After half an hour at 37 °C, culture medium (D/H containing 10% FCS) was added. The outgrowths were subcultured and passaged 10 times. Although these MF may be derived from vascular smooth muscle cells, they are similar to other MF, for instance to MC and activated fibroblasts.

Bovine endothelial cells (EDC) were isolated from lung arteries. 10- to 12-cm long blood vessels were closed on an end with the aid of a clamp and placed into a breaker. Collagenase solution (Worthington, 0.2 mg/ml PBS) were put into the blood vessel. The breaker was placed in a water bath at 37°C and incubated for 12 min. Subsequently, the solution was removed, and the cells were separated and washed by centrifugation. A second cell fraction was obtained by desintegration using a higher collagenase concentration (1 mg/ml; 15 min). Cells from both cell fractions were cultured separately in culture dishes with D/H medium supplemented with 10% FCS. For cell culture experiments passages 10–20 were used.

Cellular Proliferation and Protein Synthesis Induced by Cytokines

The cells were replated onto multiwell dishes and incubated with suitable concentrations of the mediators in the presence of low amounts of FCS in the culture medium (EPC with 0.5%, others with 1.0% FCS). To obtain higher differences in the promoting or inhibiting action on cell proliferation or protein synthesis, the cultures were initially preincubated with mediators. After medium change and second mediator application, the tritium-labelled substances (thymidine, leucine, proline) were added, and the cultures were further incubated for at least 14 h. Cell proliferation was determined using the [³H]thymidine incorporation. Cells grew to 30–50% density in 10% FCS-containing medium and subsequently starved for 1 day in 1% FCS-containing medium in order to make the cells quiescent. At this point, new medium with or without growth factors/cytokines were added. The concentrations of the mediators were 10 ng/ml, only TGFβ1 and TNFα had the concentrations of 2 and 6 ng/ml, respectively. After a further 24 h, the medium was changed and fresh medium with or without mediators and 0.6 μCi/well (methyl-³H)-thymidine (5 Ci/mmol; Amersham Pharmacia, Little Chalfont, England) were added. After an incubation period of 14 h, cell proliferation was arrested by placing the culture plates in a refrigerator at 4°C. The cells were washed three times using PBS at this temperature, fixed with 10% trichloroacetic acid (TCA), washed 2 times with 5% TCA, solubilized with 0.3 M sodium hydroxide containing 1% SDS, and counted in a liquid scintillation counter (LSC) to measure cellular [³H]thymidine incorporation. Cells incubated with control medium were assigned a relative value for [³H]thymidine uptake of 100%. Incorporation of thymidine into cells treated with mediators was calculated as percentage of control. Only EPC grew under altered conditions: This medium contained 0.5% FCS and the incubation times were doubled.

De novo protein synthesis was determined under the same conditions as the thymidine uptake with some differences: The culture medium was supplemented with sodium ascorbate (50 μg/ml) and β-aminopropionitrile (80 μg/ml). In 24-well plates, the starved cells were incubated with control medium supplemented with 1% (EPC with 0.5%) FCS or with this medium containing growth factors/cytokines. After 24 h, the medium was changed and new medium containing the same mediators, and 0.6 μCi/well [³H]-labelled leucine (Leu) or proline (Pro) (L-(4,5-³H) leucine, 157 Ci/mmol, L-(2,3-³H) proline, 48 Ci/mmol; Amersham Pharmacia) were added. Pro incorporation into TCA-precipitable protein was used as a marker for collagen synthesis, since Pro is a major component of and is preferentially incorporated in collagen. The uptake of amino acids (AA) was stopped by placing the plates in a refrigerator at 4°C. For the detection of AA incorporation into secreted proteins, the medium of three or four wells each, containing the same mediator, was removed. Ali-

quots were used for the precipitation of proteins. After addition of 10 mg BSA/ml medium using a 20% BSA solution, proteins were precipitated by 4 ml 10% TCA. The precipitates were washed threefold using 6 ml of 5% TCA containing 0.5 mM of the respective unlabelled AA. Finally, the protein pellet was solubilized in 0.3 M sodium hydroxide containing 1% SDS and counted in a liquid scintillation counter.

For determination of the AA uptake in cellular proteins, the cells were washed with ice-cold PBS three times, fixed with 10% TCA, washed 2 times with 5% TCA, solubilized with 0.3 M sodium hydroxide containing 1% SDS, and placed in a liquid scintillation counter to measure the radioactivity, which allowed the calculation of molar amounts of incorporated AA in proteins. The ratio Pro/Leu increases with elevation of the collagen content of proteins and can serve to assess the collagen synthesis induced by cytokines.

Transdifferentiation of Endothelial Cells

Confluent cultures of endothelial cells (EDC and GEDC) growing in D/H medium supplemented with 10% FCS were incubated with TNFα (1–6 ng/ml) and bFGF (10 ng/ml). The medium was changed every third day. The concentrations of TNFα were chosen in dependence of cell viability, so that not more than 10% of the cells were detached from the surface of the culture dishes. Normally, after 10–14 days most cells were transdifferentiated into myofibroblast-like cells that were resistant to TNFα but were growth-stimulated by PDGF-BB, bFGF or their combination with IL-4. The transdifferentiated EDC (TEC) reacted negatively against anti-von Willebrand factor VIII antibodies and positively against anti-α-smooth muscle actin (αSMA) antibodies. To exclude that TEC grew from a very low amount of myofibroblast in endothelial cell cultures, transdifferentiation experiments were performed also with EDC of passages 17–23 and the homogeneity of the cultures was checked by immunocytochemistry (anti-factor VIII antibody).

Identity of Cells, Immunocytochemistry

The identity of the cells were assessed by their shape, growth behavior and reactions induced by cytokines/growth factors. Furthermore, cells were characterized by immunocytochemistry using FITC-labelled secondary antibodies (FITC-conjugated goat anti-mouse IgG, FITC-conjugated F(ab')₂ fragment swine antirabbit IgG; Dako, Glostrup, Denmark). Cells were plated onto cover glasses (or in the case of GEDC onto glasses coated with collagen) (24 × 24 mm) placed in round culture dishes (Falcon, Plymouth, England). After reaching semi-confluency, they were fixed with ice-cold methanol at –20°C for 8 min and dried shortly at environmental temperature. The primary antibodies were used to determine the identity of EDC/GEDC, EPC and myofibroblast-like cells (MFLC = MC, MF, TEC), respectively: rabbit antihuman von Willebrand factor (Dako); anti-pan cytokeratin monoclonal antibody (MOAB) (Becton Dickinson, Bedford, Mass., USA); antihuman cytokeratin 19 MOAB (Dako); rabbit anti-αSMA (Dako), antihuman vimentin MOAB (Boehringer).

Light Microscopy

Cultured cells on plastic dishes (Falcon) were fixed with buffered formaldehyde (4%) and thereafter with 92% methanol. After drying, cells were stained with 0.05% Coomassie in 10% acetic acid containing 25% methanol and then with Giemsa (Boehringer, Mannheim, Germany).

Statistical Analysis

Values were expressed as means $\pm$ SD. Statistical analyses were performed using the two-tailed t test. $p < 0.05$ was considered statistically significant. Each experiment was performed as least three times, and single values of cell cultures result from three or four wells.

Results

There was only a minor effect of the mediators on the proliferation of tubular EPC although in these experiments longer incubation times were used than in those with other cells (fig. 1a). Only EGF and, to a somewhat lesser extent, bFGF stimulated cell proliferation as compared to the control. Moreover, only a relatively high concentration of TNF α was able to inhibit the proliferation of EPC significantly. TGF β 1 diminished the growth-stimulating effect of EGF. Other mediators were relatively ineffective. The proliferation of EDC was stimulated by bFGF, VEGF and a combination of the two (fig. 1b). A significant growth inhibition was caused by TGF β 1, IL-4 and TNF α and a combination of these with noninhibitory mediators. Thus, the inhibiting effects of TNF α and IL-4 were not compensated in the presence of bFGF and VEGF. Since the isolation of renal interstitial microvascular EDC is not practicable in a large number of EPC in primary cultures, we cultured glomerular endothelial cells from homogenous glomerular fractions and compared the influence of mediators on the two endothelial cells (EDC and GEDC). Figure 1b and c demonstrates that the same mediators stimulate or inhibit the proliferation of GEDC and EDC.

A different pattern of cell proliferation was found in experiments with MC and MF, summarized as MFLC (fig. 1d, e). In comparison to EDC, GED, and EPC, the proliferation of these cells was stimulated to a much higher extent and only sparsely inhibited by growth factors like TNF α and TGF β 1. Furthermore, this inhibiting action was overcompensated by PDGF-BB and bFGF. The effect of both growth factors was additive in the absence of other mediators. Remarkably, IL-4 elevated the stimulating action of PDGF-BB and bFGF about twofold, but increased the proliferation itself only slightly. To demonstrate that EDC-derived MFLC (obtained by cytokine-mediated transdifferentiation of EDC and termed as TEC), react similarly to other myofibroblasts, we incubated TEC cultures with mediators and found a comparable profile of induction and only an insignificant inhibition (fig. 1f). The bFGF causes a higher cell proliferation than PDGF-BB. Figure 2 shows photomicrographs of confluent growing EPC and endothelial cells (EDC and GEDC and those of MFLC (MC, MF, TEC).

Incubation of the cell cultures with tritium-labelled Leu and Pro was performed to determine protein synthesis. The content of Pro and its secondary modification product hydroxyproline is much higher in collagens than in other proteins [17].

Figure 3a shows that only EGF stimulated the cellular uptake of Pro in EPC significantly, as compared to the controls, whereas TNF α caused a decrease in the uptake of the two AA. In EDC cultures, the hypertrophic effect of TGF β 1 and the growth-promoting action of bFGF may be responsible for the increased AA uptake, whereas PDGF-BB was ineffective (fig. 3b). The AA uptake in MC, MF, and TED was similar (fig. 3c–e). The differences between the high thymidine uptake and the comparably low AA uptake results from the higher nuclear/cytoplasmic ratio in dividing cells. The ratios of incorporated Pro and Leu in cellular proteins varied only slightly, even in the presence of mediators (table 1). Compared to other cells, EPC revealed the lowest Pro/Leu ratio. Table 1 gives the ratios of the secreted proteins obtained from control cultures as well as the means of the ratios from cultures incubated in the presence of mediators. These ratios differ significantly from those measured in cellular proteins and can be explained by a higher collagen content in secreted proteins than that found in cellular proteins. Figure 4a, b shows the influence of cytokines on the protein secretion of MC and MF in cell culture. PDGF-BB and bFGF caused higher amounts of secreted proteins than TGF β 1, which is considered to have a strongly stimulating effect on the ECM synthesis under in vivo conditions [18, 19].

Table 1. Pro/Leu-ratio in cellular and secreted proteins

Cells	Cellular protein ¹	Secreted protein ²
MC	0.471 $\pm$ 0.033 (8)	0.700 $\pm$ 0.080 (4)
MF	0.525 $\pm$ 0.075 (9)	0.763 $\pm$ 0.073 (4)
TEC	0.405 $\pm$ 0.007 (6)	n.d.
EDC	0.384 $\pm$ 0.029 (6)	0.591 $\pm$ 0.071 (4)
EPC	0.202 $\pm$ 0.013 (7)	0.266 $\pm$ 0.029 (4)

¹ Means $\pm$ SD resulting from cell cultures incubated with different mediators and from controls (see fig. 2).

² Means $\pm$ SD resulting from cell cultures, incubated with mediators (TGF, PDGF-BB, TGF + PDGF-BB) and from unstimulated control cultures; in the case of EPC, cells were cultured with EGF instead of PDGF-BB; number of independent experiments, each performed in triplicate, is given in parentheses.

Fig. 1. Effect of growth factors and cytokines on the proliferation (^3H -thymidine incorporation). a Renal tubular epithelial cells (EPC). b Endothelial cells from the lung artery (EDC). c Glomerular endothelial cells (GEDC).

Fig. 1. d Mesangial cells, e Myofibroblasts (MF), f Endothelial cells derived myofibroblast-like cells (TEC). The cells were cultured in 24-well plates with D/H medium containing 1% FCS (in the case of EPC only 0.5% FCS) and the following mediators: TGF β 1 (T), PDGF-BB (P), bFGF (F), EGF (E), IL-4 (L), IL-1 β (I), TNF α (N), VEGF (V) (concentration: (T) 2 ng/ml; (N) 6 ng/ml; others 10 ng/ml) for 24 h. After the addition of ^{3} H-thymidine, cells were further incubated for at least 14 h. Values are given in percentage of control cultures grown in the absence of mediators. The data represent the means $\pm$ SD of 3–6 independent experiments each conducted in triplicates or quadruplicates. Statistical significance was analyzed by the two tailed t test. * $p < 0.05$ vs. control, ° $p < 0.05$ vs. cells incubated with one mediator only.

Fig. 2. Light micrographs of bovine cells cultured in D/H medium. Fixed cells were stained with Coomassie and after drying with Giemsa. a Renal tubular epithelial cells (EPC). b Endothelial cells from the lung artery (EDC). c Glomerular endothelial cells (GEDC). d Mesangial cells (MC). e Myofibroblasts (MF). f Endothelial-cell-derived myofibroblast-like cells (TEC) obtained by endothelial-mesenchymal transdifferentiation (induced by $\text{TNF}\alpha$ and bFGF). While untreated endothelial cells exhibit a cuboidal, cobblestone-like morphology in confluent cultures, the transdifferentiated cells are similar to MC and MF, show an elongated, stellate morphology, grow in multiple layers of cells in confluent cultures, and are comparably large. Original magnification $\times 100$.

For Fig. 3d and e see next page

Fig. 3. The effects of growth factors and cytokines on the protein synthesis. The synthesis of cellular proteins was assessed by ^3H -Pro- (gray columns) and ^3H -Leu- (hatched columns) incorporation in the presence of ascorbic acid and β -aminopropionitrile. For abbreviations and experimental conditions, see the legend to figure 1. The data represent the means $\pm$ SD of 3–6 independent experiments each conducted in triplicate or quadruplicate. Values were obtained from wells of the same cultures and expressed in percentage of controls to allow a direct comparison. * $p < 0.05$ versus control. The molar Pro/Leu ratio is depicted above the columns. a Renal tubular epithelial cells. b Endothelial cells from the lung artery. c Mesangial cells.

Fig. 3. d Myofibroblasts. e Endothelial cells derived myofibroblast-like cells.

In table 2 the relative amounts of secreted proteins are given as a percentage of newly synthesized cellular proteins, determined by both Pro and Leu uptake. The means resulting from the two values (Pro and Leu uptake) show a secretion capacity of MC and MF that is about 4- to 6-times higher than that of EDC and EPC, respectively. The slight differences between unstimulated and stimulated cells confirm the minimal influence of mediators on the composition of secreted proteins. The differences are cell-specific, and mediators had only a minor influence on the protein composition of cellular and secreted proteins.

Discussion

It has already been shown that there are no cytokines and growth factors that are fibrosis-specific [1, 3, 5, 8]. Changes in cytokine concentrations during the development of fibrosis are similar to those seen in normal wound healing [20]. While repair processes in wound healing are self-limiting, these regulative mechanisms are disturbed in progressive fibrosis [9, 21, 22]. An understanding of the reasons for the progression of fibrosis remains, today, the main topic of current investigations. In addition to histological and immunohistochemical examinations of fibrot-

Fig. 4. Secreted proteins into the culture medium. After incubation with ^3H -Pro (gray columns) and ^3H -Leu (hatched columns) the media from mesangial cell and myofibroblast cultures (see fig. 2d, e) were separated. After addition of BSA to aliquots of combined media from identical wells, the precipitated protein by 10% TCA was washed 3-fold using 5% TCA, and the pellets were prepared to measuring radioactivity (see legend to fig. 1). $p < 0.05$ vs. controls. a Mesangial cells. b Myofibroblasts.

ic kidneys from laboratory animals, cell culture experiments may contribute to identifying the influence of individual or combined mediators on cell function. Suitable mediators for these experiments are primarily growth factors like PDGF-BB, bFGF, VEGF, TGF β 1, EGF, TNF α , and some interleukins. As early as 1993, Fine et al. [6] concluded that ischemia, as a result of primary cell damage, will cause secondary damage to tubules and consequently expose antigens that lead to secondary inflammatory cell infiltrates in the affected areas. This concept of 'chronic hypoxia hypothesis' has been confirmed [23, 24], and finally these authors could verify that hypoxia per se

may be a fibrogenic stimulus [25]. Compared to EPC, EDC seem to be more resistant to hypoxia as shown in the remnant kidney model [26]. It is suggested that damage of microvascular endothelial cells causes hypoxia, inflammation, and fibrogenic responses, leading to the obliteration of further capillaries, thereby setting up a vicious cycle [23, 24].

Our results revealed that endothelial cells are most sensitive to mediators which are secreted in inflammatory areas after kidney damage [3]. These mediators also stimulated the proliferation and protein secretion of ECM-synthesizing myofibroblast-like cells. In this respect, IL-4

Table 2. De-novo synthesis of secreted proteins expressed as a percentage of synthesized cellular proteins¹

Cells	Unstimulated ²	Stimulated ²
MC	48.0	51.4 ± 5.9 (4)
MF	46.2	43.6 ± 5.1 (4)
EDC	10.7	9.9 ± 1.2 (4)
EPC	7.6	7.6 ± 0.4 (4)

¹ Measured by ³H-labelled amino acid incorporation.

² Means ± SD resulting from Pro and Leu incorporation in cell cultures, incubated with mediators (TGF, PDGF-BB, TGF + PDGF-BB) and from unstimulated control cultures; in the case of EPC, cells were cultured with EGF instead of PDGF-BB; number of independent experiments, each performed in triplicate, is given in parentheses.

may play a special role. Besides IL-10 and IL-13, IL-4 is also considered to be an anti-inflammatory cytokine, which is able to inhibit the expression of inflammatory mediators and macrophage functions [27]. IL-4 is a pleiotropic cytokine that has multiple cell-type specific activities [28, 29], and has also been proposed as playing a role in glomerulonephritis [30]. Pretreatment with IL-4 decreased crescent formation and improved renal function [31, 32]. Postlethwaite et al. [33] and Salmon-Ehr et al. [34] demonstrated that IL-4 is able to stimulate the synthesis of ECM compounds by fibroblasts (collagens type I, III and fibronectin) in vivo. According to Sempowski et al. [35] elevated levels of IL-4 may lead to the development of fibrosis by enhancing fibroblast subset proliferation and collagen synthesis. Our results represent IL-4 as a cytokine without any stimulating effects on the proliferation of bovine cells (MC, MF, TEC). In the presence of the growth factors PDGF-BB and bFGF, however, IL-4 acts in a co-stimulating manner. The special role of PDGF-BB in progressive fibrotic disease has been previously discussed [36]. Its causative role in renal scarring could be shown by the inhibition of the PDGF-BB effect in animal models of RIF and glomerulonephritis [37]. The assumption that only pro-inflammatory cytokines secreted by Th1-lymphocytes promote fibrosis should be amended so that cytokines liberated by Th2-lymphocytes are likewise implicated in this process [38].

The question concerning the origin of ECM-synthesizing cells in RIF still remains open. Different sources have been discussed: constitutive interstitial fibroblasts could be transformed into metabolically active myofibroblasts; smooth muscle cells from the adventitia of renal vessels

could migrate as myofibroblasts into the damaged interstitium [39, 40]; and finally cells like EPC and microvascular EDC are able to transdifferentiate into mesenchymal cells [11, 12, 41, 42]. In our experiments bovine EDC and GEDC transdifferentiated into myofibroblasts under cell culture conditions in the presence of TNF α or the combination of TNF α with other growth factors. Apart from the in vivo transdifferentiation of tubular EPC into MFLC as discussed by Strutz et al. [43] and convincingly demonstrated by Fan et al. [12], EDC can also transdifferentiate into MFLC in the presence of a suitable mediator cocktail in inflamed tissue. In view of the high sensitivity of EDC to TNF α and other cell growth inhibiting mediators, we assume that the transdifferentiation of EDC may be involved in RIF. In histological examinations of kidney slices from patients with RIF, Bohle et al. [44] confirmed a decrease in tubulointerstitial capillaries. So far, it is not known whether EDC from obstructed capillaries are completely destroyed by apoptosis or if microvascular EDC transdifferentiate into MFLC that take part in the ECM-synthesis. In organs other than kidney, an endothelial cell-myofibroblast transdifferentiation has already been established [45, 46]. Madri et al. [47] described changes of EDC near the site of injury and the role of dysfunctional endothelium in the pathogenesis of atherosclerosis. Moreover, they proved that cultured EDC may synthesize α SMA in the presence of TGF β [47]. In addition to the embryonic EDC transdifferentiation into mesenchymal cells described by DeRuiter [48] and Nakayima et al. [46], Baranek et al. [39] assumed that MFLC may originate from the vascular endothelium in inflammatory disease.

The presence of TNF α seems to be essential for the transdifferentiation of EDC into MFLC. TNF α and IL-1 β are cytokines that mediate the onset of fibrosis and maintain inflammation in the damaged areas [49]. TNF α exerts a variety of biological effects on glomerular MC and EPC and is believed to contribute to the severity of renal lesions [50, 51]. Sato et al. [52] described the specific toxicity of TNF α to EDC. It may be assumed that the transdifferentiation of EDC into TEC in damaged areas could protect these cells from apoptosis or necrosis. Moreover, these cells can be used for ECM-synthesis in repair processes.

TGF β 1 is considered to be a major fibrogenic molecule in many experimental and human diseases [18, 19]. In our cell culture experiments, only a minor stimulating effect of TGF β 1 on protein secretion was seen, but rather the dependence of protein secretion on the growth behavior of cells. Under cell culture conditions resting cells do not

secrete significantly more proteins than proliferating cells. The comparison of the Pro/Leu ratio between cellular and secreted proteins can provide information only on the relative changes of the collagen content. Yet, this method is sufficient to detect different effects of mediators on ECM synthesis. The relatively low amount of proteins secreted by EDC and EPC as compared to MFLC suggest that EPC and EDC may contribute to fibrogenesis only marginally whereas the mainly ECM-synthesizing cells (MC, MF, and TEC) can be stimulated strongly by growth factors and cytokines. We compared MC and EDC with MF obtained as outgrowths from arteries. These cells may originate from vascular smooth muscle cells (VSMC) having lost their original characteristics.

As found by Alpers et al. [53], the expression of PDGF β -receptors seems to characterize MFLC more than smooth muscle cells. In our experiments, the proliferation of MF was highly stimulated by PDGF-BB, suggesting that VSMC transdifferentiate into MF under pro-

longed cell culture conditions. Fine and co-workers [24, 25, 54] and Yamamoto [19] also emphasize the particular importance of the impaired microvasculature during progressive RIF. We assume that damaged EPC are the primary reason for inflammation, and EDC damage plays a significant role. This takes into account that, for the focal development of fibrosis, primarily damaged areas must exceed a certain threshold to cause progressive fibrosis. The ongoing presence of stimulated ECM-synthesizing cells of different origin in damaged renal tissue is assumed to be decisive for the disease and should be the subject of further investigation.

Acknowledgements

This work was supported by a grant from the BMBF (Bundesministerium für Bildung und Forschung) No. 01ZZ9602/7.

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Commentary

Renal Fibrosis: Origin and Potential of Myofibroblasts

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Fibrosis arises in the kidneys when there has been damage to the glomeruli and tubules, and so there is atrophy of the normal architecture, and fibroblasts that produce new extracellular matrix expand the interstitial space. There are many causes of tubulo-interstitial fibrosis (TIF), ranging from the effects of hypertension, glomerulonephritides and pyelonephritis to causes of heavy pro-

teinuria, and any process that incites proximal tubules to produce pro-inflammatory mediators as occurs in chronic rejection. From the time of injury both interstitial cells and proximal tubules are activated and myofibroblasts appear. Myofibroblasts, defined by their expression of α -SMA (smooth muscle actin) often correlate with the degree of renal impairment and histological damage [1].

They play a key role in the development of glomerulosclerosis and interstitial fibrosis [2]. Table 1 summarises the processes that trigger fibrosis.

Fibroblasts are not homogenous [14] for they include functionally different interstitial cells called fibroblastlike cells (FbLCs). In cortex [15] and outer medulla [16] or kidneys there are pericytes that become interstitial fibroblasts, and there are unique interstitial fibroblasts in the inner medulla that contain lipid droplets and react with the lectin *Bandeira simplicifolia*-1 (BSL-1). Shortly, I will gather the evidence that myofibroblasts (MF) arise from tubular epithelial cells [17] and we know that they are formed from mesangial cells [18], and in their current article Sommer et al. [19] suggest that they also arise from endothelial cells that are involved in inflammation. Granulation tissue MFs of skin, since they are accessible, were soon shown to produce muscle related phenotypic markers like α -SMA, vimentin (Vim), desmin and myosin heavy chain. Fibroblasts in murine kidneys stain with fibroblasts specific protein FSP-1, and the mesenchymal markers α ASM and Vim. In normal kidneys Vim+ fibroblast like cells occur as in dermis. Yet is this the case in diseased kidneys? Okada et al. [20] looked at Goodpasture syndrome in rats and found α -SMA+ FbLCs negative for Vim and negative for TGF β , and other fibroblasts that were negative for α -SMA and for Vim that were producing α 1(I)procollagen! So they have reminded us that the exact contribution of α -SMA+ FbCLs to fibrogenesis still requires appraisal [21]. In their study, SMA-negative fibroblasts were producing type I collagen. The MF is a motile cell and further gene changes may be required before collagen is synthesised. That renal tubular epithelial cells can transdifferentiate into MFs expressing FSP-1, α SMA and Vim has been proposed for some time and the evidence is persuasive [22–24]. (i) There is loss of epithelial cell adhesion, and indeed TGF β suppresses expression of E-cadherins. (ii) The tubular cells express α -SMA and undergo actin reorganisation. (iii) There is disruption of tubular basement membrane. (iv) There is cell migration and invasion of the interstitium by MFs. MFs are morphologically intermediate between fibroblasts and smooth muscle cells and they have the ability to contract. Moreover, they form fibronectin and collagens I and III to add to the extracellular matrix.

Epithelial-mesenchymal transformation (EMT) is produced most efficaciously by TGF β acting with epidermal growth factor (EGF) [22]. PDGF-BB administered to rats induces a dose dependent proliferation of tubulointerstitial cells associated with expression of α -SMA [26]. Therefore, PDGF-BB actin along with TGF β may be the usual

Table 1. Processes leading to tubulo-interstitial fibrosis

- 1 Hydraulic pressure damaging glomeruli and postglomerular capillaries with ischaemia and hypoxia [3] affecting the tubules
Along with hypertension, angiotensin II [4] has a key role
- 2 Leakage of proteins into the tubules [5]
That means exposure of the tubules internally to arachidonic acid products [6], to lysophosphatidic acid [7] and complement components [8]
- 3 Inflammatory cells in nonimmune [9] and immune reactions, producing oxidative stress [10], exacerbated by oxidise LDL [11] and available cholesterol
Calcium is often deposited in tubular cells
- 4 Liberation of metalloproteinases [12] that damage tubular basement membranes
Mast cells producing [13] tryptase

cause of EMT. PDGF-BB receptors are plentiful on MFs and TGF β receptors are upregulated on all cells in areas of renal damage. Connective tissue growth factor is strongly expressed in resident cells of the kidney at sites of tubulointerstitial damage but not by monocyte-macrophages. CTGF helps cell migration and formation of collagen and fibronectin, but it does not aid proliferation of cells and it does not induce MFs [27]. The products of Th-2 lymphocytes like interleukin-4 (IL-4) and the chemokine monocyte-chemotactic protein MCP-1 have an established place in pulmonary fibroses [28]. Therefore, it has been suspected that although IL-4 administration may reduce proteinuria experimentally, it could go on to induce renal fibrosis. So it is interesting that Sommer et al. [19] find that IL-4 costimulates PDGF-BB or bFGF mediated proliferation of mesangial cells and MFs. Also interesting is their demonstration that PDGF-BB or bFGF support TNF α mediated transdifferentiation of endothelial cells into MFs [19].

What can be done about pulmonary fibrosis [29] could impart some ideas to those studying kidneys, for example the use of pirfenidone. Nephrology has yielded some surprises too, for now we are contemplating the use of hepatocyte growth factor (HGF) to offset renal fibrosis [30–31] and relaxin could have something to offer [32]. For those who prefer drugs that are available, there is the news that fluvastatin suppresses interstitial expansion and MF formation in obstructed kidneys of mice [33]. That is supported by evidence from Melbourne that lovastatin can decrease MF proliferation and growth. It also decreases collagen synthesis [34]. The theoretical background to such wondrous effects has recently been reviewed [35], but we shall have to look out for side effects.

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