Kidney International, Vol. 67 (2005), pp. 543–553

Human renal epithelial cells produce the long pentraxin PTX3

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Human renal epithelial cells produce the long pentraxin PTX3.

Background. Pentraxin 3 (PTX3) is a prototypic long pentraxin with structural similarities in the C-terminal domain to the classical short pentraxins C-reactive protein (CRP) and serum amyloid P component. PTX3 is suggested to play an important role in the innate resistance against pathogens, regulation of inflammatory reactions, and clearance of apoptotic cells. Unlike the classic pentraxins, PTX3 is mainly expressed extrahepatically. The present study was designed to investigate the expression of PTX3 by human proximal renal tubular epithelial cells (PTECs).

Methods. PTECs were cultured in the presence or absence of inflammatory cytokines. PTX3 mRNA expression was measured by reverse transcription-polymerase chain reaction (RT-PCR) in human kidney and PTECs. PTX3 protein levels in PTEC cultures were quantified by enzyme-linked immunosorbent assay (ELISA).

Results. PTX3 mRNA was shown to be constitutively expressed in human kidney. Constitutive expression and production of PTX3 was shown in primary mesangial cells, in primary PTECs, and in renal fibroblasts. Further analysis showed that interleukin (IL)-1 and tumor necrosis factor-α (TNF-α) stimulation strongly enhanced the expression and production of PTX3 in PTECs in a dose- and time-dependent manner. In addition, activation of PTECs with IL-17 and CD40L, respectively, but not with IL-6 or IL-4, resulted in strongly increased production of PTX3, whereas granulocyte macrophage-colony-stimulating factor (GM-CSF) inhibited IL-1–induced PTX3 production. PTX3 produced by PTEC is functionally active in binding C1q.

Conclusion. These results indicate that PTX3 is expressed and released by PTECs and that in proinflammatory conditions PTX3 production is up-regulated. Local expression of PTX3 may play a role in the innate immune response and inflammatory reactions in the kidney.

Pentraxins are acute-phase proteins, characterized by a cyclic pentameric structure, that show a strong interspecies homology [1]. The classical short pentraxins, C-reactive protein (CRP), and serum amyloid P component (SAP) are produced in the liver in response to inflammatory mediators, most prominently interleukin (IL)-6 [2, 3]. Short pentraxins contribute to innate immune responses to microbes and are involved in the scavenging of cellular debris [4, 5].

PTX3, also called tumor necrosis factor (TNF)-stimulated gene 14, is the first cloned long pentraxin, structurally related to, yet distinct from, classic short pentraxins. The C-terminal half of the protein aligns with the full-length sequence of CRP and SAP, whereas the N-terminal region of the protein does not show any significant homology with other proteins. PTX3 was originally cloned as a gene highly induced by IL-1 in endothelial cells [6] and by TNF-α in fibroblasts [7]. PTX3 is produced by a variety of cell types, mainly endothelial cells and cells of the monocyte/macrophage lineage, upon stimulation with inflammatory mediators [8].

We have previously demonstrated that PTX3 binds C1q and activates the classical complement pathway [9, 10]. Furthermore, PTX3 participates in the clearance of apoptotic cells and several micro-organisms [11, 12]. Transgenic mice overexpressing the PTX3 gene showed an excessive inflammatory response to endotoxic shock and sepsis, resulting in a more effective control of infection and longer survival [13]. However, during ischemia/reperfusion (I/R) injury the increased inflammatory response in PTX3 transgenic mice resulted in a higher degree of tissue damage and enhanced lethality [14]. Serum levels of PTX3 are elevated in a number of human disorders, such as myocardial infarction [15], rheumatoid arthritis [16], and sepsis [17]. Taken together, these findings suggest an important role for PTX3 in the regulation of inflammatory reactions and innate immunity.

Many renal diseases are characterized by an influx of monocytes and T lymphocytes in the tubulointerstitium. These infiltrating cells contribute to the inflammatory response in the kidney by the release of inflammatory mediators, such as IL-1 and TNF-α, thereby activating

Key words: pentraxin 3, tubular epithelial cells, inflammation.

Received for publication July 31, 2003
and in revised form June 27, 2004, and August 12, 2004
Accepted for publication October 26, 2004

resident renal tubular epithelial cells. Several studies have shown that activated tubular epithelial cells produce large amounts of chemokines, cytokines, and complement components [18–23]. Therefore, tubular epithelial cells are considered to play an important role in inflammatory processes within the kidney.

In the present study we examined whether PTX3 is expressed in the kidney. We observed that PTX3 mRNA is constitutively expressed in normal human kidney. We also investigated the constitutive and inducible expression and production of PTX3 in different renal cells, including tubular epithelial cells. Stimulation with IL-1, TNF-α, IL-17, and CD40L increases the expression and production of PTX3 by renal epithelial cells. The production of PTX3 in the kidney may contribute to local innate resistance and inflammation.

METHODS

Immunohistochemistry

Immunostaining was performed on rejected kidneys from patients with acute allograft rejection. Renal tissue was fixed in formalin and embedded in paraffin. Tissue sections (4 μm thick) were dewaxed and rehydrated. Endogenous peroxidase activity was blocked by incubation in methanol and 0.3% hydrogen peroxide. Sections were microwave-heated at 100°C for 10 minutes in 0.1 mol/L sodium citrate (pH 6), and subsequently rinsed with water and phosphate-buffered saline (PBS). The sections were incubated with 10% normal goat serum, followed by affinity-purified rabbit anti-PTX3 polyclonal antibody (1μg/mL) at a dilution of 1/1000, washed in PBS, incubated with goat antirabbit IgG-poly-horseradish peroxidase antiserum (Immunologic, Duiven, The Netherlands), and peroxidase activity was visualized using diaminobenzidine and hydrogen peroxide.

Cell culture

Proximal tubular epithelial cells (PTECs). Primary human PTECs were isolated from kidneys not suitable for transplantation and from pretransplantation biopsies [24]. Cell monolayers were cultured on a matrix of heat-inactivated fetal calf serum (FCS) (Gibco BRL/Life Technologies, Inc., Paisley, UK) in Dulbecco's modified Eagle's medium (DMEM)/Ham-F12 (Bio-Whittaker, Walkersville, MD, USA), supplemented with insulin (5 μg/mL), transferrin (5 μg/mL), selenium (5 ng/mL), hydrocortisone (36 ng/mL), trio-iodothyronine (40 pg/mL), epidermal growth factor (EGF) (10 ng/mL) (all from Sigma Chemical Co., St. Louis, MO, USA), and 100 U/mL penicillin/100 μg/mL streptomycin (P/S) (Gibco/Life Technologies, Inc.) [25]. Specific outgrowth of PTECs was confirmed by morphologic appearance and immunofluorescent staining as described [26]. For passage of the culture, cells were harvested by trypsinization with 0.05% (wt/vol) trypsin/0.02% (wt/vol) ethylenediaminetetraacetic acid (EDTA) (Sigma Chemical Co.). PTECs obtained between passage 2 and 6 of culture were used for experiments.

Human kidney 2 (HK-2). The immortalized PTEC-derived cell line HK-2 was kindly provided by M.J. Ryan, University College Dublin, Ireland [27]. HK-2 cells are being widely used for in vitro studies of human PTECs displaying most characteristics of primary cells. Cells were cultured in DMEM/Ham-F12 supplemented as described above.

L cells. Mouse fibroblast L cells, stably transfected with human CD40L (L-CD40L), have been described previously [28]. Nontransfected L cells (L orient) were used as a negative control. Cells were cultured in RPMI 1640 supplemented with 10% inactivated FCS and P/S. L cells were irradiated (80 Gy) before coculture experiments to prevent overgrowth of the cultures.

TK173. The human fibroblast cell line TK173 was kindly provided by F. Strutz, Department of Nephrology and Rheumatology, Georg-August University, Germany. Cells were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% inactivated FCS and P/S [29].

Adult mesangial cells (AMC). Primary AMCs were isolated from human kidneys not suitable for transplantation. AMCs were cultured and characterized as described previously [30].

Cell stimulation

PTEC or HK-2 were trypsinized and transferred to 48-well plates (Costar, Corning, NY, USA) at a cell concentration of 0.5×10^5 cells per well. After 24 hours, stimulation experiments were performed using the following cytokines: IL-1α, IL-1β (1 ng/mL) (both from Preprotech, Rocky Hill, NJ, USA), TNF-α (40 ng/mL) (R&D Systems, Minneapolis, MN, USA), IL-17 (50 ng/mL) (R&D Systems), IL-4 (10 ng/mL) (Peprotech), IL-6 (80 ng/mL) (Sanbio, Uden, The Netherlands), granulocyte macrophage-coloncy stimulating factor (GM-CSF) (80 ng/mL) (Leucomax®) (Novartis Pharma BV, Arnhem, The Netherlands), macrophage colony stimulating factor (M-CSF) (80 ng/mL), interferon-γ (IFN-γ) (80 ng/mL) (Boehringer Ingelheim, Alkmaar, The Netherlands) for 48 hours unless indicated otherwise. Irradiated L cells were added in a 1:1 ratio.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from cells and from tissue was isolated using RNAzol (Campro, Veenendaal, The Netherlands), according to the description of the manufacturer. Tissue samples were first homogenized using an ultraturrax (IKA Labortechnik, Staufen, Germany). Alternatively,

Table 1. Primer sequences

Human gene	Primer sequence	Corresponding base	Product size	Accession number
PTX3				X63613
Forward	'5-CAT CCA GTG AGA CCA ATG AG-3'	656–675	287 bp	
Reverse	'5-GTA GCC GCC AGT TCA CCA TT-3'	923–942		
GAPDH				M33197
Forward	'5-ACC ACA GTC CAT GCC ATC AC-3'	585–604	452 bp	
Reverse	'5-TCC ACC ACC CTG TTG CTG TA-3'	1017–1036		

Abbreviations are: PTX3, pentraxin 3; GAPDH, glyceraldehyde-3-phosphate dehydrogenase

total RNA was isolated by means of RNeasy Mini Kit (Qiagen, Venlo, The Netherlands). The quantity and purity of the isolated RNA was determined by measuring the ratio of optical densities at 260 and at 280 nm.

Fixed amounts of total RNA (1 μg) were reverse transcribed into cDNA by oligo-dT priming using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Gibco/Life Technologies). The amplification of cDNA by PCR was performed using the primers as described in Table 1. Expression of glyceraldehyde-3-phosphatate dehydrogenase (GAPDH) mRNA was used as a control. PCR was performed under standard conditions in a final volume of 40 μL [50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.4, 2 mmol/L $MgCl_2$, 0.06 mg/mL bovine serum albumin (BSA), 0.25 mmol/L deoxynucleoside triphosphate (dNTP), 25 pmol of each primer, and 1 U of Taq polymerase] (Perkin Elmer, Norwalk, CT, USA). Amplification of cDNA started with 5 minutes at 95°C, followed by 35 cycles of 1 minute at 95°C, 1 minute at 55°C, 1 minute at 72°C, and the final primer extension for 7 minutes at 72°C. PCR products were analyzed on a 1% agarose gel containing ethidium bromide. The intensity of the bands was determined by densitometry, using EaglySight software (Stratagene, La Jolla, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

The concentration of PTX3 secreted in culture supernatants was measured using a sandwich ELISA [17]. For the detection of PTX3, 96-well Nunc Maxisorb microtiter plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 1 μg/mL of the rat monoclonal antibody MNB4 antihuman PTX3 in coating buffer (0.1 mol/L sodium carbonate, pH 9.6). All reaction volumes were 100 μL and plates were washed after each step in PBS containing 0.05% vol/vol Tween. Plates were incubated for 1 hour at 37°C with PBS containing 1% wt/vol BSA to block uncoated sites. Cell supernatants or recombinant human PTX3 standards (purified from supernatant of Chinese hamster ovary cells stably and constitutively expressing the protein [9]), for quantification, were added to the wells, diluted in PBS containing 0.05% Tween and 1% BSA. Following 1 hour of incubation at 37°C, bound PTX3 was detected using biotin-conjugated polyclonal rabbit anti-PTX3 antibody, followed by incubation with streptavidin conjugated to poly horseradish peroxidase (CLB, Amsterdam, The Netherlands). Finally, 2,2′-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (Sigma Chemical Co.) was added for color development and optical density at 415 nm was assessed. Alternatively, wells were coated with 5 μg/mL serum-purified C1q, prepared as described previously [31]. PTX3 binding was detected as indicated above. Production of IL-6 was measured in the supernatant by specific ELISA as described previously [24].

Western blotting

For Western blot analysis, supernatants of stimulated cells (concentrated 40-fold by Speedvac) were boiled for 5 minutes in 3 × sodium dodecyl sulfate (SDS) sample buffer (New England Biolabs, Beverly, MA, USA), separated by SDS-polyacrylamide gel electrophoresis (PAGE), and blotted on polyvinyldene fluoride (PVDF) membranes (Immobilon-p) (Millipore, Bedford, MA, USA). Membranes were incubated with 2% casein in PBS containing 0.05% Tween for blocking and then the primary antibody biotin-conjugated monoclonal rat anti-PTX3 antibody MNB4 was added. After incubation with horseradish peroxidase-conjugated streptavidin (Bio-Rad, Hercules, CA, USA) as a secondary reagent, detection was performed with Supersignal (Pierce, Rockford, IL, USA) and the blots were exposed to Hyperfilm™ films (Amersham Pharmacia Biotech, UK).

Statistical analysis

Protein production data are presented as mean ± SD of absolute values of representative experiments performed in triplicate. Alternatively, data are presented as relative increases as compared to control cultures, using the mean ± standard error of the mean (SEM) of multiple experiments performed in triplicate. Statistical analysis of relative increases of protein production upon cell stimulation was performed using two-way analysis of variance (ANOVA). *P* values were considered statistically significant when *P* was below 0.05.

RESULTS

Expression of PTX3 in renal biopsies

Renal tissue from patients with acute allograft rejection was analyzed for expression of PTX3 by immunohistochemistry. Intense staining for PTX3 was mainly observed in tubulointerstitial areas of leukocytic infiltration (Fig. 1A). In detail, staining was especially present at

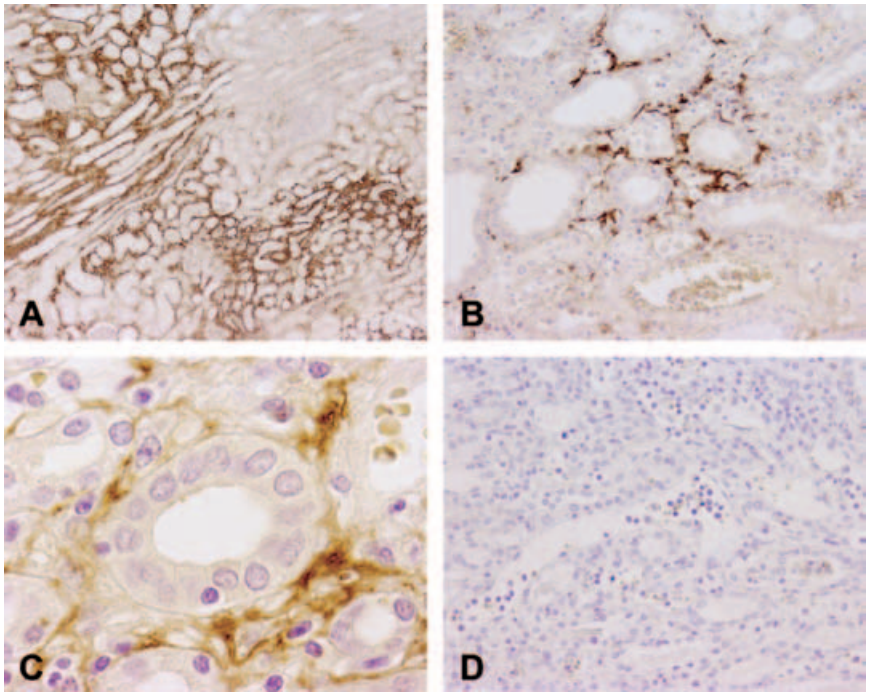

Fig. 1. Expression of pentraxin 3 (PTX3) in renal allografts with acute rejection. Renal tissue sections were stained for PTX3 as described in the **Methods** section. PTX3 staining is detectable in the inflamed interstitium (*A*), often at sites of cell-cell and cell-matrix interaction (*B* and *C*). Tubular epithelial cells show a faint staining for PTX3 (C). Control staining, in the absence of the primary antibody, is negative (*D*) [original magnification (A) ×40; (B and D) ×200; (C) ×800].

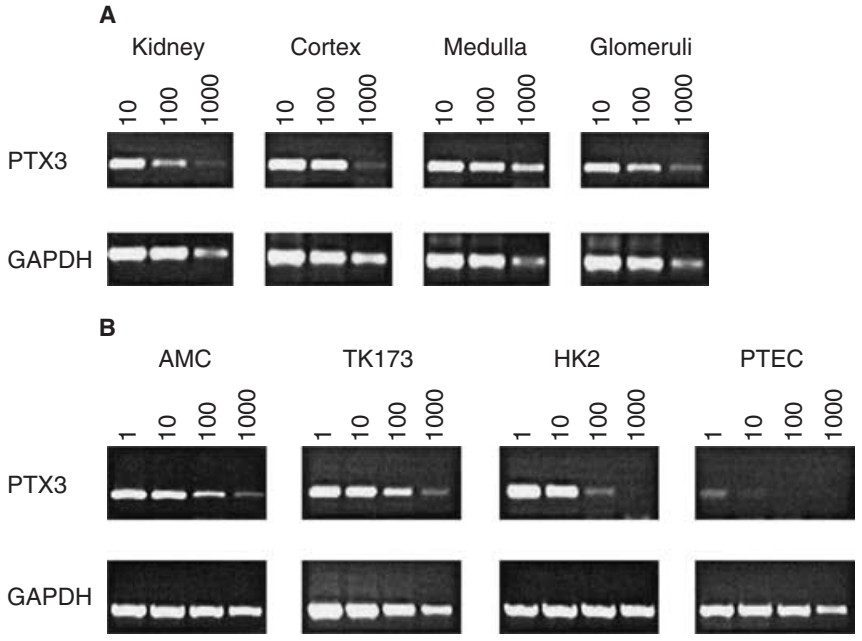

Fig. 2. Pentraxin 3 (PTX3) is expressed in the kidney. (*A*) Total RNA was isolated from adult human kidney, from the cortex, from the medulla and from isolated glomeruli, and reverse transcription-polymerase chain reaction (RT-PCR) was performed for PTX3 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). (*B*) Expression of PTX3 by primary mesangial cells, renal fibroblasts, epithelial-derived cell line human kidney 2 (HK-2), and primary renal tubular epithelial cells was analyzed by RT-PCR. For PTX3 and GAPDH, cDNA was diluted up to 1000 times, as indicated.

cell-cell and cell-matrix interaction sites (Fig. 1B and C). Focal weak staining was observed on tubular epithelial cells (Fig. 1C). Control staining was negative (Fig. 1D). In contrast to this positive staining in renal tissue showing acute rejection, normal renal tissue stained negative for PTX3 (results not shown).

Expression of the PTX3 gene in kidney

Adult human kidney was tested for the mRNA expression of the PTX3 gene. Total mRNA was isolated from total kidney as well as from renal cortex, medulla and glomeruli. By RT-PCR analysis we observed PTX3 mRNA to be present in all the different compartments of the kidney (Fig. 2A). We next examined the ability of different renal cells to express the PTX3 transcript by RT-PCR. PTX3 gene expression was detected in primary mesangial cells and in renal fibroblasts, in accordance with previous publications [7, 32]. In addition, we observed expression of PTX3 mRNA by renal proximal tubular epithelial cells (Fig. 2B).

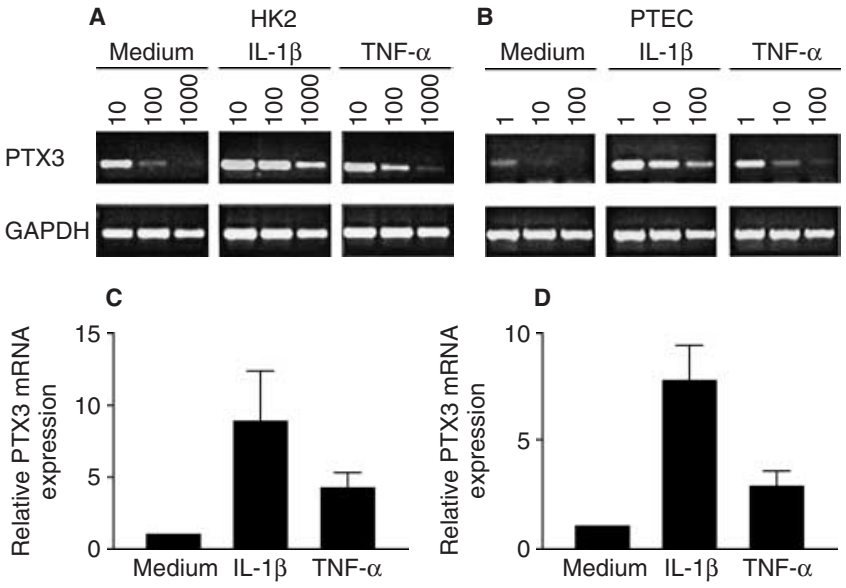

Fig. 3. Pentraxin 3 (PTX3) mRNA expression by interleukin (IL)-1β- and tumor necrosis factor-α (TNF-α)-stimulated renal epithelial cells. Human kidney 2 (HK-2) (*A* and *C*) or primary human proximal tubular epithelial cells (PTECs) (*B* and *D*) were stimulated with medium, IL-β (1 ng/mL) or TNF-α (40 ng/mL). (A and B) After 6 hours, RNA was isolated and a semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) was performed for PTX3 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH). (C and D) Quantification of the mRNA expression of PTX3 by calculating mRNA levels of PTX3 relative to those of GAPDH using densitometry analysis. Measurements of ratios in control cultures were assigned a relative value of 1, representing control values. Results are expressed as mean ± SD of two independent experiments.

Cytokine modulation of PTX3 gene expression in renal epithelial cells

The observed expression of PTX3 mRNA by renal tubular epithelial cells, as well as the proposed role of tubular epithelial cells in inflammatory processes in the kidney [33], prompted us to further investigate the expression of PTX3 by human PTECs and to define its modulation by cytokines in vitro. Primary PTECs or the PTEC-derived cell line HK-2 were stimulated with human recombinant IL-1β or TNF-α. These proinflammatory cytokines have been described to induce PTX3 expression in monocytes and endothelial cells [8]. As shown in Figure 3, both IL-1β and TNF-α strongly increased PTX3 mRNA expression in HKs2 cells (Fig. 3A) and in primary PTECs (Fig. 3B). Compared to basal PTX3 expression, PTX3 mRNA expression was about eightfold increased by IL-1β and threefold increased by TNF-α, both in HK-2 cells and in primary PTECs (Fig. 3C and D). Affymetrix gene chip analysis confirmed our findings, demonstrating that PTX3 is constitutively expressed by primary PTECs. Stimulation with IL-1α for 20 hours resulted in a 2.4-fold increase in PTX3 expression level (Simone de Haij, unpublished observations).

PTX3 protein production

Having established that PTX3 mRNA is expressed in PTECs, we examined whether PTX3 protein was indeed released. Western blot experiments were performed under reducing conditions with supernatant from nonstimulated and IL-1β–stimulated HK-2 cells. The results presented in Figure 4 show that under basal conditions a specific signal for PTX3 protein is detectable at the correct molecular weight (45 kD) [9], indicating that PTX3 is secreted. Stimulation with IL-1β strongly increased the production of PTX3.

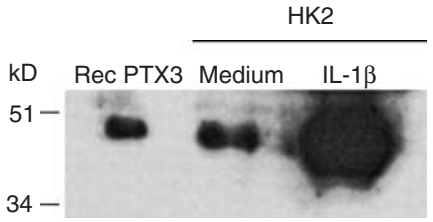

Fig. 4. Detection of pentraxin 3 (PTX3) secreted by renal epithelial cells. Human kidney 2 (HK-2) cells were stimulated with medium or interleukin (IL)-1β (1 ng/mL) for 48 hours. Supernatant was harvested, concentrated 40-fold, and analyzed by Western blotting with a biotinylated mouse anti-PTX3 monoclonal antibody. Recombinant PTX3 purified from Chinese hamster ovary cells was used as a positive control, as indicated. Molecular weight markers (ovalbumin, 52 kD, and carbonic anhydrase, 34 kD) are indicated. PTX3 production measured by enzyme-linked immunosorbent assay (ELISA): HK-2 medium, 35 ± 15 ng/mL; HK-2 IL-1β, 3622 ± 364 ng/mL.

PTX3 production by renal epithelial cells after activation by cytokines

The production of PTX3 by renal cells was more specifically quantified by ELISA. The production of PTX3 by primary PTECs under basal conditions showed a high variability within the different cell lines, ranging from 21 pg/mL to 321 pg/mL PTX3 per 48 hours ($N = 6$). As previously reported, both IL-1β and TNF-α can stimulate PTECs to produce a large variety of inflammatory mediators, including IL-6 [34]. The production of PTX3 by primary PTECs (Fig. 5A) and HK-2 cells (Fig. 5B) was clearly up-regulated in the presence of IL-1β and TNF-α. Exposure to IL-1β and TNF-α also increased the

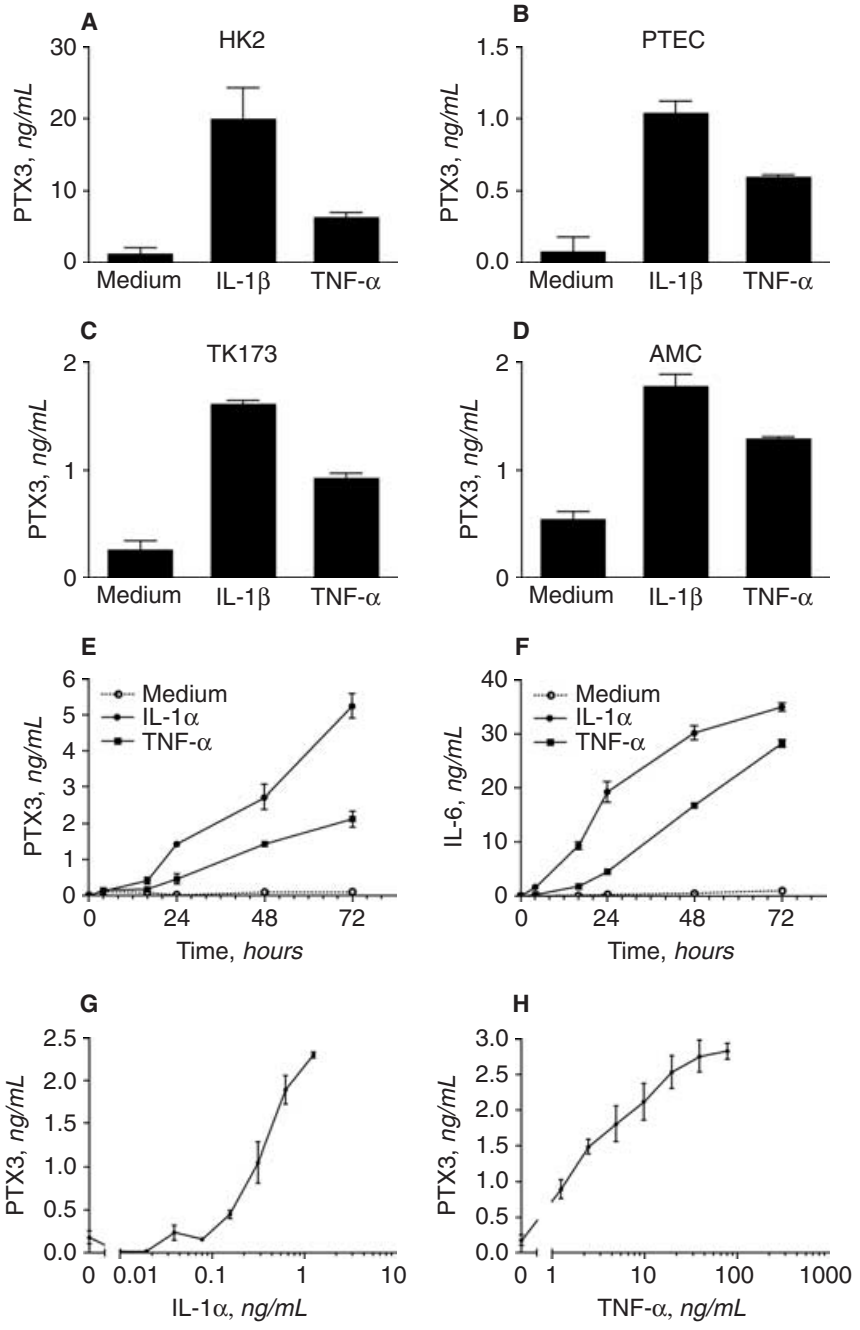

Fig. 5. Pentraxin 3 (PTX3) production by renal epithelial cells stimulated with interleukin (IL)-1β or tumor necrosis factor-α (TNF-α). Human kidney 2 (HK-2) cells (*A*), primary human proximal tubular epithelial cells (PTECs) (*B*), TK173 cells (*C*), and adult mesangial cells (AMC) (*D*) were stimulated with IL-1β (1 ng/mL) or TNF-α (80 ng/mL). Supernatants were harvested and were tested for PTX3 and IL-6 production. Primary PTECs were incubated for various time-periods in the absence or presence of IL-1β or TNF-α. Supernatants were harvested and assessed for PTX3 (*E*) and IL-6 (*F*) production. PTECs were activated with increasing concentrations of IL-1β (*G*) or TNF-α (*H*). After 48 hours, supernatants were harvested and tested for PTX3 and IL-6 production. Results are expressed as mean of triplicate cultures and are representative for three independent experiments.

production of PTX3 by renal fibroblast cell line TK173 (Fig. 5C) and primary AMCs (Fig. 5D).

The production of PTX3 by primary PTECs was time-dependent and already after 16 hours of culture, the stimulatory effect of IL-1β and TNF-α on PTX3 production could be observed (Fig. 5E). Comparable with the regulation and kinetics of PTX3 production, both IL-1β and TNF-α showed a time-dependent increase in IL-6 production, as assessed in parallel in the same supernatants (Fig. 5F). A significant increase, compared with background productions, was found with 20 pg/mL of IL-1β, and increasing amounts of IL-1β showed a dose-dependent increase in PTX3 production (Fig. 5G). Similar results were obtained with IL-1α, another molecular form of IL-1, binding to the same set of specific IL-1 receptors [34] (data not shown). Furthermore, also TNF-α enhanced the production of PTX3 in a dose-dependent fashion, showing a significant increase at 2.5 ng/mL (Fig. 5H). Similar dose-response curves were found for IL-6 and with HK-2 cells (data not shown).

Since previous studies have demonstrated that next to IL-1β and TNF-α, also IL-17 can stimulate PTECs to

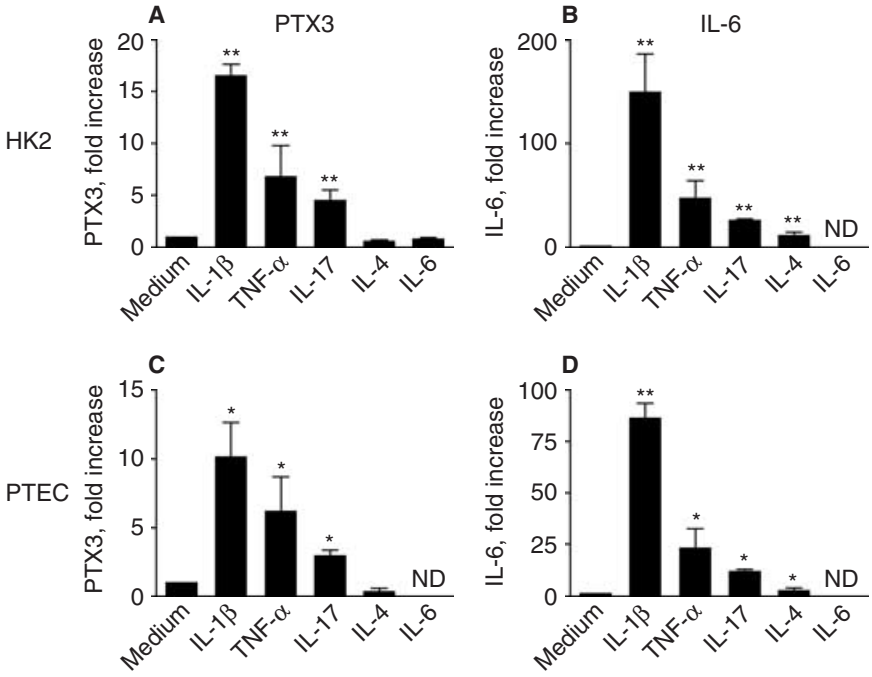

Fig. 6. Pentraxin 3 (PTX3) production in response to different cytokines. Human kidney 2 (HK-2) cells (*A* and *B*) or primary human proximal tubular epithelial cells (PTECs) (*C* and *D*) were stimulated for 48 hours with interleukin (IL)-1β (1 ng/mL), tumor necrosis factor-α (TNF-α) (40 ng/mL), IL-17 (50 ng/mL), IL-4 (40 ng/mL), or IL-6 (80 ng/mL). After 48 hours, supernatants were harvested and tested for PTX3 (A and C) and IL-6 (B and D) production. The stimulation index was calculated using ratio of production by stimulated cells and production by nonstimulated cells. Data represent the mean stimulation index ± SEM of four independent experiments performed in triplicate. $^{*}P < 0.01$; $^{**}P < 0.001$ compared with medium. ND = not done.

produce various inflammatory mediators [35], we examined the effect of this stimulus on the production of PTX3. A significant increase of PTX3 production by PTECs upon stimulation with IL-1β, TNF-α, and IL-17 (Fig. 6A and C) was observed, comparable with the regulation of IL-6 production (Fig. 6B and D). No effects were found with IL-4 on PTX3 production, whereas a stimulatory effect was observed on IL-6 production.

IL-1 and CD40L synergistically enhance PTX3 production

Our group previously demonstrated that combined activation of PTECs with IL-1 and CD40L resulted in synergistic effects on IL-6, IL-8, and regulated upon activation, normal T cell expressed and secreted (RANTES) (CCL5) production [34]. Therefore, we examined whether PTX3 production was modified after stimulation with CD40L-transfected L cells (L-CD40L). Production of PTX3 was strongly increased after stimulation with CD40L, both in HK-2 cells (Fig. 7A) and primary PTECs (Fig. 7B). Next, we investigated the effect of combined stimulation of PTECs on the production of PTX3. Stimulation of primary PTECs with a combination of IL-1β and CD40L resulted in synergistic effects on PTX3 production (Fig. 7C). A 89% ± 35% increase was found for the combination, as compared to the sum of the stimuli alone, in three out of four primary PTEC lines.

Negative regulation of PTX3 production

IL-6 is an important mediator of the acute phase response and induces the expression of SAP and CRP. In contrast, the expression of PTX3 was not up-regulated by addition of exogenous IL-6 (Fig. 6A). Furthermore, IL-4, IFN-γ, GM-CSF, and M-CSF were unable to induce the expression of PTX3 in primary PTECs (data not shown), which is in line with the previously described effect of these mediators on the expression of PTX3 by human monocytes [8]. It was of interest to investigate whether cytokines or growth factors could down-regulate the production of PTX3. A modest downregulation of the IL-1–stimulated production of PTX3 was observed by IFN-γ (23.8% ± 3.7%). Interestingly, GM-CSF strongly inhibited the IL-1β–induced PTX3 protein production by HK-2 cells (Fig. 8A), whereas it had no effect on the production of IL-6 (Fig. 8B). The inhibitory effect of GM-CSF was dose-dependent and complete inhibition was reached with 80 ng/mL GM-CSF.

PTX3 produced by renal epithelial cells can bind C1q

To demonstrate the functional activity of PTX3 produced by PTECs its capacity to bind C1q was examined. Therefore, wells coated with C1q were incubated with culture supernatant from nonstimulated and IL-1β–stimulated HK-2 cells, and PTX3 binding was analyzed. Both PTX3 in HK-2 supernatant and purified PTX3 showed strong binding to C1q (Fig. 9).

DISCUSSION

PTX3 is suggested to be important in the regulation of inflammatory reactions and innate immunity and, in view of this role, we have examined the expression of PTX3 in the kidney. Immunohistochemistry on renal tissue of

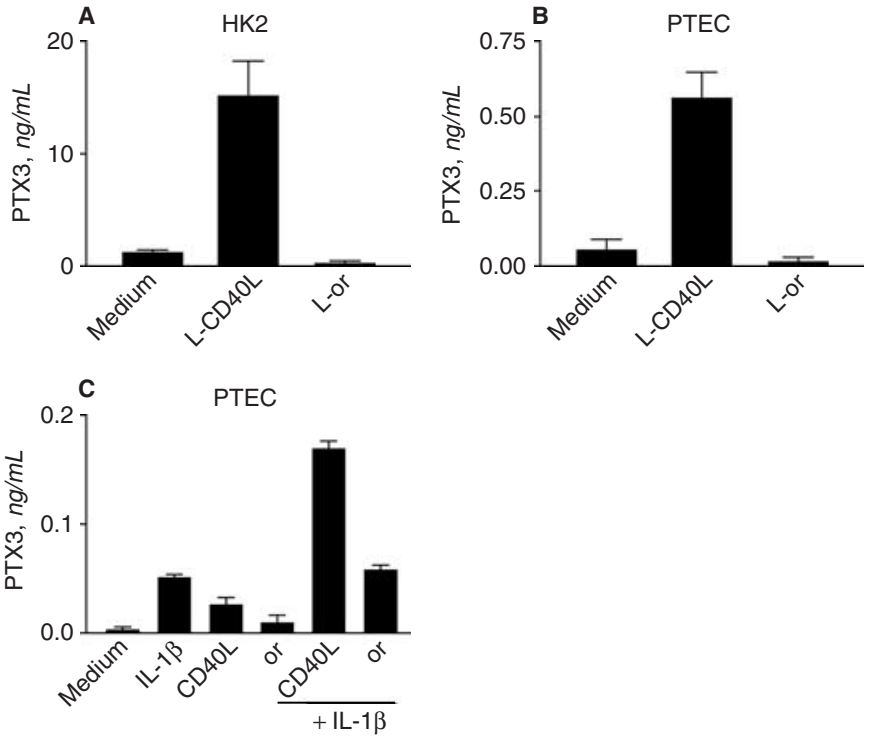

Fig. 7. Effect of CD40L stimulation on pentraxin 3 (PTX3) production by renal epithelial cells. Human kidney 2 (HK-2) cells (*A*) or primary human proximal tubular epithelial cells (PTECs) (*B*) were cultured with CD40L-transfected (CD40L) or nontransfected (or) L cells (10^5 cells/well) and after 48 hours supernatant was harvested and assessed for PTX3 production. (*C*) Primary PTEC were cultured with either transfected- (CD40L) or nontransfected (or) L cells and stimulated with interleukin (IL)-1β (1 ng/mL). After 48 hours, supernatant was harvested and tested for PTX3 production. Data are representative for three experiments, and shown is the mean production of triplicate cultures of one representative experiment.

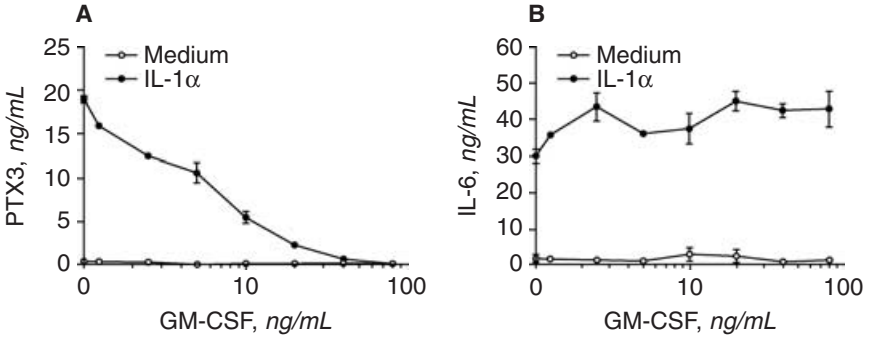

Fig. 8. Granulocyte macrophage-colony stimulating factor (GM-CSF) inhibits pentraxin 3 (PTX3) production by interleukin (IL)-1β-stimulated human kidney 2 (HK-2) cells. HK-2 cells were cultured with different concentrations of GM-CSF in the presence or absence of IL-1β (1 ng/mL). After 48 hours, supernatants were harvested and tested for PTX3 (*A*) and IL-6 (*B*) production. Data indicated are representative for three experiments, and shown are the mean production of triplicate cultures.

patients with acute allograft rejection showed that PTX3 was predominantly present in the inflamed tubulointerstitium in close proximity of tubular epithelial cells. Furthermore, focal weak staining for PTX3 was observed on tubular epithelial cells. Moreover, constitutive expression of PTX3 mRNA was shown in primary mesangial cells, in renal fibroblasts and in primary PTECs using RT-PCR. Activation of PTECs with the proinflammatory cytokines IL-1β, TNF-α, and IL-17, and stimulation with CD40L, resulted in strongly increased expression and production of PTX3 that is able to interact with C1q.

PTX3, a prototypic long pentraxin, has been shown to be expressed by several cell types, most prominently by endothelial cells and monocytes [6, 8], in response to inflammatory stimuli including IL-1, TNF-α, and lipopolysaccharide (LPS). In the present study we demonstrate that PTX3 mRNA is expressed in human renal cortex, medulla, and in the glomeruli. Previous studies demonstrated that PTX3 is expressed and produced by mesangial cells [32] and that the expression of PTX3 can be induced in fibroblasts upon stimulation with TNF-α [6]. Next to glomerular mesangial cells and renal fibroblasts, we now demonstrate that also renal PTECs may contribute to the local production of PTX3 in the kidney. PTECs have been described to be an important source of inflammatory mediators and are therefore considered as a central cell type in the renal inflammatory response [33]. The expression and production of PTX3 by PTECs is increased by proinflammatory signals provided by IL-1, TNF-α, IL-17, and CD40L. Furthermore, combined activation of PTECs with IL-1 and CD40L resulted in synergistic effects on the production of PTX3, in agreement with previous studies on the production of IL-6, IL-8, and RANTES (CCL5) [34]. Inflammatory mediators

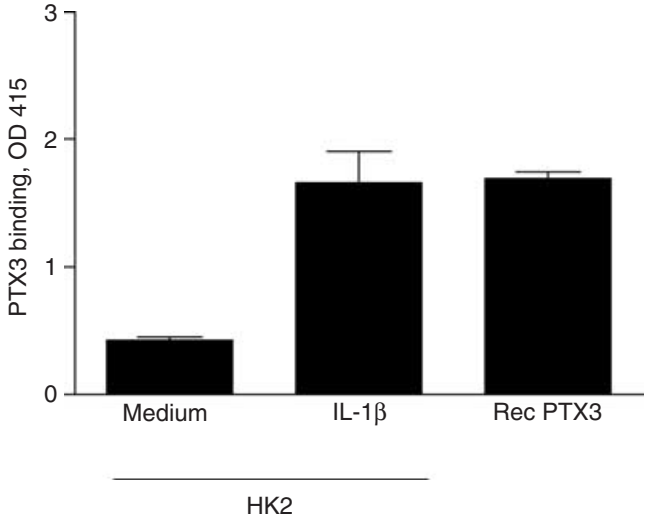

Fig. 9. Pentraxin 3 (PTX3) produced by renal epithelial cells binds to C1q. Microtiter wells coated with C1q were incubated with supernatant from nonstimulated and interleukin (IL)-1β–stimulated human kidney 2 (HK-2) cells. The amount of PTX3 quantified in enzyme-linked immunosorbent assay (ELISA) was 1.2 ng/mL (medium) and 12.3 ng/mL (IL-1β). Recombinant PTX3 (10 ng/mL) was used as a positive control. PTX3 binding was detected. Results are expressed as mean of triplicate cultures and are representative for three independent experiments.

such as IL-1 and TNF-α, derived from both infiltrating cells and activated resident renal cells, and CD40L-expressing cells have been demonstrated to be present in the kidney during renal inflammation [34, 35]. Therefore, we hypothesize that the local production of these inflammatory mediators and the presence of CD40L subsequently increases PTX3 production by PTECs.

We found that other cytokines, including IL-6, IL-4, IFN-γ, M-CSF, and GM-CSF were ineffective in increasing the expression and production of PTX3 by PTECs, as previously reported for monocytes [8]. In sharp contrast, stimulation of PTECs with IL-6 was reported to induce the expression and production of CRP, whereas incubation with IL-1 and TNF-α had no stimulatory effect [36]. Moreover, GM-CSF specifically inhibits the secretion of PTX3, but not IL-6, by PTECs stimulated with IL-1. Recent studies have reported that certain cytokines can inhibit PTX3 expression by monocytes (IL-4 and IFN-γ), or synoviocytes [transforming growth factor-β (TGF-β)] [8, 16, 37]. We observed that IFN-γ was partially able to inhibit PTX3 production by IL-1–stimulated PTECs. So far no inhibitory effect of GM-CSF on PTX3 expression has been described. Since GM-CSF can be produced by PTECs after stimulation with inflammatory mediators, including IL-1 and TNF-α [38], it is tempting to speculate that the production of PTX3 can be down-regulated by endogenously produced GM-CSF as a feedback mechanism. Such mechanisms may also influence PTX3 production in inflamed renal tissue in vivo.

PTX3 is the first identified member of the long pentraxins that are structurally related to the classic short pentraxins, such as SAP and CRP. However, the long pentraxins differ from short pentraxins for the presence of an unrelated long N-terminal domain, gene organization, chromosomal localization, cellular source, and inducing stimuli. SAP and CRP have been described to be mainly produced in the liver in response to IL-6 [2, 3], whereas the major site of synthesis of PTX3 is extrahepatic. However, in a recent study, CRP was described to be also produced locally in the kidney by renal epithelial cells, and was suggested to play a role in the local inflammatory response [36].

The function of PTX3 has not been completely defined. The regulated expression of PTX3 by cells present in peripheral tissue [15–17], as well as its capacity to bind C1q and activate complement [10], suggest that PTX3 plays a role in the regulation of inflammatory reactions and innate resistance. This hypothesis is supported by studies in transgenic mice overexpressing PTX3 and studies with PTX3-deficient mice [11, 13]. PTX3 transgenic mice were more protected against endotoxic shock and sepsis, whereas PTX3-deficient mice were more susceptible to microbial pathogens. The increased expression of PTX3 under inflammatory conditions may support the clearance of damaged self-material, based on the capacity of PTX3 to bind apoptotic cells [12]. Several studies reported the presence of apoptotic cells in the kidney in renal disease [39]. Cell death by apoptosis is thought to play an important role in the recovery after renal injury by the clearance of excess numbers of resident renal cells and potentially injurious infiltrated leukocytes [40]. PTX3 could be involved in mediating the clearance of apoptotic cells, thereby contributing to the resolution of inflammation.

Alternatively, increased expression of PTX3 during inflammation may be pathogenic. The localization of PTX3 at cell-cell and cell-matrix sites in renal tissue of patients with acute allograft rejection suggests that PTX3 may play a role in adhesion. Recently, PTX3 was shown to play an important role in the organization of extracellular matrix by cross-linking of hyaluronan via interaction with TNF-α–stimulated gene 6 (TSG6) [41]. TSG6 has been reported to enhance interaction between CD44 and hyaluronan, implicated in the primary adhesion of leukocytes to endothelium at sites of inflammation [42]. Therefore, PTX3 could facilitate the CD44-mediated recruitment of inflammatory cells, and contribute to inflammation.

PTX3 may also amplify complement-mediated tissue damage in pathologic situations such as I/R injury [14]. CRP has previously been reported to be an important mediator of tissue damage in myocardial infarction, via activation of complement [43]. Recently, PTX3 was shown to enhance tissue factor expression by endothelial cells,

which is involved in the occurrence of thrombogenesis and vascular ischemia [44]. In addition, PTX3 can activate mesangial cells leading to cell contraction and synthesis of the proinflammatory lipid mediator platelet-activating factor [32]. PTX3 might contribute to the local inflammatory process by activating resident renal cells. So far, no specific membrane receptor for PTX3 has been described.

CONCLUSION

We have demonstrated that renal tubular epithelial cells are able to produce PTX3 that is up-regulated under inflammatory conditions. Future studies are required to define the exact role and significance of the local expression and production of PTX3 in the kidney.

ACKNOWLEDGMENTS

The authors thank Nike Claessen (Department of Pathology, AMC, Amsterdam, The Netherlands) for excellent technical assistance. These studies were financially supported by The Netherlands Organization for Scientific Research (NWO 901–12–095), by the Dutch Kidney Foundation (PC95 and NSN C98.1749), and by the European Union (QLG1 CT 2002–01215).

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