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Regulation and production of IL-8 by human proximal tubular epithelial cells *in vitro*

J. S. J. GERRITSMA, P. S. HIEMSTRA*, A. F. GERRITSEN, W. PRODJOSUDJADI, C. L. VERWEIJ†, L. A. VAN ES & M. R. DAHA *Department of Nephrology, *Pulmonology and †Rheumatology, Leiden University Hospital, Leiden, The Netherlands*

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SUMMARY

A number of inflammatory kidney diseases are associated with interstitial nephritis and influx of leucocytes in the renal interstitium. Potentially the influx of neutrophils in the interstitium may be induced by the chemotactic cytokine IL-8. In the present study we have analysed the production of IL-8 by cultured human proximal tubular epithelial cells (PTEC) in response to a number of proinflammatory cytokines. Primary cell lines of proximal tubular epithelium obtained from ten different kidneys, and cultured under serum-free conditions, were found to produce IL-8 to different degrees from not detectable levels up to $10{\cdot}8 \pm 1{\cdot}5$ ng IL-8 per 1×10^5 cells in 72 h. Gel filtration chromatography of PTEC supernatant indicated that the size of IL-8 of PTEC is 15·1 and 8·1 kD, and is chemotactically active for polymorphonuclear neutrophils (PMN). Addition of 0·5 ng/ml rIL-1α or 1000 U/ml recombinant tumour necrosis factor-alpha (TNF-α) to the culture media of PTEC induced an up-regulation of IL-8 production up to 6·3-fold and 3·0-fold, respectively. The up-regulation by IL-1α and TNF-α was dose- and time-dependent. In contrast, 500 U/ml recombinant interferon-gamma (rIFN-γ) down-regulated the production of IL-8 3·4-fold. Northern blot analysis showed that IL-1α and TNF-α increased the expression of IL-8 mRNA, whereas IFN-γ reduced IL-8 mRNA expression. Taken together, these experiments indicate that human PTEC are a potential source of IL-8 in the kidney, and that IL-8 produced in the proximal tubule can be induced by various proinflammatory cytokines.

Keywords proximal tubular epithelial cells IL-8 cytokines

INTRODUCTION

Neutrophils are a predominant cell type in inflammatory lesions. Recruitment of these neutrophils is dependent on local production of chemotactic factors at the site of inflammation. One of the chemotactic factors which may be responsible for the attraction of neutrophils is IL-8 [1], also known as monocyte-driven neutrophil chemotactic factor (MDNCF) [2] or neutrophil-activating factor (NAF) [3]. IL-8 is produced by various cell types, including monocytes but also by other lymphoid cells, endothelial cells, keratinocytes, epithelial cells and various tumour cell lines [1,4,5], and its synthesis is induced by stimuli such as lipopolysaccharide (LPS), tumour necrosis factor-alpha (TNF-α) and IL-1α. IL-8 promotes the directed migration of neutrophils, basophils and T cells along a concentration gradient to the site of inflammation. IL-8 activity is not limited to chemotaxis alone, because *in vitro* it may also induce the generation of reactive oxygen intermediates, degranulation, and an increase in cytosolic calcium in neutrophils [6,7]. The structure of crystalline IL-8 corresponds to a dimeric molecule. The secondary structure of the monomer consists of three β-sheets and one α-helix, which together form a HLA class I type structure [8,9]. The structure of this dimer and the interhelix distance may be important for the biological activity of IL-8. However, in a recent study it was shown that monomeric IL-8 also is functionally active [10].

Renal interstitial inflammation is associated with the presence of leucocytes of both mononuclear and polymorphonuclear morphology in the interstitium. The mechanism by which the leucocytes are attracted into the tissue is still unknown. Tubulointerstitial inflammation is found in a number of kidney diseases such as glomerulonephritis and pyelonephritis, but also transplant rejection and vasculitis. Inflammation at the local site in the kidney may be caused by several mechanisms, such as immune-mediated, infectionmediated or idiopathic [11]. The initiation of the inflammation and accumulation of proinflammatory cytokines may trigger the local synthesis of chemotactic factors such as IL-8. Due to the production of chemotactic factors, neutrophils are attracted to the site of inflammation and may induce renal damage. *In vitro* it has been shown that mesangial cells and

Correspondence: Jort S. J. Gerritsma, Leiden University Hospital, Department of Nephrology, Building 1 C3P, PO Box 9600, 2300 RC Leiden, The Netherlands.

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renal cell carcinomas can produce IL-8 in response to inflammatory cytokines [12–15]. Since it is known that human proximal tubular epithelial cells produce cytokines and complement components in response to inflammatory cytokines both *in vitro* and *in vivo* [16–19], we postulated that human proximal tubular epithelial cells (PTEC) potentially could be a source of IL-8. In addition, we investigated the effect of proinflammatory cytokines on the production of IL-8 by PTEC.

MATERIALS AND METHODS

PTEC cell cultures

Primary human PTEC cell lines were obtained according to the method described by Detrisac *et al.* [20] from kidney tissue not suitable for transplantation because of anatomical reasons, or from healthy nephrectomy specimens and as described previously [18]. In brief, PTEC monolayers were cultured in serum-free Dulbecco's modified Eagle's medium (DMEM)/Ham′F12 media in a 1:1 ratio (Seromed Biochem KG, Berlin, Germany) supplemented with insulin 5 μg/ml, transferrin 5 μg/ml, selenium 5 μg/ml, hydrocortisone 36 μg/ml, tri-iodothyronine 4 pg/ml, and epidermal growth factor 10 ng/ml (all from Sigma Chemical Co., St Louis, MO). The primary cell lines were grown on a matrix of bovine collagen (Vitrogen; Collagen Corporation, Palo Alto, CA) and decomplemented fetal calf serum (ΔFCS; Life Technologies Inc., Gaithersburg, MD). Explanted cultures were characterized by their morphological appearance as described by Detrisac *et al.* [20] and by immunofluorescence using various MoAbs (anti-cytokeratin; RGE53; Eurodiagnostics, Apeldoorn, The Netherlands; anti-epithelial membrane antigen: EMA; Dako, Glostrup, Denmark; and two anti-adenosine deaminase binding protein antibodies donated by Dr Dinjens, University Hospital Maastricht, The Netherlands). PTEC obtained between passages 2 and 6 were used for the experiments in either T25, T75 or 24-well tissue culture plates coated with ΔFCS alone (Costar, Cambridge, MA).

Stimulation with rIL-α, rTNF-α or IFN-γ

PTEC lines obtained from different kidney specimens grown in T25 flasks were trypsinized with 0·02% (w/v) EDTA/0·05% (w/v) trypsin (Sigma) and the cells were seeded in 24-well plates at a cell concentration of 1×10^5 cells/well. After 24 h the wells were washed and incubated with 1 ml culture medium supplemented with various concentrations of rIL-1α, rTNF-α or rIFN-γ (Genzyme, Uden, The Netherlands) for 72 h unless stated otherwise. After 72 h supernatants were harvested and assessed for IL-8 by an inhibition radioimmunoassay. The cells in all wells were trypsinized and counted using a Coulter Counter (Coulter Electronics, Mijdrecht, The Netherlands). All experiments were performed in duplicate.

In selected experiments the cells were incubated with rIL-1α, rTNF-α or IFN-γ in the presence or absence of rIL-1 receptor antagonist (12·5 μg/ml) or neutralizing polyclonal antibodies against TNF-α and IFN-γ (50 μg/ml). The supernatants were collected after 24 h and assessed for IL-8. The rIL-1 receptor antagonist was kindly provided by A. Steinkasserer (University of Oxford, UK) [21], and the neutralizing polyclonal antibodies were generated in our laboratory using rTNF-α or rIFN-γ. For this purpose, rabbits were immunized with either rTNF-α or rIFN-γ in Freund's complete adjuvant (FCA; Bacto-Difco Labs, Detroit, MI) and boosted with the cytokine in Freund;'s incomplete adjuvant (FIA). After 2 weeks the sera of the rabbits were collected and the IgG fraction was isolated by ion exchange chromatography.

RNA isolation and Northern blot analysis

Total RNA was isolated according to Chomczynski & Sacchi [22] from PTEC grown in T25 flasks in medium alone or supplemented with rIL-1α, rTNF-α or rIFN-γ after 48 h. Total RNA (15 μg) was separated on a 1% (w/v) agarose gel containing 2·2 M formaldehyde pH 4·0, MOPS buffer (0·02 M MOPS pH 7·0, 8 mM sodium acetate, 1 mM EDTA pH 8·0) and blotted to nitrocellulose as described by Maniatis *et al*. [23]. Prehybridization and hybridization were done in a hybridization mix consisting of 0·5 M sodium phosphate buffer pH 7·2, 7% (w/v) SDS, 1% (w/v) bovine serum albumin (BSA; Sigma) and 1 mM EDTA, 100 μg/ml single-stranded herring sperm DNA as described by Church & Gilbert [24]. After 2 h of prehybridization at 65°C a 1·2 kb cDNA probe specific for IL-8 (kindly provided by Dr J. Oppenheim) radiolabelled with α-^{32}P-dCTP by random primed labelling [25] was added and the blot was hybridized overnight at 65°C. After hybridization the blot was washed for 30 min with three wash buffers with decreasing molarity of the sodium phosphate buffer, respectively 0·5, 0·25, 0·1 M pH 7·2, 1% (w/v) SDS, 1 mM EDTA. Control hybridization for equal loading was performed using a 0·5 kb glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (no. 78105; American Type Culture Collection, Rockville, MD).

IL-8 inhibition radioimmunoassay

IL-8 was quantified using an inhibition radioimmunoassay (RIA) essentially as has been described for IL-1 [26]. A polyclonal rabbit IgG anti-IL-8 antibody (generated in our laboratory using rIL-8 (PeproTech Inc., Rocky Hill, NJ)) was incubated at room temperature with samples diluted in RIA buffer (0·01 M Na_2HPO_4, 0·14 M NaCl, 0·01 M EDTA, 0·02 M glycine, 0·01 M ϵ-amino-*n*-caproic acid, 0·001 MM NaN_3, 0·11% (w/v) human serum albumin (HSA; Sigma), pH 7·4). After 2 h of incubation iodinated rIL-8 was added and incubated at 4°C overnight. Complexed labelled rIL-8 was precipitated with goat anti-rabbit serum in the presence of 9% (w.v) polyethyleneglycol (PEG 6000). The assay was calibrated using a standard of known concentrations of unlabelled rIL-8. The range of the RIA was 11 ng to 350 pg IL-8 per ml.

Chemotaxis

To determine whether IL-8 produced by PTEC was biologically active, the chemotactic activity was determined using a modification of the method of Boyden [27] in a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD) [28]. In the upper chamber 1×10^5 freshly isolated polymorphonuclear neutrophils (PMN) were added per well. The upper and lower chamber were separated by a 8 μm pore size cellulose nitrate migration-filter (Sartorius AG, Göttingen, Germany; SM 113) and a 0·45 μm stop-filter (Millipore, Bedford, MA; type HA). In the lower chamber the PTEC supernatant was added in a dilution of 1:1 with incubation buffer (20 mM HEPES, 132 mM NaCl, 6 mM KCl, 1·2 mM KH_2PO_4, 1 mM $MgSO_4$ pH 7·4 and osmolarity 280–290 mOsm/kg containing a final concentration of 0·5% (w/v) HSA, 5·5 mM glucose and 1 mM $CaCl_2$). After 2 h of incubation at 37°C with humidity control the upper filter was fixed with an ethanol/butanol (20/80% w/v) fixation mixture and stained with Weigert solution. The filters were dehydrated with ethanol and made transparent with xylene followed by determination of the number of PMN per high power

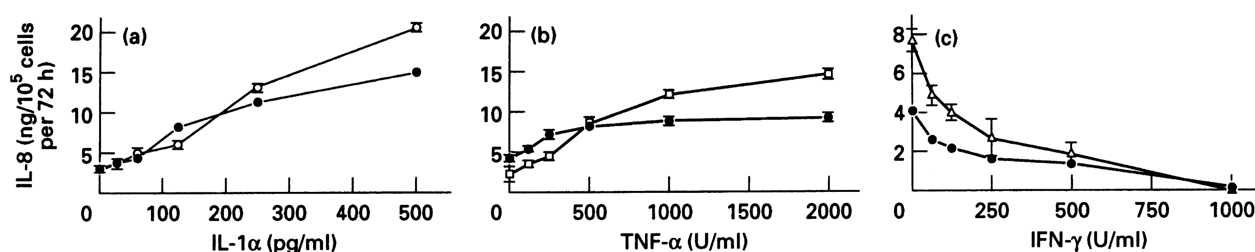

Fig. 1. Effect of increasing concentrations of recombinant (r) IL-1α (a), recombinant tumour necrosis factor-alpha (TNF-α) (b) and rIFN-γ (c) on the production of IL-8 by proximal tubular epithelial cells (PTEC) *in vitro* (cell lines no. 1 (○), 3 (□), 4 (△), and 10 (●)). The values are expressed as mean ± s.d. from two independent cultures.

field. As a positive control rIL-8 (100 ng/ml) was added in the lower chamber.

Gel filtration

Concentrated PTEC supernatant was separated on a 2·5 × 60 cm Superdex G-75 (Pharmacia Biotech Benelux, Roosendaal, The Netherlands) column that was equilibrated and run in 0·15 M NaCl, 0·05 M sodium phosphate buffer pH 7·2. The column was calibrated using proteins of known molecular weight. The fractions were tested for IL-8 using the IL-8 RIA and protein using a BCA protein assay (Pierce Chemical Co., Rockford, IL).

RESULTS

Production of IL-8 by PTEC

Cells (passages 4–5) from 10 human PTEC lines obtained from different kidneys were cultured in 24-well plates at a cell concentration of 1×10^5 cells/well and tested for their ability to produce IL-8 under basal conditions (Table 1). Most of the PTEC cell lines were able to produce IL-8 under basal conditions, while some cell lines did not show detectable IL-8 production. The presence of rIL-1α (0·5 ng/ml) or rTNF-α (1000 U/ml) for 72 h resulted in either an increase of IL-8 production or induction of production of IL-8, whereas addition of rIFN-γ (500 U/ml) down-regulated IL-8 production in the four cell lines which produced significant amounts of IL-8 under basal conditions. To determine the optimal concentrations for the effect of the different cytokines, a number of different PTEC cell lines were stimulated for 72 h with increasing concentrations of the three different cytokines. Production of IL-8 was up-regulated in a dose-dependent manner by rIL-1α and rTNF-α (Fig. 1a,b). Similar effects were also observed with eight other cell lines. rIFN-γ, however, down-regulated IL-8 production (Fig. 1c). This effect was also observed in two other cell lines. The kinetics of the increased IL-8 production by rIL-1α and rTNF-α as depicted in Fig. 2 showed an increase of 6·6-fold and 4·7-fold, respectively. To determine the specificity of the effect of the various cytokines on IL-8 production, blocking experiments were performed using neutralizing antibodies against rTNF-α and rIFN-γ (50 μg/ml) and a rIL-1 receptor antagonist (12·5 μg/ml) in the presence or absence of the corresponding cytokines (Fig. 3). The results demonstrate that the increased production of IL-8 induced by the different cytokines was blocked by the appropriate neutralizing antibody or receptor antagonist. Rabbit IgG anti-TNF-α, rabbit IgG anti-IFN-γ and rIL-1 receptor antagonist alone had no effect on IL-8 production by PTEC.

Table 1. Production of IL-8 by different proximal tubular epithelial cell (PTEC) lines in the presence or absence of inflammatory cytokines

Cell line number	Medium	IL-1α (0·5 ng/ml)	TNF-α 1000 U/ml)	IFN-γ (500 U/ml)
1	3·0 (0·42)*	20·4 (0·52)	ND	ND
2	<0·35	8·5 (0·21)	3·1 (0·50)	ND
3	2·3 (0·94)	ND	12·1 (0·16)	ND
4	7·7 (0·21)	ND	ND	1·8 (0·11)
5	<0·35	6·2 (0·30)	2·0 (0·21)	ND
6	2·4 (0·40)	15·4 (0·30)	9·7 (0·45)	ND
7	2·5 (0·50)	18·6 (0·70)	ND	ND
8	10·8 (1·5)	ND	30·0 (1·2)	<0·35
9	3·2 (0·51)	6·7 (0·60)	4·1 (0·50)	1·2 (0·30)
10	4·0 (0·42)	15·0 (0·43)	9·25 (0·55)	0·1 (0·50)

*Nanogram of IL-8 per 1×10^5 cells per 72 h. The mean ± s.d. (in parentheses) are presented.

<0·35 = lower than the detection limit of the IL-8 radioimmunoassay. ND, Not done.

Northern blot analysis

To determine whether rIL-1α, rTNF-α and rIFN-γ regulate IL-8 expression at the transcriptional level, total-RNA from a PTEC cell line cultured with or without rIL-1α (0·5 ng/ml), rTNF-α (1000 U/ml) or rIFN-γ (500 U/ml) for 48 h was analysed for the level of IL-8 mRNA expression by Northern blot analysis. The Northern blot was subsequently hybridized with a specific cDNA probe for IL-8

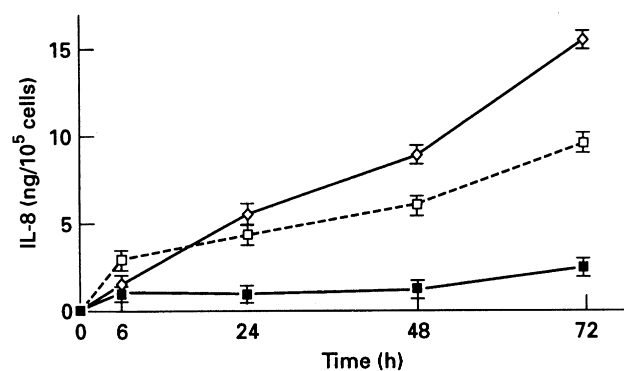

Fig. 2. Kinetics of IL-8 production by proximal tubular epithelial cells (PTEC) in the presence or absence of 0·5 ng/ml rIL-1α (◇) or 1000 U/ml recombinant tumour necrosis factor-alpha (rTNF-α) (□) (cell line no. 6). ■, Medium. The values are expressed as mean ± s.d. from two inde-dependent cultures.

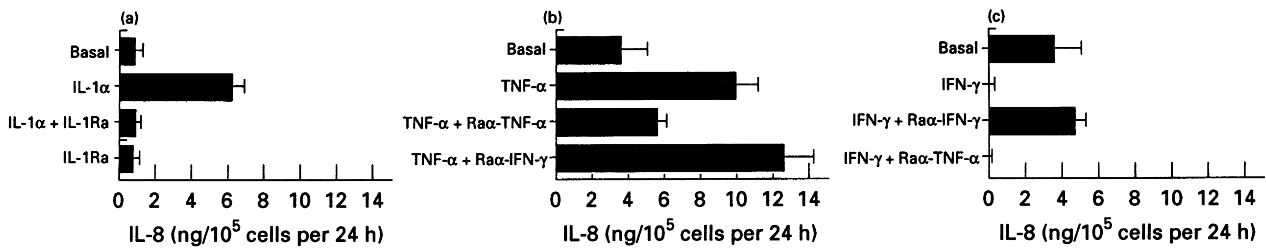

Fig. 3. Effect of rIL-1 receptor antagonist, rabbit IgG anti-tumour necrosis factor-alpha (TNF-α) and rabbit IgG anti-IFN-γ on rIL-1α-, rTNF-α- and rIFN-γ-mediated IL-8 production. Proximal tubular epithelial cell monolayers were incubated with (a) 0·5 ng/ml rIL-1α in the presence or absence of 12·5 μg/ml rIL-1 receptor antagonist; or with (b) 500 U/ml rTNF-α with or without 50 μg/ml rabbit IgG anti-TNF-α or rabbit IgG anti-IFN-γ; or with (c) 500 U/ml rIFN-γ with or without 50 μg/ml rabbit IgG anti-IFN-γ or rabbit IgG anti-TNF-α (cell line no. 7 and 8). The values represent the mean ± s.d. of two independent cultures.

and as an internal control the blot was hybridized with a cDNA probe for GAPDH. As shown in Fig. 4, PTEC cell line 9 expressed IL-8 mRNA under non-stimulating conditions. IL-8 mRNA expression was markedly up-regulated by rIL-1α and slightly by TNF-α, and the same effect was seen by cell line 5. The expression of basal IL-8 mRNA was down-regulated by rIFN-γ. The amount of mRNA blotted on the nitrocellulose was equal, as shown by hybridization with the internal control GAPDH (Fig. 4). In another cell line, which did not show production of IL-8 under non-stimulating conditions, no detectable expression of IL-8 mRNA and no effect of rIFN-γ on IL-8 mRNA were observed. In these two cell lines rIL-1α and rTNF-α enhanced the amount of IL-8 mRNA.

Characterization of IL-8

To determine the contribution of IL-8 to the neutrophil chemotactic activity of PTEC supernatant *in vitro*, chemotactic activity was determined. As shown in Fig. 5, IL-8 produced by PTEC *in vitro* had chemotactic activity comparable to rIL-8. The chemotactic activity of PTEC IL-8 could be blocked completely to background values following the addition of rabbit IgG anti-IL-8. Neither rabbit IgG anti-IL-8 nor normal rabbit IgG alone had any detectable effect on the migration of the PMN (data not shown).

To determine the molecular weight of IL-8, PTEC supernatant was subjected to gel filtration using a G-75 column, and the fractions were assessed for IL-8 antigen and protein (Fig. 6). IL-8 eluted from the column in two peaks, i.e. fractions 172–182 and 190–208, respectively, corresponding with a molecular weight of 15·1 kD for peak I and 8·1 kD for peak II.

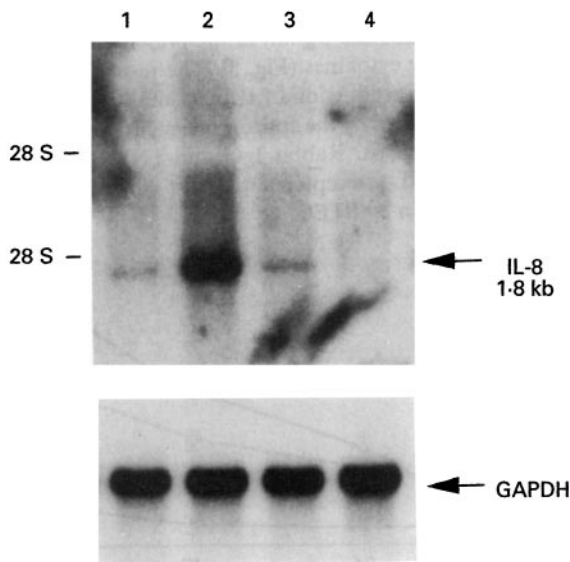

Fig. 4. Expression of IL-8 mRNA (cell line no. 9). Non-stimulated proximal tubular epithelial cells (PTEC) (lane 1) and PTEC stimulated with rIL-1α (lane 2), recombinant tumour necrosis factor-alpha (rTNF-α) (lane 3) or rIFN-γ (lane 4) for 48 h determined by Northern analysis. Equal amounts of mRNA were loaded on the gel as assessed by hybridization with a GAPDH cDNA probe.

DISCUSSION

Renal inflammation, such as found in patients with pyelonephritis, is associated with increased excretion of IL-8 in the urine. Detailed studies have shown that urinary IL-8 presumably is produced in the kidney because elevated plasma levels of IL-8 in patients with acute pyelonephritis correlate poorly with increased levels of IL-8 in the urine [29]. The cells in the kidney responsible for the production of IL-8 *in vivo* are unknown. It has been shown that human mesangial cells can produce IL-8 *in vitro* in response to LPS, IL-1 and TNF-α [12,13]. On the other hand, studies in patients with kidney transplant rejection have indicated that IL-8 staining in the tubuli is associated with renal inflammation [30]. Indeed, cultured renal cortical epithelial cells were shown to produce IL-8, though the type of epithelial cells was not defined in detail. Furthermore, these cells were cultured in medium

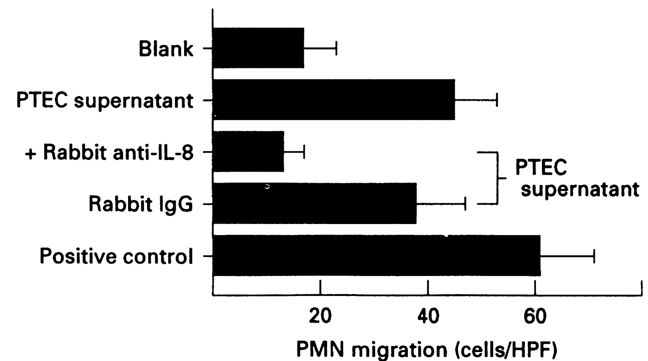

Fig. 5. Chemotactic activity of IL-8 produced by proximal tubular epithelial cells (PTEC). Normal PTEC supernatant was assessed for chemotactic activity in the presence or absence of rabbit IgG anti-IL-8 or normal rabbit IgG; as a positive control rIL-8 (100 ng/ml) was used. HPF, High power field.

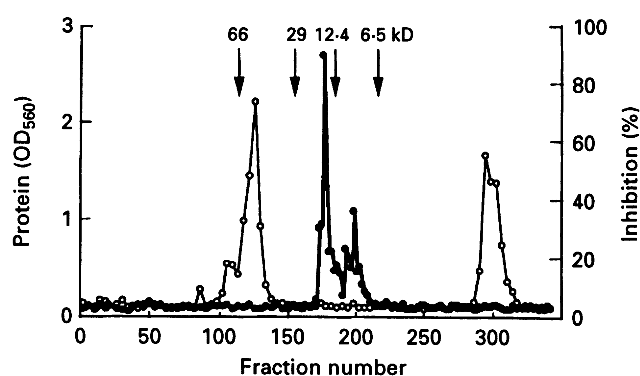

Fig. 6. Characterization of IL-8 produced by proximal tubular epithelial cells (PTEC). Gel filtration profile for IL-8 of concentrated PTEC supernatant on a Superdex G-75. The fractions were analysed for protein (○, OD_{560}) and assessed for IL-8 (●, % inhibition).

containing 10% FCS, which may result in activation of the cells [30,31].

In the present study we obtained primary cultures from a number of normal donors. These cells were characterized as proximal tubular epithelial cells using several markers and cultured under serum-free conditions. The results using these primary cells indicated that there is heterogeneity in the degree of production of IL-8 by these cultures. However, we found that cultures between passages 2 and 6 did not show a significant change in the level of IL-8 production. Therefore, all experiments were performed with cells with a maximum of six passages.

A number of cell lines did not exhibit detectable levels of IL-8 at the protein and mRNA level. The cells producing IL-8 could be stimulated to increased production of IL-8 by IL-1α and TNF-α, while the cells with no detectable baseline production could be induced to produce IL-8 both at the protein and mRNA level. These results are in agreement with the observation that renal cortex inflammation is generally associated with an increased presence of IL-8 in cortical cells. An interesting observation was the down-regulating effect of IFN-γ on the production of IL-8 by PTEC, an effect which has also been described for fibroblasts and PMN [32,33]. This in contrast to a study done by Schmouder *et al.*, where they showed that IFN-γ had no effect on IL-8 production by renal cortical epithelial cells [34], perhaps due to differences in culturing conditions. The PTEC cell lines expressing IL-8 were down-regulated by the addition of IFN-γ in a dose-dependent manner on the protein and mRNA level. The cell lines negative for IL-8 message were not influenced by IFN-γ. The effect of the cytokines on IL-8 production was specific for the different cytokines, as shown by blocking experiments using antibodies and a rIL-1 receptor antagonist.

All in all, the balance between the degree of production of IL-1α, TNF-α and IFN-γ at the local site may determine the final outcome of the inflammation by fine regulation of the production of IL-8 or other chemotactic cytokines or complement components at the local site [16–19,35]. In case of up-regulation of IL-8, the influx of neutrophils may lead to an increase of the local inflammation. We and others have shown recently that PTEC are able to produce another chemotactic factor mainly specific for monocytes, namely monocyte chemotactic protein 1 (MCP-1) [34,36]. MCP-1 production is up-regulated by IFN-γ in a dose-dependent manner. The down-regulation of IL-8 and up-regulation of MCP-1 by IFN-γ may influence the ratio of influx between neutrophils and monocytes at the local site.

The production of IL-8 *in vitro* is *de novo* because the increase of IL-8 is accompanied by an increase of IL-8 mRNA. In all cell lines the effect of IL-1α is remarkedly stronger than TNF-α, as can be seen by Northern blot analysis. In several studies it has been shown that IL-1α and TNF-α regulate IL-8 expression at the transcriptional level [37–39]. IL-8 mRNA is unstable and rapidly degraded in the absence of stimuli. IL-1α has been shown to stabilize IL-8 mRNA by preventing the degradation of mRNA and thereby inducing high levels of IL-8 mRNA [40]. The size of IL-8 produced by PTEC *in vitro* is $\approx$15·1 and 8·1 kD. This IL-8 is biologically active and is present in the dimeric and monomeric forms in culture medium. It has been demonstrated that an equilibrium between the dimeric and monomeric forms of IL-8 exists [41,42]. We assume that dimerization has occurred in the culture medium, presumably after the production of monomeric IL-8 by PTEC, as shown by gel filtration. It has been questioned whether monomeric IL-8 also has chemotactic activity. Recent studies indicate that both monomeric and dimeric are active in this regard [10]. Therefore the evidence that PTEC produce IL-8 under inflammatory conditions may be relevant in inflammation in the kidney in certain diseases.

The findings presented in this study suggest that the influx of neutrophils in the interstitium could be due to local production of IL-8 by the proximal tubular epithelial cells, which is under control of a number of proinflammatory cytokines. The cytokines IL-1α and TNF-α not only up-regulate production of IL-8, but also up-regulate the expression of intercellular adhesion molecule-1 (ICAM-1; CD54) on PTEC [43,44]. ICAM-1 is a ligand for CD11a/b and CD18 which is present on leucocytes, and CD54 also has an interaction with CD43 which is expressed on neutrophils and involved in the activation of neutrophils. Therefore the production of IL-8 by PTEC under the influence of IL-1α and TNF-α may not only result in recruitment of neutrophils but also in their activation, which leads to local inflammation.

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