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Transforming growth factor-β1 regulates chemokine and complement production by human proximal tubular epithelial cells

JORT S.J. GERRITSMA, CEES VAN KOOTEN, ARNOUT F. GERRITSEN, LEENDERT A. VAN ES, and MOHAMED R. DAHA

Department of Nephrology, Leiden University Hospital, Leiden, The Netherlands

Transforming growth factor-β1 regulates chemokine and complement production by human proximal tubular epithelial cells. Previously it has been demonstrated that human proximal tubular epithelial cells (PTEC) are able to produce chemokines (such as IL-8 and MCP-1) and complement components (such as C2, C3, C4 and factor H), and that production of these proteins is regulated by pro-inflammatory cytokines such as interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). Since TGF-β is also expressed in the renal interstitium during inflammation, we investigated the effect of TGF-β on the production of chemokines and complement components by PTEC in culture. Transforming growth factor-β1 up-regulated IL-8 production by an average of 4.11 ± 1.0-fold. macrophage chemoattractant phagocyte (MCP-1) production, on the other hand, was down-regulated by TGF-β1 by an average of 2.2 ± 0.7-fold. The production of C3 and C4 was also down-regulated after incubation with TGF-β1 (1.9 ± 0.3- and 3.0 ± 1.2-fold, respectively). All effects were dose- and time-dependent and were found to be specific for TGF-β1, as assessed by inhibition of the effect with a neutralizing antibody against TGF-β1. These data, together with the knowledge that TGF-β, chemokines and complement components play a role in several types of renal disease, suggest that TGF-β is involved in the regulation of local expression of chemokines and complement components by tubular cells.

The transforming growth factor-beta (TGF)-β family is composed of a group of pleiotropic cytokines that are involved in proliferative, inductive and regulatory effects on a wide variety of cell types [1, 2]. Transforming growth factor-β is an important immunomodulator, influencing inflammatory events during various phases of inflammation [3, 4]. It is known, for instance, that TGF-B regulates the production of acute phase proteins, and it is involved in monocyte recruitment and activation [5–7]. For monocytes, TGF-β has been shown to down-regulate the expression of CD32 (IgG Fc receptor type II) and CD89 (FcαR) [8, 9]. Furthermore, TGF-β inhibits complement C3 gene expression in astrocytes [10]. In addition, it has been shown that TGF-β regulates the induction of chemokines in neutrophils, bone marrow stromal cells, astrocytes and osteocytes [11–14]. In renal disease it has been shown that TGF-β plays an important role in the fibrogenesis of renal diseases [15, 16]. Expression of TGF-β mRNA in renal tissue has been demonstrated in biopsies of patients with IgA nephropathy [17], interstitial fibrosis [18] and in acute and chronic renal allograft rejection [19, 20]. It is thought that regulation of extracellular matrix production is one of the main effects of TGF-β in the kidney [21–25]. However, since TGF-β is involved in inflammatory processes, such as leukocyte attraction and acute phase protein production, we hypothesized that TGF-β might also be involved in the regulation of chemokine and complement production by renal cells. It has been reported that expression of these factors is found in renal tissue in different renal diseases, such as renal transplant rejection and tubulointerstitial nephritis [26–36].

Previously we have demonstrated that human proximal tubular epithelial cells are able to produce chemokines like macrophage chemoattractant phagocyte-1 (MCP-1), interleukin-8 (IL-8) and RANTES, and complement components C2, C3, C4, factor H and factor B [37–41]. In the present study we found that TGF-β1 enhances IL-8 production by PTEC in culture, while it down-regulates MCP-1 and complement synthesis.

METHODS

Proximal tubular epithelial cell cultures

Seven different primary human proximal tubular epithelial cell (PTEC) lines were obtained from kidney tissue anatomically unsuitable for transplantation or from healthy nephrectomy specimens as described previously [38, 42]. In brief, PTEC monolayers were cultured in serum-free DMEM/Ham's F12 media in a 1:1 ratio (Seromed Biochem KG, Berlin, Germany) supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), tri-iodothyronine (40 pg/ml) and epidermal growth factor (10 ng/ml) (all from Sigma Chemical Co., St. Louis, MO, USA). The primary cell lines were grown on wells coated with a matrix of bovine collagen type I (Sigma Chemical Co.) and decomplemented fetal calf serum (ΔFCS) (Life Technologies Inc., Gaithersburg, MD, USA) in serum free medium.

Explanted cultures were characterized by their morphological appearance, immunofluorescence [42–44] and by FACS analysis with antibodies directed to two surface peptidases, namely anti-adenosine deaminase binding protein antibody (ADBP/CD26;

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Table 1. Production of IL-8 and MCP-1 by different PTEC lines for 72 hours, in the presence or absence of 1.8 ng/ml TGF-β1

	IL-8 *ng/ml*		MCP-1 *ng/ml*	
Cell line	Medium	TGF-β1	Medium	TGF-β1
PTEC-1	3.1 ± 0.5	8.1 ± 0.3	4.3 ± 0.2	2.2 ± 0.1
PTEC-2	2.7 ± 0.4	16.0 ± 1.5	5.2 ± 0.3	1.8 ± 0.1
PTEC-3	2.0 ± 0.4	7.3 ± 0.7	3.1 ± 0.2	0.9 ± 0.1
PTEC-4	4.2 ± 0.3	20.1 ± 2.1	9.5 ± 0.4	6.0 ± 0.6
PTEC-5	1.0 ± 0.2	4.9 ± 0.5	2.2 ± 0.3	0.9 ± 0.2
PTEC-6	2.3 ± 0.3	7.5 ± 0.9	7.3 ± 0.5	5.4 ± 0.4
PTEC-7	3.7 ± 0.2	15.4 ± 1.3	4.5 ± 0.3	2.3 ± 0.4

Data are mean ± SD ($N = 3$). Abbreviations are: IL-8, interleukin-8; MCP-1, macrophage-1; PTEC, proximal tubular epithelial cell; TGF-β1, transforming growth factor-β1.

Table 2. Production of complement C3 and C4 by different PTEC lines for 72 hours, in the presence or absence of 1.8 ng/ml TGF-β1

	Complement C3 *ng/ml*		Complement C4 *pg/ml*	
Cell line	Medium	TGF-β1	Medium	TGF-β1
PTEC-1	34.6 ± 1.6	18.2 ± 2.1	651 ± 10.2	222 ± 10.2
PTEC-2	15.5 ± 1.5	6.1 ± 1.0	1255 ± 10.6	350 ± 9.8
PTEC-3	21.9 ± 2.3	13.1 ± 1.2	905 ± 10.3	188 ± 5.6
PTEC-4	13.5 ± 1.2	7.3 ± 0.9	< 100[a]	< 100[a]
PTEC-5	7.6 ± 1.1	3.8 ± 1.0	< 100[a]	< 100[a]
PTEC-6	18.1 ± 2.1	9.5 ± 1.3	346 ± 7.5	208 ± 8.9
PTEC-7	16.9 ± 1.6	8.7 ± 2.0	222 ± 7.3	< 100[a]

Data are mean ± SD ($N = 3$). Abbreviations are in Table 1.
[a] Lower than the detection limit of the ELISA.

kindly provided by Dr. Dinjens, University Hospital Maastricht, the Netherlands), and anti-leucine amino peptidase antibody (LAP; kindly provided by Dr. De Broe, University of Antwerp, Belgium) [40, 45]. Proximal tubular epithelial cells used for the experiments were cultured in 24-well tissue culture plates in serum free medium and were from the third to seventh passages. As judged from the expression of the two surface peptidases CD26 and LAP, no detectable changes in the phenotype of the cells occurred during passage of the cells.

Stimulation with transforming growth factor-β1

Proximal tubular epithelial cells grown in T25 flasks were trypsinized with 0.05% (wt/vol) trypsin/0.02% (wt/vol) EDTA (Sigma) and seeded in 24-well plates at a cell concentration of 1×10^5 cells per well. After 24 hours the wells were washed with serum-free culture medium and incubated with 1 ml culture medium supplemented with biologically active TGF-β1 or TGF-β2 (R&D Systems, Abingdon, UK). The supernatants were harvested and assessed for the chemokines IL-8, MCP-1 and RANTES and complement components C2, C3, C4, factor H, all by sandwich ELISA. The cells in all wells were trypsinized and counted using a Coulter Counter (Coulter Electronics, Mijdrecht, The Netherlands).

In selected experiments PTEC were incubated with TGF-β1 in the presence or absence of a neutralizing monoclonal antibody 2G7 directed against TGF-β1 [46]. After 48 hours of incubation the supernatants were harvested and assessed for chemokine and complement production. All experiments were performed in triplicate.

Chemokine sandwich ELISAs

The chemokines MCP-1, IL-8 and RANTES were quantified using sandwich ELISAs as described previously [39, 47]. In brief, wells of 96-well microtiter plates (Greiner, Alphen aan de Rijn, the Netherlands) were coated with a MoAb anti-human MCP-1 (R&D Systems), MoAb anti-IL-8 (CLB, Amsterdam, the Netherlands) or MoAb anti-RANTES (R&D Systems) overnight at room temperature. Nonspecific binding sites were blocked with PBS/0.01% Tween/2% casein. After one hour of incubation at 37°C the plates were washed with PBS/0.01% Tween and samples were added to the wells and incubated for one hour at 37°C. The plates were washed and incubated subsequently with rabbit anti-MCP-1, anti-IL-8 or anti-RANTES for one hour at 37°C, washed again and reacted with goat anti-rabbit IgG-HRP (Jackson Immuno Research Lab. Inc., West Grove, PA, USA). Bound HRP was developed using 2,2′-Azino-bis(3-Ethylbenzthiazoline-6-sulfonic acid) (Sigma) as a substrate followed by measurement of the optical density at 415 nm. A standard curve was constructed using a standard with known concentrations of MCP-1, IL-8 or recombinant human RANTES (PreproTech, London, UK).

Complement sandwich ELISAs

Complement components C2, C3, C4, and factor H were quantified by sandwich ELISA as described previously [40, 41]. In brief, wells of 96-well microtiter plates (Greiner) were coated with affinity-purified polyclonal anti-human C2, C3, C4 or factor H for two hours at 37°C, no additional saturation step were required. After washing, 100 μl samples were diluted appropriately to fall within the standard range and incubated for one hour at 37°C. The plates were washed and incubated subsequently with a corresponding digoxigenin (DIG)-conjugated affinity-purified rabbit anti-human complement C2, C3, C4, or factor H and horseradish-peroxidase conjugated sheep $F(ab)_2$ anti-DIG polyclonal antibody (Boehringer Mannheim Biochemica). The plates were developed using 2,2′-Azino-bis(3-Ethyl benzthiazoline-6-sulfonic acid; Sigma) as a substrate and the optical density was measured at 415 nm. A standard curve was constructed using pooled normal human serum with known concentrations of the various complement components.

RESULTS

Dose dependent response to transforming growth factor-β1

To determine whether the effect of TGF-β1 on chemokine and complement production was dose-dependent, three PTEC lines (numbers 1, 2 and 3 in Table 1) were exposed to increasing concentrations of TGF-β1 (0.3 to 1.8 ng/ml TGF-β1) and after 72 hours the supernatants were collected and assessed for IL-8, MCP-1, C3 and C4 production. As depicted in Figure 1A, TGF-β1 induced a dose-dependent increase of IL-8 production in all three PTEC lines. The production of MCP-1, however, was also decreased in a dose dependent manner (Fig. 1B). Similar results were obtained for C3 and C4 production (Fig. 1C, D). Suboptimal effects of TGF-β1 on chemokine and complement production were obtained after incubation with 0.6 ng/ml TGF-β1.

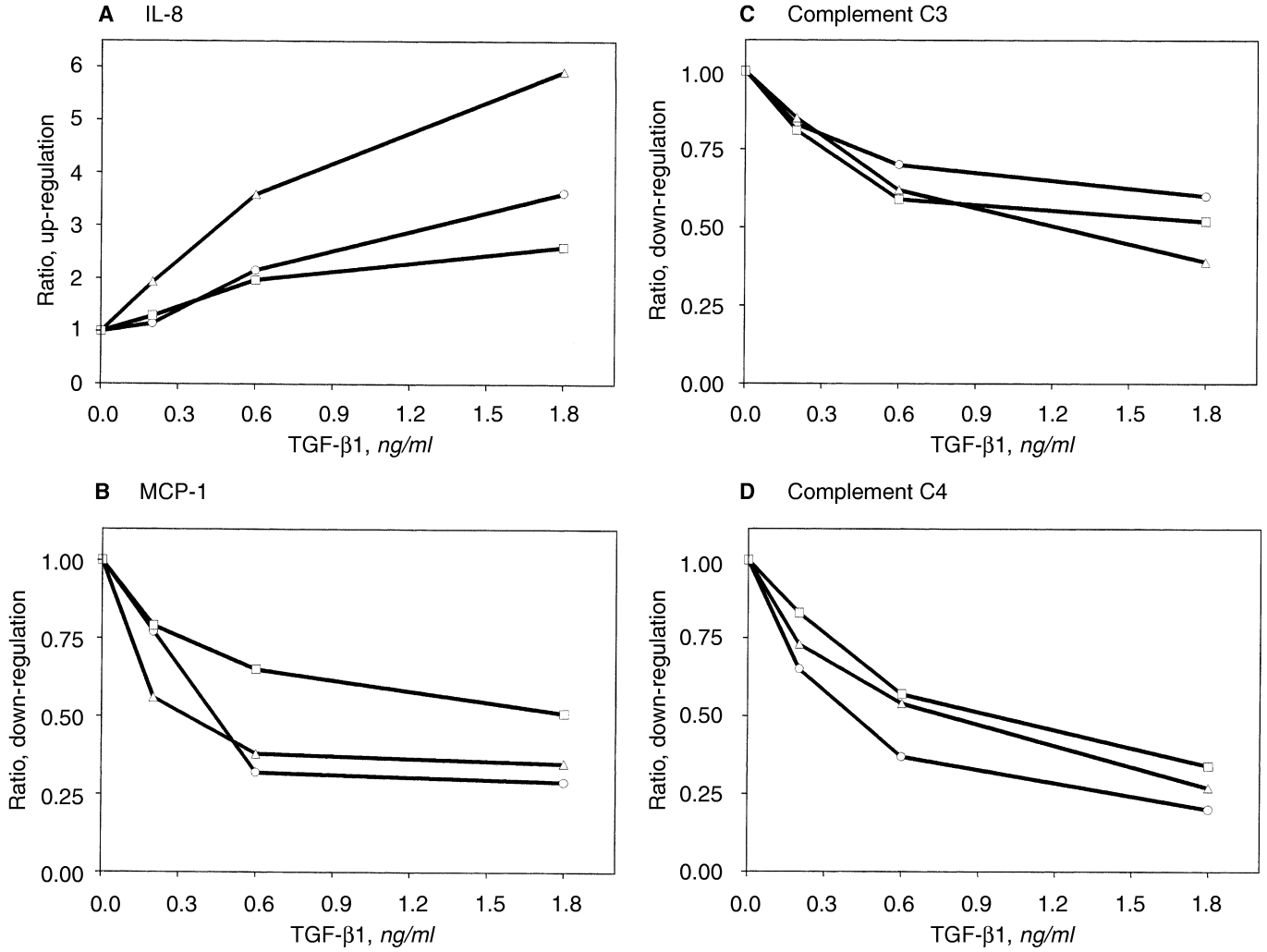

Fig. 1. Effect of increasing concentrations of transforming growth factor-B1 (TGF-β1) on chemokine and complement production by proximal tubule epithelial cells (PTEC). The PTEC lines 1 (□), 2 (△) and 3 (○) were cultured in the presence of increasing concentrations of TGF-β1, and after 72 hours the supernatants were harvested and assessed for interleukin-8 (IL-8; *A*), macrophage chemattractant phagocyte-1 (MCP-1; *B*), and complements C3 (*C*) and C4 (*D*) production by sandwich ELISA. The production in the absence of TGF-β1 for each of the cell lines was adjusted at 1.0. Increases or decreases relative to the baseline production are indicated on the ordinate, while TGF-β concentrations are depicted on the abscissa. Each point represents the mean ± SD ($N = 3$).

Effect of transforming growth factor-β1 on chemokine and complement production

To further analyze the effect of TGF-β1 on human proximal tubular epithelial cells (PTEC), subcultures of seven different primary PTEC lines were incubated with 1.8 ng/ml TGF-β1. After 72 hours the supernatants were harvested and assessed for the chemokines IL-8, MCP-1 and RANTES, and complement components C2, C3, C4, and factor H. The production of IL-8 by the different cell lines varied from 1.0 ± 0.2 to 4.2 ± 0.3 ng/ml IL-8 per 72 hours. The presence of 1.8 ng/ml TGF-β1 resulted in increased production of IL-8 in all seven PTEC lines with an average of 4.11 ± 1.0-fold increase (Table 1). The baseline production of MCP-1 ranged between 2.2 ± 0.3 and 9.5 ± 0.4 ng/ml MCP-1 per 72 hours. Incubation with TGF-β1 resulted in a decreased production with an average of 2.2 ± 0.7-fold. No effect of TGF-β1 was observed on RANTES production. In the case of complement production, the baseline production was variable between the seven lines, and again down-regulation of the production of complement C3 and C4 was observed after incubation with TGF-β1. In all PTEC lines the C3 production was decreased after incubation with TGF-β1 by an average of 1.9 ± 0.3-fold (Table 2). Production of C4 was observed in five out of seven PTEC lines. Incubation with TGF-β1 resulted in decrease of the production C4 by an average of 3.0 ± 1.2-fold. No detectable C2 production was found in either of the seven PTEC lines. Factor H production was found in six out of seven lines, however, no up- or down-regulation of production was seen with TGF-β1 (data not shown).

Kinetics of the transforming growth factor-β1 induced effects

The regulation of TGF-β1 was found to be time-dependent. After incubation with 0.6 ng/ml TGF-β1 for 24, 48 and 72 hours, a time-dependent increase of IL-8 production and a time dependent decrease of MCP-1 production was observed (Fig. 2 A, B).

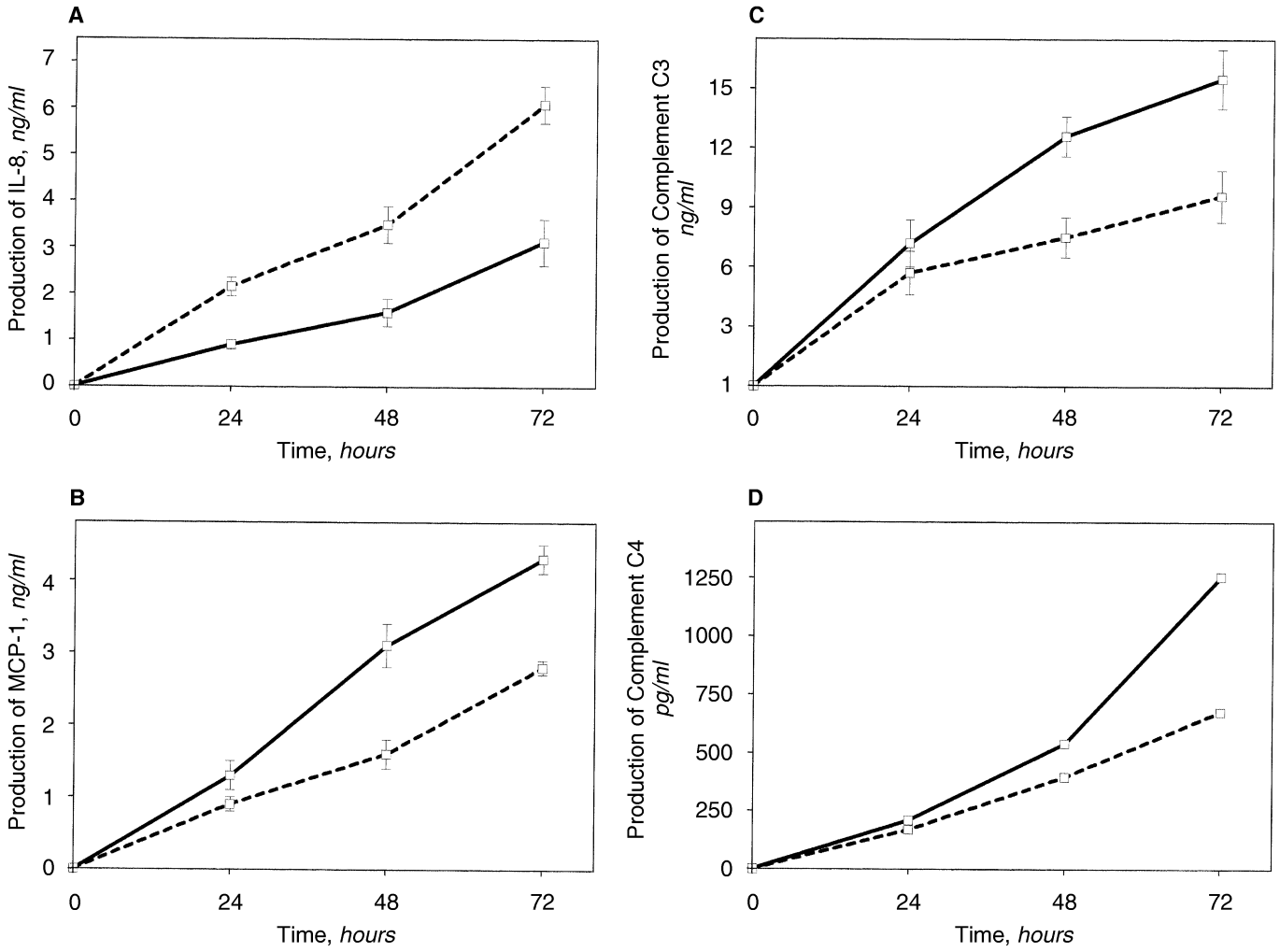

Fig. 2. Kinetics of chemokine and complement production by proximal tubule epithelial cells (PTEC) in the presence or absence of transforming growth factor-B1 (TGF-β1). The PTEC lines 1 and 2 were incubated for various time periods in the absence (solid line) or presence (dashed line) of 0.6 ng/ml TGF-β1. At each time point the supernatants were collected and assessed for the production of interleukin-8 (IL-8; *A*), macrophage chemoattractant phagocyte-1 (MCP-1; *B*), and complements C3 (*C*) and C4 (*D*). Data are depicted for PTEC-1 (A) and (B) and for PTEC-2 (C) and (D). Each point represents the mean ± SD ($N = 3$).

Already at 24 hours a 2.4-fold increase in the IL-8 production could be observed, whereas the maximal decrease in MCP-1 production was observed at 48 hours. The results of the kinetic experiments for complement components C3 and C4 are depicted in Figure 3, C and D. For both C3 and C4 a maximal decrease in the production was observed at 72 hours with an 1.7- and 1.8-fold decrease, respectively.

Inhibition of the effect of transforming growth factor-β1 by a monoclonal antibody against TGF-β1

To determine the specificity of the observed effects of TGF-β1 on chemokine and complement production, PTEC lines 1 and 2 were incubated with 0.6 ng/ml TGF-β1 in the presence or absence of a neutralizing anti-TGF-β1 monoclonal antibody 2G7 [41]. After 48 hours the supernatants were harvested and assessed for IL-8, MCP-1, C3 and C4 production. The effect of TGF-β1 on IL-8, MCP-1, C3 and C4 production was completely abrogated by the neutralizing anti-TGF-β antibody, as depicted for IL-8 and MCP-1 in Figure 3. A control antibody had no detectable effect on the TGF-β1 induced effects. Results similar to that for MCP-1 were obtained for C3 and C4, namely, that the neutralizing antibody abrogated the TGF-β1 down-regulation. No effect was observed with a control antibody.

DISCUSSION

Progressive renal disease such as glomerulonephritis and diabetic nephropathy are characterized by glomerulosclerosis and tubular atrophy. Several studies have suggested that TGF-β has an important role in the pathogenesis of these diseases [15, 18, 48]. Transforming growth factor-β has been described as a modulator of the synthesis of extracellular matrix components [49, 50]. In the present study we analyzed the effect of TGF-β1 on the production of chemokines and complement by renal cells *in vitro*. Previously we described the production and regulation of chemokine and complement components by PTEC [37–39, 41, 51]. The pro-inflammatory cytokines IL-1α and TNF-α were shown to

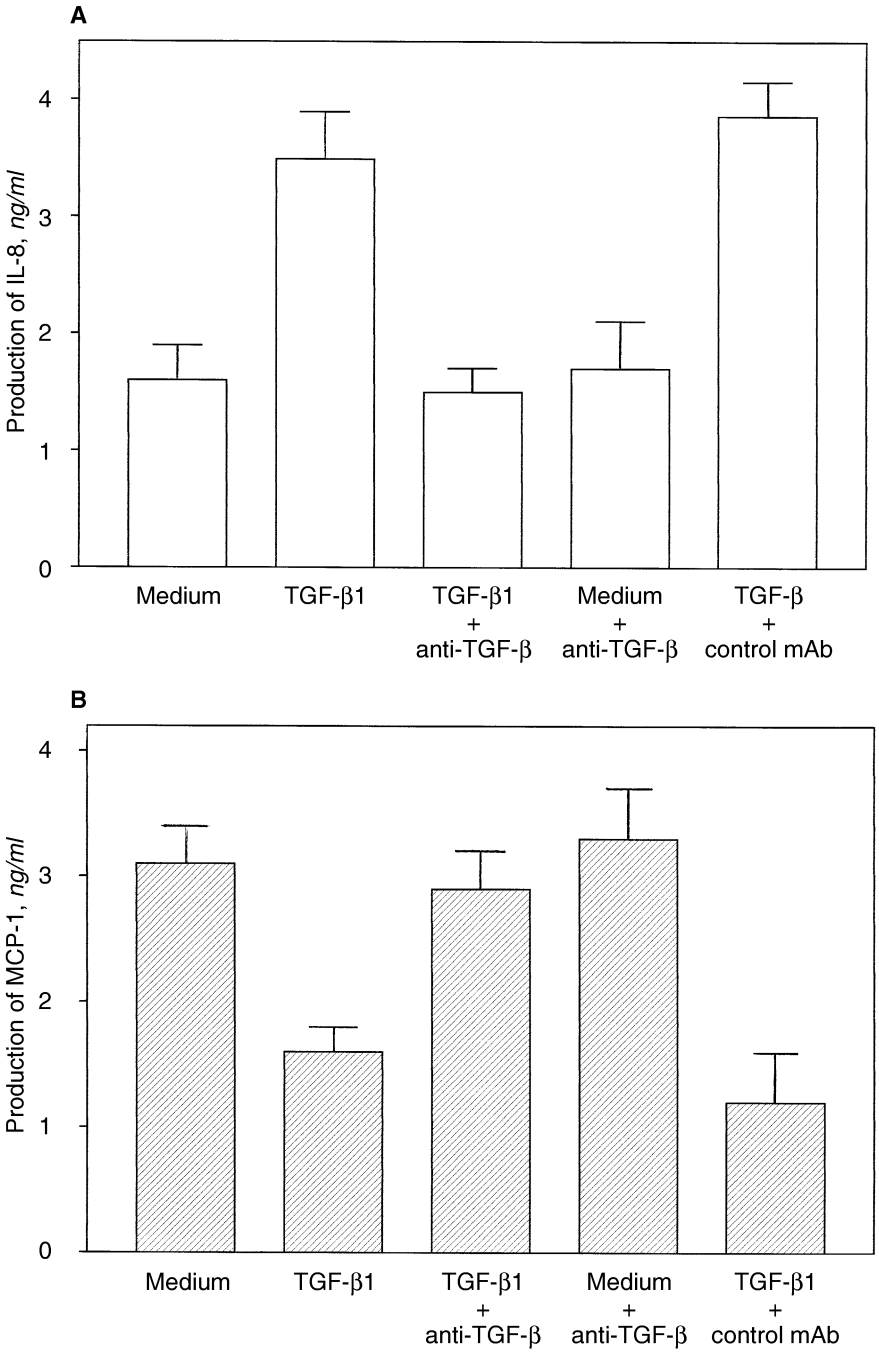

Fig. 3. Effect of a monoclonal antibody (mAb) anti-TGF-β on the transforming growth factor-B1 (TGF-β1) mediated production of interleukin-8 (IL-8; *A*) and macrophage chemoattractant phagocyte-1 (MCP-1; *B*). Proximal tubular epithelial cells-1 (PTEC-1) were incubated in the absence or presence of 0.6 ng/ml TGF-β1 alone or together with anti-TGF-β mAb 2G7 or control mAb. After 48 hours the supernatants were collected and assessed for chemokine production. Each bar represents the mean ± SD (N = 3).

up-regulate the production of IL-8 and MCP-1, whereas IFN-γ down-regulated IL-8 production and up-regulated MCP-1 production by PTEC. These data demonstrate that there is a tight balance between the production of IL-8, MCP-1 and the influx of monocytes or polymorphonuclear cells (PMNs).

It is known that TGF-β has a dual effect, for instance, it inhibits the proliferation of mesangial cells at high concentrations, it has a mitogenic effect at lower concentrations [52], and inhibits monocyte chemotaxis at picogram concentrations [7]. In the present study we found that over a dose response range of 0.2 to 1.8 ng/ml TGF-β1 up-regulated IL-8, while in the same dose response range a suppression of MCP-1 production was found, suggesting that the concentration of TGF-β1 at a certain site may influence the type of infiltrating inflammatory cells. At higher concentrations it was shown that TGF-β was able to inhibit the production of IL-8 by endothelial cells [53]. At higher concentrations of TGF-β1, namely at 3.0 and 6.0 ng/ml, plateau levels of effect on PTEC were seen for the various parameters studied. There were no significant differences compared to the use of the 1.8 ng/ml concentration of TGF-β1. Kinetic experiments revealed that there was a time-dependent effect of TGF-β1. Moreover, the time point at which the response to TGF-β1 was maximal differed

per protein, suggesting a differential regulation by TGF-β1. Incubation of PTEC with TGF-β1 in the presence of a neutralizing antibody against TGF-β supported the notion that the effects observed were specific for TGF-β1. Interesting was the observation that anti-TGF-β1 did not alter the spontaneous production of IL-8 or MCP-1. Since significant effects of TGF-β1 on chemokine production by PTEC were observed at doses higher than 0.6 ng/ml, we feel that the contribution of TGF-β1 produced in the system does not substantialy influence the effect of exogenously added TGF-β1. In accordance with that hypothesis, supernatants of PTEC cultured under serum free conditions contained less than 50 pg/ml of TGF-β1.

In previous studies we demonstrated that cytokines, such as IL-1α, IL-2 and interferon-gamma (IFN-γ), were able to up-regulate the production of either C3 or C4 [40–42, 54]. Since TGF-β has been shown to be present in the various forms of renal disease [15, 17–19], one could speculate that the production of the complement components can be regulated by TGF-β1 or by other cytokines. In the present study TGF-β1 reduced the production of C3 and C4 in a dose- and time-dependent fashion. No effects were observed for the other complement components known to be produced by PTEC such as C2 and factor H. Expression of complement C3 and C4 has been found in various renal diseases such as systemic lupus erythematosis (SLE), membranoproliferative glomerulonephritis, tubulointerstitial nephritis and renal allograft rejection [33, 55–57]. A dual effect of TGF-β on complement C3 production was demonstrated for two different cell types. Transforming growth factor-β was shown to induce C3 synthesis in monocytes, whereas in astrocytes an inhibition of cytokine-induced C3 expression was found [10, 58]. In this respect a number of the effects of TGFβ-1 on PTEC are in agreement with the expectation upon down-regulation of MCP-1 and up-regulation of IL-8. However, the effects on complement production are different from that observed in monocytes. Additional studies are required to analyze the reason for these differences.

The role of TGF-β on tubular cells is not only restricted to chemokine and complement production. Transforming growth factor-β has also been shown to play an role on the production of extracellular matrix proteins by several tubular cells of non-human origin [59, 60]. Furthermore, it has been shown that rabbit proximal tubular epithelial cells possess a receptor for TGF-β [61]. Since mRNA expression of TGF-β occurs in several types of glomerulonephritis and renal allograft rejection, and because the expression of chemokines and complement component mRNAs *in situ* in tubular cells in several types of glomerular nephritis and renal allograft rejection have been reported [26, 33, 55, 62–64], it might be possible that TGF-β is involved in the local regulation of chemokine and complement component production by tubular cells. In the present study the major emphasis was placed on the effect of TGF-β1 on PTEC. Studies employing TGF-β2 indicated similar degrees of up-regulation of IL-8 as that seen with TGF-β1. Optimal effects of TGF-β2 were found in a dose range of 0.3 to 2.6 ng/ml, both for IL-8 up-regulation and for MCP-1 down-regulation. Unfortunately, we did not have access to TGF-β3.

The data obtained in this study, together with our previous studies on cytokine regulated expression of chemokines and complement production by PTEC [37, 38, 40, 41], suggest that a relationship between the expression of chemokines and complement by tubular cells and the cytokines expressed during this inflammation might exist together to regulate the influx of inflammatory cells and degree of local inflammation.

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Reprint requests to Dr. Mohamed R. Daha, Department of Nephrology, Leiden University Hospital, PO Box 9600, 2300 RC Leiden, The Netherlands. E-mail: M.R.Daha@Nephrology.MedFac.LeidenUniv.nl

APPENDIX

Abbreviations used in this study are: ADBP/CD26, anti-adenosine deaminase binding protein antibody; CD32, IgG Fc receptor type II; CD89, FcαR; ΔFCS, decomplimented fetal calf serum; IL-8, interleukin-8; LAP, anti-leucine amino peptidase antibody; MCP-1, macrophage chemoattractant phagocyte-1; MoAb, monoclonal antibody; PMN, polymorphonuclear; PTEC, proximal tubular epithelial cells; SLE, systemic lupus erythematosis.

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