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Synergistic effect of interleukin-1 and CD40L on the activation of human renal tubular epithelial cells

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Synergistic effect of interleukin-1 and CD40L on the activation of human renal tubular epithelial cells.

Background. Renal tubular epithelial cells are a central cell type in tubulointerstitial inflammation because they can produce inflammatory mediators such as cytokines and chemokines. Several signals derived from either monocytes or activated T cells have been reported to regulate the activation of tubular epithelial cells. We studied this regulation in more detail by combined treatment with CD40 ligand and the proinflammatory cytokine interleukin-1 (IL-1) *in vitro.*

Methods. The regulation of cytokine and chemokine production was studied in primary cultures of human proximal tubular epithelial cells (PTECs). PTECs were activated by coculture with CD40L-transfected murine fibroblasts in combination with recombinant human cytokines. The production of IL-6, IL-8, monocyte chemoattractant protein-1 (MCP-1), and RANTES were measured by specific enzyme-linked immunosorbent assay.

Results. The combined activation of PTECs with CD40L and IL-1 resulted in strong synergistic effects on the production of IL-6, IL-8, and RANTES, whereas only an additive stimulation of MCP-1 production was observed. The effects were specific for IL-1 and could be neutralized by the addition of the IL-1R antagonist. Both IL-1α and IL-1β showed similar effects on cytokine production by PTECs. The effects of IL-1 were dose dependent, and kinetic experiments showed that synergistic effects were observed after 24 hours of activation and remained present for at least five days. Reverse transcription-polymerase chain reaction analysis showed that human PTECs could express both IL-1α and IL-1β. The activation of PTECs with IL-1 resulted in an up-regulation of CD40 expression on these cells.

Conclusions. A complex network of regulation exists for the production of cytokines and chemokines by PTECs. The combined treatment results in strong synergistic effects on IL-6, IL-8, and RANTES production. This strengthens the potential role of tubular epithelial cells in inflammatory responses within the kidney.

Key words: cytokines, chemokines, CD40, inflammation, tubulointerstitial disease, progressive renal disease.

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It has been proposed that the progressive loss of kidney function observed in many renal diseases results from a pathogenic process that is independent of the original cause, functioning as a final common pathway [1]. This common pathway is characterized by the triggering of interstitial infiltration and the induction of tubular damage. Indeed, the decline of renal function is associated with mononuclear cell infiltration in the interstitium, tubular atrophy, and interstitial fibrosis [2]. The interstitial infiltrate consists of both monocytes/macrophages and T lymphocytes, with $CD4^+$ cells generally being more prominent than $CD8^+$ cells. Infiltrating T cells and monocytes supply the local environment with a wide variety of cytokines that regulate numerous pathophysiological processes, including inflammation [1, 3, 4].

Resident renal cells, such as tubular epithelial cells, might also have an important role in the inflammatory response. *In vitro* studies of human tubular epithelial cells have demonstrated that these cells can produce large amounts of cytokines [interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α)] [5–7], chemokines [IL-8, monocyte chemoattractant protein-1 (MCP-1), RANTES, and ENA-78] [8–14], and complement components (C3, C4, factor-B) [15–17]. Therefore, tubular epithelial cells might be considered as a central cell type in the renal inflammatory response, which contributes to the regulation of interstitial infiltration via the production of chemokines [18].

Previous studies have shown that the production of most inflammatory mediators by PTECs can be stimulated by the proinflammatory cytokines IL-1 and TNF-α. Of these mediators, IL-1 was the most active. IL-1 is a cytokine with a wide range of proinflammatory and immunological effects, including activation of endothelial cells, stimulation of T and B lymphocytes, increased production of cytokines and chemokines, and increased leukocyte adhesion [19]. There are two distinct molecular forms of IL-1 (IL-1α and IL-1β) that have similar biological activities and that bind to the same set of

specific IL-1 receptors. Next to activated monocytes/macrophages, many other cells may serve as a source of IL-1 production [19]. The increased expression of IL-1 in the kidney has been demonstrated in various forms of both human and experimental glomerulonephritis [20–24]. Furthermore, the treatment with agents that neutralize IL-1, such as the natural IL-1R antagonist (IL-RA), blunts the inflammatory processes in various forms of experimental glomerulonephritis [25–27]. These data suggest a causal role for IL-1 in renal inflammation, with activation of resident kidney cells, such as PTECs, as a possible mechanism.

Recently, we demonstrated the expression of a functional CD40 receptor on the surface of renal epithelial cells [13]. The ligand for this receptor is CD40L (CD154), which is mainly expressed on activated $CD4^+$ T cells. The interaction between CD40 and CD40L plays a pivotal role in immune regulation [28]. *In vitro* cross-linking of the CD40 receptor on tubular epithelial cells resulted in a strongly increased production of the chemokines IL-8 and MCP-1 [13]. Interestingly, a strong production of RANTES was also induced by this single CD40L stimulus. This seems in contrast to activation of PTECs with cytokines, where a combination of at least two cytokines [IL-1 + interferon-γ (IFN-γ) or TNF-α + IFN-γ] seems to be required [29].

It is anticipated that under inflammatory conditions, activated T cells will express CD40L and secrete cytokines. Also, infiltrating monocytes will contribute to the local production of proinflammatory cytokines. Therefore, we have investigated the combined activation of PTECs *in vitro* using both CD40L and the proinflammatory cytokine IL-1.

METHODS

Cell cultures

Proximal tubular epithelial cells. Primary human PTEC lines were cultured from cortex tissue of human kidneys not suitable for transplantation because of anatomical reasons or from healthy parts of nephrectomy specimens [30]. PTEC monolayers were cultured on a matrix of collagen type I (Sigma Chemical Co., St. Louis, MO, USA) and heat-inactivated fetal calf serum (FCS; Life Technologies Inc., Gaithersburg, MD, USA), in a selective medium, consisting of a 1:1 ratio of Dulbecco's modified Eagle's medium (DMEM) and Ham-F12 (both from 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). For passage of the cultures, cells were harvested by trypsinization with 0.02% (wt/vol) ethylenediaminetetraacetic acid (EDTA)/0.05% (wt/vol) trypsin (Sigma). The specific outgrowth of PTECs was confirmed by morphological appearance and immunofluorescence staining (CD26 positive), as described [16, 31]. Primary cell lines were used for experiments between passages 2 and 7 of culture.

L cells. The generation of mouse fibroblast L cells stably transfected with the complete human CD40L coding sequence has been previously described [32]. Non-transfected cells were used as a negative control. Both types of L cells were cultured in IMDM glutamax (GIBCO/Life Technologies) supplemented with 10% heat-inactivated FCS and penicillin/streptomycin (GIBCO/Life).

Culture and activation experiments

For induction of cytokine production, PTECs were trypsinized and seeded in complete DMEM/Ham-F12 medium in a volume of 1 ml and in a final PTEC concentration of 1×10^5 cells in 24-well plates. PTECs were activated with recombinant human cytokines at the following final concentrations: IL-1α (1 ng/ml), IL-1β (1 ng/ml), TNF-α (15 ng/ml), IL-4 (10 ng/ml), and IL-10 (10 ng/ml) unless indicated otherwise. IL-1 and TNF-α cytokines were obtained from Genzyme (Uden, the Netherlands), and IL-4 and IL-10 were a kind gift of Dr. F. Briere (Schering Plough, Dardilly, France). Supernatants were harvested after 72 hours of culture and were frozen prior to analysis of cytokine production.

For simultaneous CD40 activation, trypsinized PTECs and trypsinized L cells were mixed in suspension in a 1:1 ratio, as described before [13]. Cells were cultured in complete DMEM/Ham F12 medium in a volume of 1 ml and in a final PTEC concentration of 1×10^5 cells/ml. L cells were irradiated (80 Gy) to prevent overgrowth of the cultures. Experiments with B lymphocytes have shown that irradiation does not inhibit the CD40L activating capacity [32, 33]. Supernatants were harvested after 72 hours of culture, unless indicated otherwise, and were frozen prior to analysis of cytokine production.

To investigate the specificity of the IL-1 effects, cultures were performed in the presence of recombinant IL-1RA at a concentration of 120 ng/ml (a kind gift of Dr. A. Steinkasserer, University of Oxford, Oxford, UK) [34].

Determination of cytokine production

The production of cytokines by cultured epithelial cells was measured in the culture supernatants by immunoassays specific for human cytokines. Details on the measurement of IL-6 [35], IL-8 [9], MCP-1 [10], and RANTES [13] have been described in previous reports.

The stimulation index of the different stimuli (cytokines, CD40L, combinations) on different cytokine productions was calculated as the ratio of the production in the presence of this stimulus divided by the basal production in the absence of this stimulus.

Table 1. Sequence of primers used for RT-PCR

Gene	Primer	Size	Reference
β-actin	Forward 5′-CTACAATGAGCTGCGTGTGG-3′ Reverse 5′-AAGGAAGGCTGGAAGAGTGC-3′	527	[62]
IL-1α	Forward 5′-ATGGCCAAAGTTCCAGACATGTTT-3′ Reverse 5′-GTGACTGCCCAAGATGAAGACCAA-3′	600	[63]
IL-1β	Forward 5′-ATGATGGCTTATTACAGTGGCAAT-3′ Reverse 5′-TTCACCATGCAATTTGTGTCTTCC-3′	777	[63]

RNA isolation and reverse transcription-polymerase chain reaction analysis

For polymerase chain reaction (PCR) analysis of growing cells, total RNA was isolated using RNAzol (Campro, Veenendaal, the Netherlands), a method based on the guanidium chloride isolation [36], according to manufacturer's instructions. The quantity and purity of RNA preparations were determined by measuring the outer diameter (OD) at 260 and 280 nm. In these experiments, PTECs were activated with a soluble chimeric protein consisting of the extracellular parts of murine CD8 and human CD40L [32]. This protein allows the activation of CD40 without the introduction of another source of mRNA.

Fixed amounts of total cellular RNA (1 μg) were reverse transcribed into cDNA by oligo-dT priming using M-MLV reverse transcriptase (RT; GIBCO/Life Technologies). The amplification of cDNA by PCR was performed using the primers as described in Table 1. PCR amplification was performed under standard conditions [50 mM KCl, 10 mM Tris-HCl, pH 8.4, 2 mM $MgCl_2$, 0.06 mg/ml bovine serum albumin (BSA), 0.25 mM dNTPs, 25 pmol of each primer, and 1 U of Taq polymerase; Perkin Elmer, Norwalk, CT, USA] by 35 cycles of the following scheme: 1.5 minutes at 95°C, 2.5 minutes at 60°C, and 1.5 minutes at 72°C, followed by 10 minutes of primer extension at 72°C.

Polymerase chain reaction products were analyzed on a 1% agarose gel containing ethidium bromide. Final results were registered using the Eagle eye (Stratagene, San Diego, CA, USA), and for reasons of clarity, the images were black/white inverted.

Fluorescent activated cell sorter staining

For fluorescent activated cell sorter (FACS) analysis, PTECs were cultured in the absence or presence of 1 ng/ml IL-1α. After 48 hours, the cells were harvested, and CD40 expression was determined. PTECs were harvested with 2.5 mM EDTA in PBS to prevent proteolysis of the surface receptors. The whole staining procedure was performed in staining buffer containing 1% BSA (Sigma), 1% human serum, and 0.02% sodium azide in PBS. CD40 antibody (mAb89) was added in a final concentration of 5 μg/ml, incubated for 30 minutes, washed three times with staining buffer, followed by incubation with a secondary antibody goat-antimouse Ig-PE (Dako, Glostrup, Denmark). Cell fluorescence was measured on a FACScan (Becton Dickinson, Mountain View, CA, USA), and data were analyzed with the LYSIS program.

Statistical analysis

Data on the stimulation of cytokine production induced by CD40L or different cytokines were analyzed by the two-tailed Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

To determine whether combined stimulation with CD40L and cytokines resulted in synergistic effects, indices of synergy were determined (that is, the quotient of the cytokine production after costimulation and the sum of the cytokine productions after single stimulation). The Wilcoxon matched-pairs signed-ranks test was used, and costimulatory effects were considered significantly synergistic when indices of synergy are above 1 and when *P* values are less than 0.05.

RESULTS

Interleukin-6 production by proximal tubular epithelial cells after simultaneous activation by CD40L and cytokines

We studied in detail the activation of PTECs by monocyte/macrophage- and T-cell–derived signals. Human primary PTECs were stimulated either by cytokines alone (proinflammatory cytokines IL-1α and TNF-α; anti-inflammatory cytokines IL-4 and IL-10) or by combinations of these cytokines with CD40L. Both IL-1α

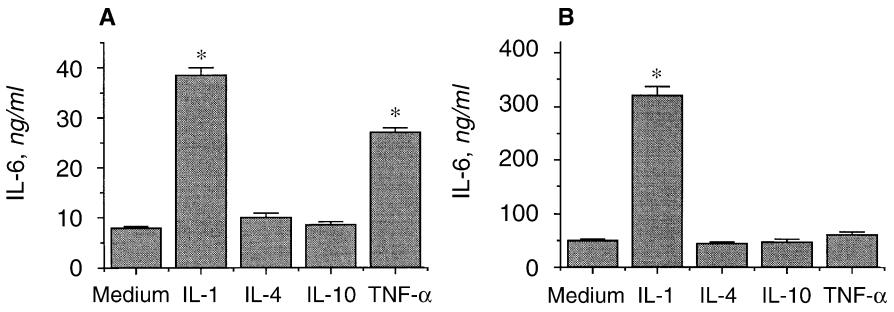

Fig. 1. Effect of cytokines in combination with CD40L on interleukin-6 (IL-6) production by proximal tubular epithelial cells (PTECs). PTECs (10^5 cells/ml) were cultured as described in the **Methods** section. Cells were cocultured with either nontransfected (*A*) or CD40L-transfected (*B*) L cells (10^5 cells/ml), as indicated. In addition, cells were activated with recombinant human cytokines: IL-1α, IL-4, IL-10, or tumor necrosis factor-α (TNF-α). After 72 hours, supernatants were harvested and tested for IL-6 production by specific enzyme-linked immunosorbent assay (ELISA). Data indicated are representative of experiments with seven different PTEC lines, and shown are the mean production (± SD) of duplicate cultures. $*P < 0.05$ compared with medium (A) or to CD40L (B).

and TNF-α increased IL-6 production by PTECs (Fig. 1A), which is in accordance with previous publications [5, 6]. In addition, stimulation with CD40L also increased IL-6 production. Compared with basal IL-6 production, a mean 21.6-fold stimulation by IL-1α, a 4.9-fold stimulation by TNF-α, and a 5.1-fold stimulation by CD40L were found in seven independent experiments. However, when combined with CD40L, only IL-1α and not TNF-α showed a strong synergy in the augmentation of IL-6 production (Fig. 1B). Combined treatment resulted in a mean 143.2-fold (CD40L/IL-1α) and 9.5-fold (CD40L/TNF-α) stimulation, respectively. No effects were found of IL-4 or IL-10 on IL-6 production by PTECs, either when tested alone or in combination with CD40L (Fig. 1B).

Chemokine production by proximal tubular epithelial cells after simultaneous CD40L and cytokine activation

To investigate whether the observed effects were specific for IL-6 production, we also investigated the production of the chemokines IL-8, MCP-1, and RANTES, which are known to be produced by PTECs. Comparable with the regulation of IL-6 production, again both IL-1α, TNF-α, and CD40L showed an increase in IL-8 production, whereas only IL-1α showed a strong synergy when combined with CD40L (Fig. 2 A, B). The mean stimulation indices of seven independent experiments are given in Table 2.

Interestingly, differences in the regulation of MCP-1 and RANTES production were found. For MCP-1, again IL-1α, TNF-α, and CD40L showed a stimulation of production by PTECs. However, in contrast to the production of IL-6 and IL-8, the combined activation with CD40L and IL-1α only resulted in additive effects (Fig. 2, C, D and Table 2).

The treatment of PTECs with single cytokines (IL-1α, TNF-α, IL-4, and IL-10) did not result in detectable RANTES production (Fig. 2E), whereas activation with CD40L did result in significant RANTES production, as published before [13]. A combination of CD40L with IL-1α or TNF-α resulted in a strongly increased RANTES production (Fig. 2F and Table 2). Although IL-4 showed no effect on the CD40L-induced IL-6, IL-8, or MCP-1 production by PTECs, it strongly increased RANTES production, as shown recently [37].

Effects of interleukin-1 are inhibited by interleukin-1RA

In view of the strong stimulatory effects of combinations of CD40L and IL-1α, we focused on the IL-1α effect. To confirm the specificity of the observed IL-1α effects, we used a recombinant form of the natural IL-1RA. The addition of IL-1RA completely neutralized the effects of exogenous IL-1α on the CD40L-induced production of IL-6 (Fig. 3A), IL-8 (Fig. 3B), MCP-1 (Fig. 3C), and RANTES (Fig. 3D). In all cases, cytokine production is reverted to the levels induced by CD40L alone. IL-1RA also completely neutralized the effects of exogenous IL-1α in the absence of CD40L, and no effects of the IL-1RA are seen on the basal cytokine production by *in vitro* cultured PTECs (data not shown).

Both interleukin-1α and interleukin-1β have synergy with CD40L

Interleukin-1 consists as two molecular forms (IL-1α and IL-1β), with overlapping biological activities. We tested the effect of both recombinant IL-1α and IL-1β on the activation of PTECs. Both molecular forms showed a similar induction of IL-6 production by PTECs, and both were able to cooperate with CD40L stimulation (Fig. 4). In addition, the effects of exogenous IL-1β were also completely neutralized by the addition of the IL-1RA (data not shown).

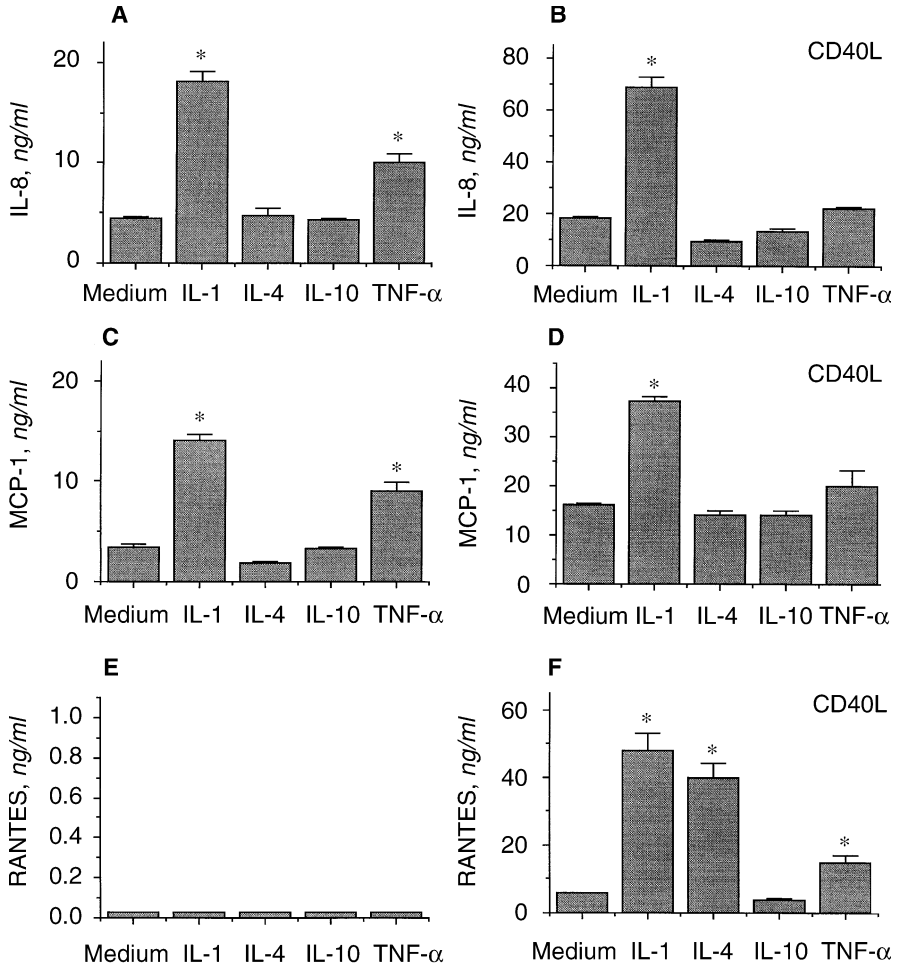

Fig. 2. Effect of cytokines in combination with CD40L on interleukin-8 (IL-8), monocyte chemoattractant protein-1 (MCP-1), and RANTES production by proximal tubular epithelial cells (PTECs). PTECs (10^5 cells/ml) were cocultured with either nontransfected (*A, C, E*) or CD40L-transfected (*B, D, F*) L cells (10^5 cells/ml), as described in Figure 1. After 72 hours, supernatants were harvested and tested for IL-8 (A, B), MCP-1 (C, D), and RANTES (E, F) production by specific enzyme-linked immunosorbent assay. Data indicated are representative of experiments with seven different PTEC lines, and shown are the mean production (± SD) of duplicate cultures. Note that the figures shown for IL-6, IL-8, MCP-1, and RANTES production are obtained from different experiments with different PTEC lines. *$P < 0.05$ compared with medium (A) or to CD40L (B). In all cases, CD40L stimulation was also significant compared with medium.

Table 2. Index of stimulation of IL-6, IL-8, MCP-1 and RANTES production by human PTEC

	Mean fold increase over baseline			
	IL-6	IL-8	MCP-1	RANTES[a]
Medium	1.0	1.0	1.0	1.0[a]
IL-1	21.6	17.9	4.5	1.0[a]
IL-4	1.1	0.6	0.8	1.0[a]
IL-10	1.2	0.6	1.4	1.0[a]
TNF-α	4.9	1.9	6.0	1.0[a]
CD40L	5.1	3.0	6.5	59
CD40L IL-1	143.2[b]	113.3[b]	9.8	354[b]
CD40L IL-4	4.5	1.9	6.0	396[b]
CD40L IL-10	4.0	3.0	8.2	53
CD40L TNF-α	9.5	5.8	10.7	107[b]

[a] Stimulation of PTEC with cytokines alone does not result in detectable RANTES production. For calculation of the stimulation index, we have therefore used the detection limit of the RANTES ELISA (0.1 ng/ml). Data included are the mean of seven independent experiments.

[b] $P < 0.05$ synergistic effects in combination with CD40L as determined by the Wilcoxon matched-pairs signed-ranks test.

Dose- and time-dependent increase in interleukin-6 production by proximal tubular epithelial cells after stimulation with CD40L and interleukin-1α

We tested the dose of IL-1α necessary to stimulate IL-6 production by PTECs. A significant increase, compared with background production, was found with 50 pg/ml of IL-1α, and increasing amounts of IL-1α showed a dose-dependent increase in IL-6 production (Fig. 5). In combination with CD40L, production levels of IL-6 were higher, but the dose response kinetics remained the same. Logarithmic presentation of the production levels on the *y*-axis revealed that the lines were parallel, suggesting that the dose response of IL-1 is independent of the CD40L stimulation. Similar dose response curves were found for the chemokines IL-8, MCP-1, and RANTES (data not shown).

Next, we investigated the time relationship between IL-6 production and CD40L and/or IL-1 stimulation. Supernatants were harvested at various time points as indicated. Already after 24 hours of culture, the synergistic effect between IL-1α and CD40L on IL-6 produc-

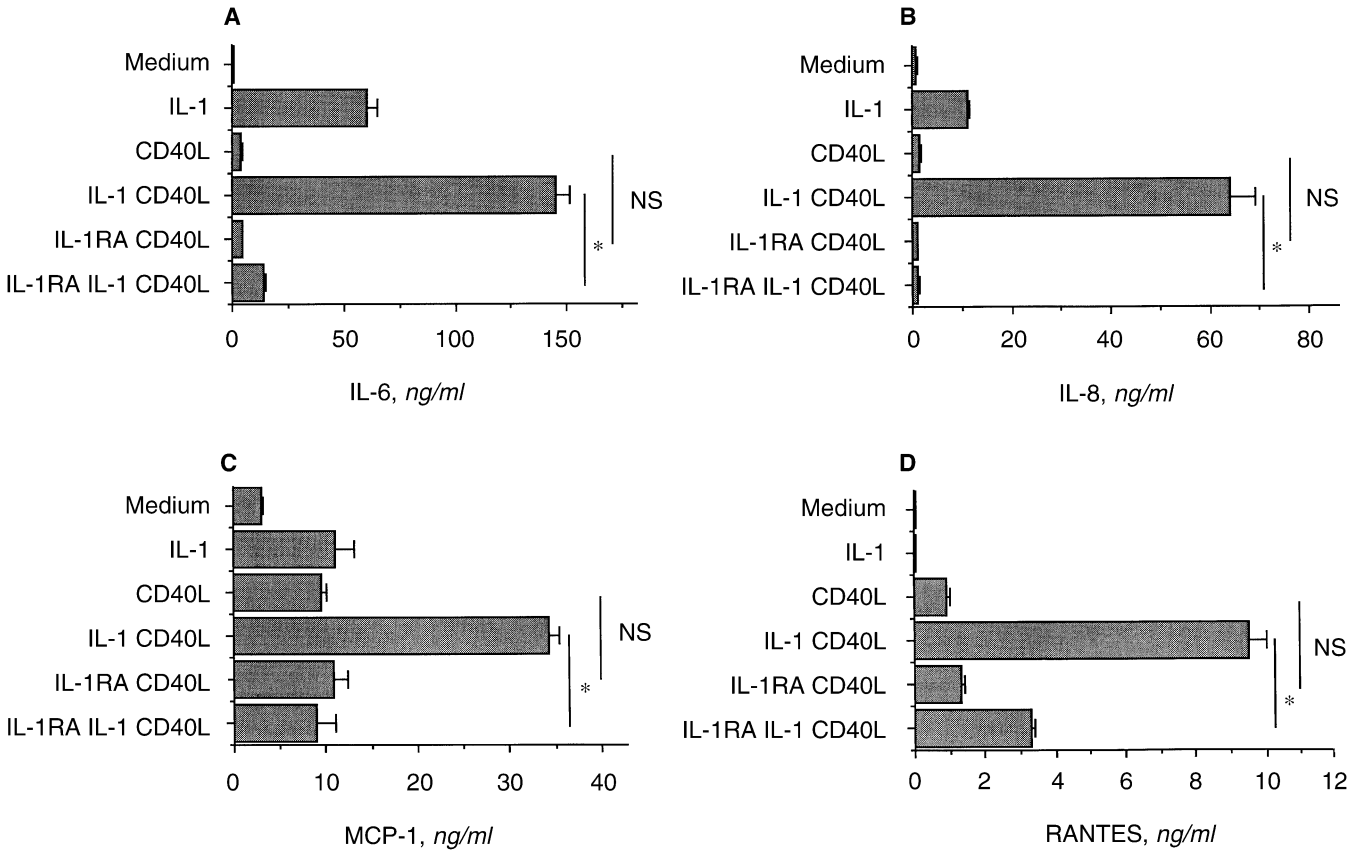

Fig. 3. Interleukin (IL)-1α–induced effects on cytokine production are completely inhibited by recombinant IL-1R antagonist. PTECs (10^5 cells/ml) were cultured as described and were activated with IL-1α (1 ng/ml) and/or CD40L as indicated. Recombinant IL-1R antagonist was added at the beginning of the cultures at a concentration of 120 ng/ml. After 72 hours, supernatants were harvested and tested for IL-6 (*A*), IL-8 (*B*), MCP-1 (*C*), and RANTES (*D*) production. Data indicated are representative of experiments with three different PTEC lines, and shown are the mean production (± SD) of duplicate cultures. $*P < 0.05$ effect of IL-1RA on CD40L + IL-1 induced production; NS is not significant.

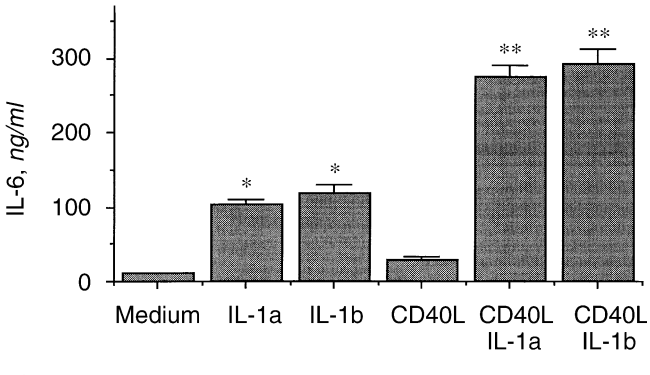

Fig. 4. Both IL-1α and IL-β stimulate IL-6 production by PTECs. PTECs were cocultured with either nontransfected or CD40L-transfected L cells (10^5 cells/ml), as indicated. In addition, cells were activated with recombinant human IL-1α or IL-1β (both at 1 ng/ml). After 72 hours, supernatants were harvested and tested for IL-6 production by specific enzyme-linked immunosorbent assay. Data indicated are representative of experiments with three different PTEC lines, and shown are the mean production (± SD) of duplicate cultures. $*P < 0.05$ compared with medium or $**P < 0.05$ compared with CD40L.

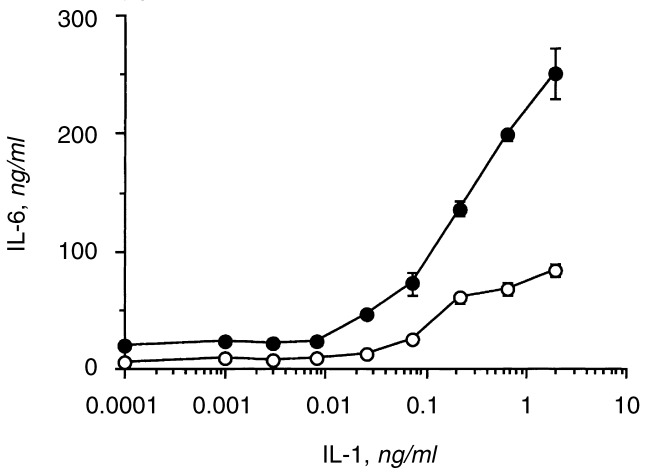

Fig. 5. Dose-dependent effects of IL-1α on IL-6 production by PTECs. PTECs (10^5 cells/ml) were cultured as described and were activated with increasing concentrations of IL-1α in the absence (○) or presence (●) of CD40L stimulation. After 72 hours, supernatants were harvested and tested for IL-6 production. Data indicated are representative of experiments with four different PTEC lines, and shown are the mean production (± SD) of duplicate cultures.

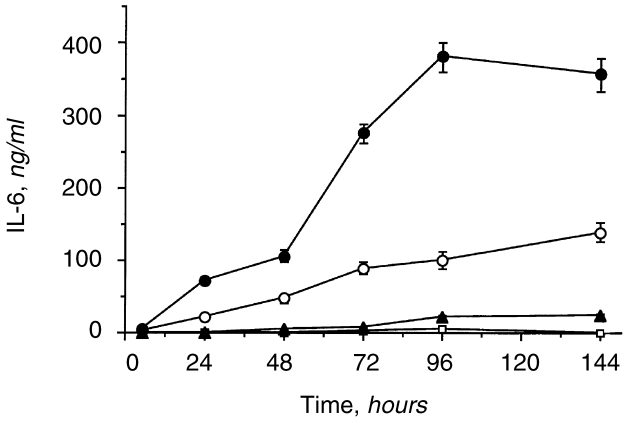

Fig. 6. Kinetics of IL-6 production by PTEC after stimulation with IL-1α and/or CD40L. PTECs (10^5 cells/ml) were cultured as described in the **Methods** section, and were activated with IL-1α in the absence or presence of CD40L stimulation. Supernatants were harvested at the indicated time points and were tested for IL-6 production. Data are representative of experiments with three different PTEC lines, and shown are the mean production (± SD) of duplicate cultures. Symbols are: (□) med; (○) IL-1; (▲) CD40L; (●) IL-1 + CD40L.

tion could be observed (Fig. 6). Production levels increased in time and reached a plateau level at 72 to 96 hours and remained stable until 144 hours.

Expression of interleukin-1α and interleukin-1β by proximal tubular epithelial cells

Some immunohistochemical studies have suggested that PTECs are a possible source of IL-1 production [20, 23, 24]. Therefore, we performed RT-PCR analysis using specific primers for IL-1α and IL-1β (Table 1). We used cDNA from nonstimulated or CD40L-stimulated PTECs. Semiquantitative RT-PCR for β-actin confirmed that equal quantities of cDNA have been generated (Fig. 7A). Under both conditions, expression of IL-1β mRNA was observed. Interestingly, although absent in nonstimulated PTECs, after activation with CD40L expression of IL-1α mRNA also could be observed (Fig. 7B).

Interleukin-1 increases CD40 expression on proximal tubular epithelial cells

Previously, we demonstrated the expression of CD40 on cultured PTECs [13]. In these experiments, we investigated the effect of stimulation of PTECs with IL-1 on the expression of CD40 as a possible mechanism to explain the synergy between IL-1 and CD40L. Incubation of PTECs with IL-1α for 48 hours resulted in a 2.9-fold increase of CD40 expression (mean of five independent experiments, range 2.3 to 5.6; Fig. 8). These data suggest that local inflammation may exert a direct effect on CD40–CD40L interactions by up-regulation of CD40 on PTECs.

DISCUSSION

In this study, we found a strong stimulatory effect of combinations of CD40L and IL-1 on the production of IL-6, IL-8, MCP-1, and RANTES by primary cultures of human PTECs. Increased production of these mediators *in situ* may contribute to local inflammatory responses in the renal interstitium. The different chemokines (IL-8, MCP-1, and RANTES) exert preferential chemoattractant effects on different cell types: IL-8 is a chemoattractant for neutrophils. MCP-1 is a chemoattractant for monocytes, and RANTES is a chemoattractant for T cells [18, 38]. Therefore, differential regulation of the various chemokines will have a direct impact on the composition of the cellular infiltrate. These results underline the potential importance of the PTECs as regulators of local inflammation. Interestingly, the synergistic effects were specifically observed for combinations of IL-1 and CD40L and were not found with combinations of CD40L and TNF-α, another major proinflammatory cytokine. However, because CD40L and TNF-α belong to the same superfamily of ligands [39], it might be that these two factors induce overlapping sets of signal transduction pathways.

These data show an intimate interplay between IL-1 and CD40L *in vitro.* We found not only a strong synergy between IL-1 and CD40L on cytokine production by PTECs, but also an induction of IL-1α message by PTECs after CD40L activation and increased CD40 expression on PTECs after IL-1 activation. Previous studies have described the cooperation between IL-1 and CD40L. The combination of CD40L and IL-1 results in an increased granulocyte macrophage-colony stimulating factor (GM-CSF) production by thymic epithelial cells [40], increased GM-CSF and MIP-1α production by synoviocytes [41], and an increased leukemia inhibitory factor (LIF) production by endothelial cells [42]. However, these studies did not reveal the strong synergy as observed with PTECs. In addition, using peripheral blood monocytes, CD40L stimulation increases IL-1 production by monocytes [43–45], which could be downregulated by IL-4 and IL-10 [46]. Like on PTECs, IL-1 increased CD40 expression on thymic epithelial cells and endothelial cells, but not on synoviocytes or keratinocytes [40, 41, 47, 48]. Finally, it was recently demonstrated that CD40L stimulation of smooth muscle and endothelial cells activates IL-1β–converting enzyme (ICE; caspase-1) and thus increases the generation of bioactive IL-1 [49]. In conclusion, these results show that IL-1 and CD40L are interacting with each other at different levels of regulation and that these effects are different for specific cell types.

Currently, the mechanism(s) behind the synergy between IL-1 and CD40L remains unexplained. Although a positive effect of IL-1 on CD40 expression is observed,

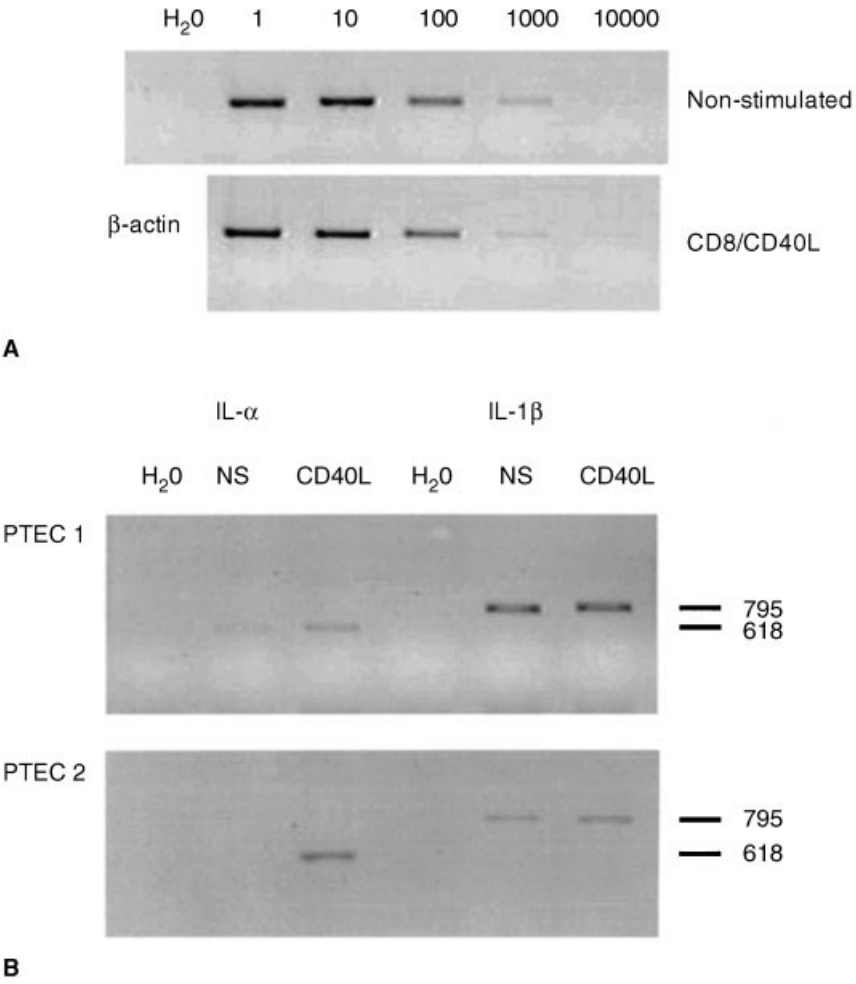

Fig. 7. Polymerase chain reaction (PCR) analysis of IL-1α and IL-1β expression in cultured PTECs. Total RNA was isolated from cultured PTECs, reverse transcribed, and PCR for β-actin, IL-1α, and IL-1β was performed. Shown is the ethidium bromide staining of PCR products analyzed on a 1% agarose gel. (*A*) Semiquantitative analysis of the amount of cDNA obtained either from nonstimulated PTECs or from PTECs stimulated with CD40L. Titration of the cDNA demonstrates equal expression of β-actin in both cases. (*B*) Expression of both IL-1α and IL-1β in PTECs either nonstimulated (ns) or after CD40L stimulation. H20 shows the negative control of the PCR reaction.

it remains to be established whether this increased expression (which is already high in the absence of IL-1) contributes to the higher cytokine production by PTECs. It is probably more likely that different signal transduction pathways are converging intracellularly. Although several intracellular signals transduced via IL-1 receptors and CD40 have been described, no specific information is available on signaling in tubular epithelial cells.

The important role of PTECs as a source of inflammatory mediators (cytokines and chemokines) is widely accepted. Using our *in vitro* culture system, we found distinct differences in the regulation of the different cytokines produced by PTECs. The production of MCP-1 showed only additive effects between IL-1 and CD40L compared with the synergistic effects on the production of the other factors. RANTES production was strictly dependent on CD40 signaling or on combinations of cytokines [29], whereas other factors were already produced under basal conditions. In addition, IL-4 specifically showed an effect on RANTES production and not on the other factors. Finally, in our experiments, the regulation of IL-6 and IL-8 production appeared to be

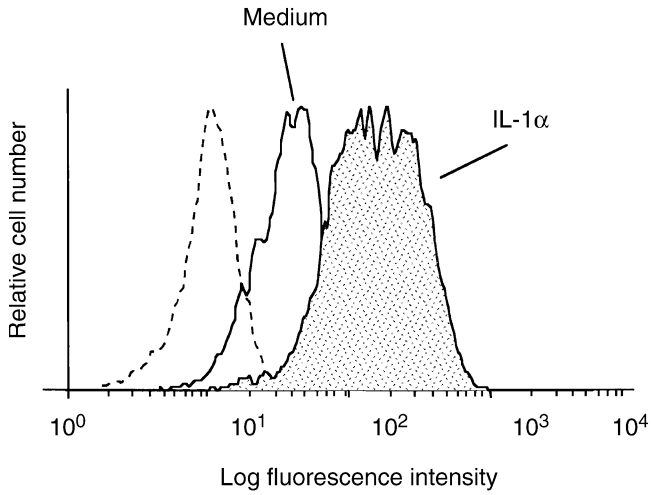

Fig. 8. Interleukin-1 (IL-1) increases CD40 expression on PTECs. PTECs were cultured as described, in the absence or presence of IL-1 stimulation (1 ng/ml). After 48 hours, cells were harvested with EDTA and stained for CD40 (mAb89). Staining was developed with a goat-antimouse-PE conjugate and compared with an irrelevant antibody (represented by the dotted line). The histogram of the cells cultured in medium is left open, whereas the histogram of the cells stimulated with IL-1 is filled.

similar. However, previous studies have demonstrated that IL-8 production, but not IL-6 production, is inhibited by IFN-γ [9, 11, 31]. Therefore, these data clearly indicate that, at least *in vitro*, the production of cytokines and chemokines by PTECs is regulated and fine tuned by the presence of local cytokines.

Several lines of investigation have demonstrated that the ligand for CD40 (CD40L) is mainly expressed on activated $CD4^+$ T cells, and plays a pivotal role in B-cell activation and the development of humoral immune responses [33]. More recently, it has become clear that CD40 might also play an important role in the activation of other hematopoietic (monocytes, dendritic cells) and nonhematopoietic (endothelial cells and fibroblasts) cell types. The functional consequence of CD40 activation on these cells is diverse and includes enhanced survival, phenotypic changes, expression of adhesion and costimulatory molecules and the induced/enhanced production of different cytokines [28, 50].

Using FACS analysis, strong and homogeneous expression of CD40 could be demonstrated on *in vitro* cultured human PTECs [13]. In contrast, in normal kidneys, the expression of CD40 is restricted to a limited number of tubules, which can be both of distal and proximal origin [13, 51, 52]. However, CD40 expression is increased on tubular epithelial cells under inflammatory conditions. In line with this, we found an increased expression of CD40 on PTECs after IL-1 stimulation. This suggests that the expression of CD40 on tubular epithelial cells is subject to regulatory mechanisms.

Recently, the presence of CD40L-expressing cells has been demonstrated in the renal interstitium in patients with either allograft rejection (Van Kooten et al, manuscript submitted) or with class IV lupus nephritis [51]. Therefore, it is tempting to speculate that CD40–CD40L interactions contribute to the inflammation observed in these disease situations.

The previously described experiments raise important questions about the possible *in vivo* role of both the CD40–CD40L pathway and the IL-1–IL-1R pathway in renal inflammation.

The administration of antibodies to CD40L prevents the development of lupus nephritis in lupus prone mice [53, 54]. More importantly, dramatically diminished inflammation, fibrosis, and vasculitis were seen in mice with established lupus nephritis (5 to 7 months of age) after treatment with anti-CD40L antibodies [55]. These data suggest that next to the inhibition of autoantibody formation, also the effector arm of the disease is inhibited by anti-CD40L treatment. In patients with systemic lupus erythrematosus (SLE), an increased expression of CD40L was observed on circulating lymphocytes [56, 57], and CD40L-positive cells could also be demonstrated in kidney sections of these patients [51].

As well, a causal role for IL-1 in renal inflammation has been suggested. Increased expression of IL-1 in the kidney has been demonstrated in various forms of glomerulonephritis [20–24]. Increased expression of IL-1 was also found in mice with lupus nephritis [58]. Immunohistochemistry showed that both infiltrating cells and resident kidney cells, including tubular epithelial cells, contribute to the increased IL-1 production. It has been demonstrated that anti-glomerular basement membrane nephritis in rats can be aggravated by injection of IL-1, whereas it can be inhibited by passive immunization against IL-1β [59, 60]. In addition, treatment with a recombinant form of the natural IL-1RA was also shown to blunt the inflammatory processes in experimental forms of glomerulonephritis [25–27, 61].

In conclusion, we have demonstrated that combinations of IL-1 and CD40L exert a very strong activation of cytokine and chemokine production by PTECs. The presence of IL-1, CD40L, and CD40 has been demonstrated in various forms of renal inflammation, including allograft rejection. This suggests an important role for these proinflammatory signals in the regulation of local inflammation. The inhibition of either the IL-1 pathway or the CD40L pathway has the potential to blunt the inflammatory response. Future experiments should be aimed at combining the targeting of these pathways in experimental forms of inflammatory kidney diseases, to establish whether the observed synergy between IL-1 and CD40L *in vitro* translates into enhanced inflammation *in vivo*.

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