Effects of mycophenolic acid on IL-6 expression of human renal proximal and distal tubular cells in vitro

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Abstract

Background. Interleukin-6 (IL-6) is a multifunctional cytokine which regulates immune responses and host defence mechanisms. IL-6 has been found to be increased in certain inflammatory conditions of the kidney, in which tubular epithelial cells play a pivotal role. Human renal tubular cells express IL-6. Until now no data about the effect of the immunosuppressant drug mycophenolic acid (MPA) on IL-6 expression were available.

Methods. Proximal and distal tubular epithelial cells (PTC/DTC) have been isolated immunomagnetically. Confluent monolayers were stimulated with interleukin-1β (IL-1β; 25 U/ml), IL-1β+ MPA (0.25–50 μM) or MPA alone for 48 h. Release of IL-6 protein into the supernatant was evaluated with an enzyme immunoassay, IL-6 mRNA expression was evaluated using the Quantikine mRNA kit.

Results. After IL-1β stimulation, a highly significant 2.6- (PTC) and 3.8-fold (DTC) upregulation of IL-6 expression was detectable. IL-6 mRNA was upregulated by IL-1β [1.57- (PTC) and 2.03-fold (DTC)]. MPA inhibited this cytokine-induced IL-6 expression in a dose-dependent manner. Incubation with the lowest MPA concentration had no effect on the stimulated upregulation, whereas all higher doses significantly decreased IL-6 expression. Dexamethasone significantly inhibited the cytokine-induced IL-6 protein release in PTC, but not in DTC.

Conclusions. In this study we demonstrated for the first time an inhibitory effect of MPA on the stimulated IL-6 expression of renal tubular epithelial cells. In contrast to older data, which showed a synergistic upregulation of the expression of a CC-chemokine by a combination of cytokines and MPA, in the present study we could demonstrate an immunosuppressive effect of MPA on the expression of an important cytokine.

Keywords: distal tubule; epithelial cells; IL-6; kidney; mycophenolic acid; proximal tubule

Introduction

In vitro culture of human renal tubular cells of well defined nephron origin is an important basis in the research of various physiological and pathophysiological mechanisms in the kidney. Although the physiological role of tubular epithelial cells has been extensively evaluated, many processes in the tubular segments under pathophysiological conditions are poorly understood. In general, all renal diseases and functional renal decline are accompanied with tubulointerstitial changes. During renal allograft rejection, tubulointerstitial changes are a prominent feature of the rejection process [1]. Expression of chemokines and adhesion molecules on the surface of renal epithelial cells plays a pivotal role in chemoattraction and infiltration by lymphocytes, monocytes and granulocytes in renal allograft rejection, inflammatory kidney diseases and autoimmune disorders.

Interleukin-6 (IL-6) is a pleiotropic cytokine which is released by a variety of different cells upon stimulation, usually at sites of tissue inflammation [2]. IL-6 shows multiple biological functions, most often associated with immune responses and host defence mechanisms. It has been found to be increased in certain inflammatory conditions such as allograft rejection, in which it exerts multiple effects on infiltrated inflammatory cells and on resident tissue cells. IL-6 has been implicated in the development of glomerular and tubular injury in various forms of immune-mediated renal diseases. The expression of the cytokine by human renal tubular epithelial cells has further been shown in different studies [3].
Mycophenolic acid (MPA) is a powerful immunosuppressive drug, which has been shown to be effective for the prevention and treatment of renal allograft rejection [4]. Rejection episodes were found to be associated with an infiltration of lymphocytes and macrophages/monocytes into the diseased kidney. MPA, the active metabolite of mycophenolate mofetil, is a potent, non-competitive and reversible inhibitor of inosine-5'-monophosphate dehydrogenase, the rate-limiting enzyme for de novo purine synthesis [5], and thus interferes with cellular biosynthesis of guanosine and deoxyguanosine nucleotides. One metabolic side effect of guanosine depletion mediated by MPA is a decrease in the transfer of mannose and fucose to glycoproteins (e.g. adhesion molecules) [6]. Most cells are able to utilize the salvage pathway which basically recycles free purine bases. Since proliferating B and T lymphocytes are dependent on the de novo pathway for purine biosynthesis rather than on the salvage pathway, MPA is described to inhibit lymphoid cell metabolism more effectively than metabolism of other cell types. It is, however, becoming increasingly evident that this drug may also act on non-lymphoid cells.

At present, no data about the effect of MPA on the expression of IL-6 are available, and there exists only a scant knowledge about the effects of MPA on the renal tubular epithelium. Therefore, the objective of this study was to investigate the effect of MPA on cytokine-induced IL-6 expression on highly purified proximal and distal tubular cells from human kidney.

Materials and methods

Materials

Tissue culture media were obtained from Gibco (Eggenstein, Germany) and Sigma Chemicals (Deisenhofen, Germany), and MACS microbeads from Miltenyi (Bergisch-Gladbach, Germany). Recombinant interleukin-1β (IL-1β) was provided by Strathman (Hamburg, Germany). MPA was obtained from Hoffmann LaRoche (Grenzach-Wyhlen, Switzerland), dexamethasone from Ratiopharm (Ulm, Germany).

For the enzyme-linked immunoassay an anti-IL-6 capture antibody, a biotinylated anti-IL-6 detection antibody and an antibody-coated magnetic beads as described previously [7]. Cells were prepared after tumour nephrectomies from those portions of the human kidney not involved in renal cell carcinoma. The tissue was minced into 1-mm² pieces and digested with RPMI 1640 containing collagenase/dispase (1 mg/ml) at 37°C for 1 h. The digested tissue fragments were passed through a 106 μm mesh and incubated for 45 min with RPMI 1640 supplemented with collagenase IV (1 mg/ml), DNase (100 μg/ml) and MgCl₂ (5 mM). After Percoll density gradient centrifugation, the cell pellet was preincubated in 10 ml of ice-cold RPMI 1640 for 20 min with human immunoglobulin G added. Cells were incubated for 20 min on ice with the primary antibody. To enrich PTC we used a monoclonal antibody (mAb) against aminopeptidase M (CD13), specific for the proximal tubule. DTC were isolated through a mAb recognizing a specific antigen of the thick ascending limb of Henle’s loop and the early distal convoluted tubule. Then, cells were washed with PBS containing 5 mM EDTA and 0.5% bovine serum albumin (BSA). Finally, cells were incubated with secondary antibody for 20 min, washed, passed through a fine mesh (pore size 70 μm) and isolated by immunomagnetic separation applying the Mini-MACS system [7].

After immunomagnetic isolation, cells were seeded in six-well plates precoated with human collagen IV [20 μg/ml; 1 h followed by fetal calf serum (FCS) for 15 min]. The cells were grown in medium 199 with 10% FCS at 37°C and 5% CO₂ in a humidified atmosphere. The culture medium was supplemented with the antibiotic meropenem (100 μg/ml) during the first 2–3 days after isolation. Culture medium was changed every 3–4 days.

Primary isolated and cultured cells were characterized by different methods, and the purity of the isolation method was shown [7,8]. Stainings for desmin, smooth muscle actin, fibroblast-specific marker and von Willebrand factor to exclude the possibility of contaminating cells were negative. In vitro differentiation of PTC and DTC was shown by scanning electron microscopy and flow cytometry. Cultured cells displayed a stimulation pattern like in vivo as shown by hormonal stimulation followed by cAMP measurements.

Assay for cytotoxicity

As an indicator of cytotoxicity, the activity of lactate dehydrogenase (LDH) in cell culture supernatants of PTC and DTC was determined by a commercial assay, measuring the decrease of absorbance at 340 nm, resulting from conversion of NADH to NAD in the presence of pyruvate. Supernatants from cell cultures treated with MPA for 48 h were collected and assayed for LDH release.

Stimulation of IL-6 expression and treatment with mycophenolic acid

Cells were grown in 24-well culture plates to confluence. Thereafter, cells were washed and kept in serum-free medium 199 for 24 h. For stimulation of IL-6 expression, the cells were treated with IL-1β (25 U/ml) in serum-free medium 199 for 48 h. The time-dependent IL-6 release and the influence of IL-1 receptor antagonist (IL-1RA, 30 ng/ml) on IL-1β-stimulated IL-6 expression were tested in 24-well cell culture plates. In the experiments with IL-1RA, the cells were pretreated with IL-1RA in medium 199 for 30 min and then cultured for the indicated period of time in the presence of IL-1β plus IL-1RA. The effects of γ-interferon (γ-IFN; 200 U/ml), tumour necrosis factor-α (TNF-α; 10 ng/ml) and a combination of the cytokines (γ-IFN, 200 U/ml; IL-1β, 25 U/ml; and TNF-α, 10 ng/ml) were tested in separate stimulation experiments in 24-well cell culture plates.
To test the influence of MPA on the induced expression of IL-6, cells were treated with media containing IL-1β + MPA (0.25, 2.5, 25, 50 μM) or MPA alone (0.25, 25 μM) at 37°C for 48 h. As a control, cells were incubated with medium 199 alone (basal) or with IL-1β plus dexamethasone (10 μM). Supernatants were harvested after 48 h for IL-6 quantification, and assessed for the cytokine or stored at −20°C for later measurement.

**Protein measurement and cell count**

Total protein content was determined according to the bicinchoninic acid (BCA) protein assay [9] using BSA as a standard (31.5–1000 μg/ml). Cells were lysed using SDS/SSC [0.02% (w/v) SDS, 150 mM NaCl, 15 mM sodium citrate] or Triton X-100 [1% (v/v)] as a lysis buffer and incubated for 10 min on ice. After centrifugation, probes were assayed by the BCA assay or stored at −20°C for later measurements. The total amount of IL-6 produced per 24 wells was related to the total protein per well.

The total cell count of confluent cell monolayers per well was determined by counting in an improved Neubauer haemacytometer. In addition, cell count was measured by a fluorometric assay with the fluorochrome 4,6-diamidino-2-phenylindole (DAPI) [10].

**Quantification of IL-6 expression**

The cytokine IL-6 was quantified using a laboratory-made sandwich enzyme-linked immunosorbent assay. In brief, wells of 96-well microtitre plates were coated with an anti-human IL-6 mAb overnight at room temperature. Non-specific binding sites were blocked with PBS/1% BSA/5% sucrose. After 1 h of incubation, the plates were washed with PBS/0.05% Tween. Standard and samples were added to the wells and incubated for 2 h at room temperature. All samples were diluted in assay buffer (1:25–1:100) and run in duplicate. The plates were washed and incubated with biotinylated anti-IL-6 for 2 h at room temperature, with horseradish-peroxidase-streptavidin for 20 min. After washing, the substrate reaction was followed by measurement of the optical density at 450 nm (vs 650 nm). Recombinant human IL-6 was used as a standard (10–500 pg/ml). The typical sensitivity of the assay is 0.6 pg/ml (published sensitivity). Data were represented as pg of IL-6 in the supernatant per μg of total cell protein (pg/μg).

IL-6 mRNA was determined by the use of a commercially available assay kit. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was determined as a housekeeping gene. The IL-6 standard curve was from 11.7–750 amol/ml, GAPDH from 9.4–600 amol/ml. Quantitation of IL-6 mRNA was normalized with GAPDH mRNA [represented as quotient (IL-6/GAPDH)].

**Statistical analysis**

The data were expressed as mean ± standard deviation (SD). All samples were run in duplicate, the number n indicated experiments with cells from different nephrectomies. For statistical analysis, Student’s t-test for unpaired samples and one-way analysis of variance (ANOVA) were used. Normality was tested by Kolmogorov–Smirnov’s test. P-values < 0.05 were considered significant; P-values < 0.01 were considered highly significant.

**Results**

**Cytotoxicity of MPA**

The measurement of LDH release into culture supernatant as a marker of cell cytotoxicity revealed no cytotoxic potential of MPA in the concentrations used in the experiments (up to 50 μM). Compared with the stimulation with cytokines alone (PTC, 19.5 ± 6.8 units LDH/ml; DTC, 20.6 ± 12.0 units LDH/ml), no statistically significant differences were detectable by the addition of MPA (0.25 μM MPA: 16.5 ± 10.1 (PTC) and 12.5 ± 0.7 (DTC); 2.5 μM MPA: 21.0 ± 3.5/14.0 ± 1.0; 25 μM MPA: 23.7 ± 3.8/16.5 ± 0.7; 50 μM MPA: 19.5 ± 6.8/not done).

**Effect of cytokines on IL-6 expression**

IL-6 protein release into the culture supernatant of PTC and DTC was significantly increased by the addition of IL-1β for 48 h, as determined by enzyme-linked immunosorbent assay (Table 1). Time-dependent expression of IL-6 and the influence of IL-1RA were assessed in separate experiments. These experiments indicated a significant stimulation of IL-6 after 2 h (P < 0.01), while the highest level of IL-6 release was reached after 48 h. The induction of IL-6 by IL-1β was significantly inhibited by the addition of IL-1RA (Figure 1).

Neither TNF-α (10 ng/ml) nor γ-IFN (200 U/ml) alone induced significant IL-6 expression in PTC and DTC [P > 0.1 vs basal expression (PTC and DTC), n = 4–5]. The mix of all three cytokines together (cytomix: IL-1β, TNF-α and γ-IFN) induced IL-6 expression in the same amount as IL-1β alone [cytomix vs IL-1β: P = 0.41 (PTC), P = 0.39 (DTC); data not shown]. Analysis of IL-6 mRNA expression revealed

**Table 1. Effect of MPA on the stimulated IL-6 protein expression**

<table>
<thead>
<tr>
<th></th>
<th>PTC</th>
<th>DTC</th>
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<tbody>
<tr>
<td>Medium 199 (basal)</td>
<td>107.6 ± 18.6</td>
<td>74.9 ± 26.7</td>
</tr>
<tr>
<td>IL-1β + 50 μM MPA</td>
<td>280.1 ± 82.9</td>
<td>285.1 ± 50.4</td>
</tr>
<tr>
<td>+25 μM MPA</td>
<td>144.7 ± 24.7</td>
<td>124.2 ± 11.0</td>
</tr>
<tr>
<td>+5 μM MPA</td>
<td>142.1 ± 20.6</td>
<td>123.5 ± 30.9</td>
</tr>
<tr>
<td>+0.25 μM MPA</td>
<td>159.6 ± 24.8</td>
<td>158.1 ± 31.9</td>
</tr>
<tr>
<td>+10 μM dexamethasone</td>
<td>273.6 ± 47.1</td>
<td>244.6 ± 59.9</td>
</tr>
<tr>
<td>25 μM MPA</td>
<td>204.7 ± 52.9</td>
<td>253.8 ± 45.7</td>
</tr>
<tr>
<td>+0.25 μM MPA</td>
<td>50.1 ± 24.1</td>
<td>Not done</td>
</tr>
<tr>
<td>10 μM dexamethasone</td>
<td>36.7 ± 16.0</td>
<td>Not done</td>
</tr>
</tbody>
</table>

The cytokine IL-6 was quantified in cell culture supernatant using an enzyme-linked immunosorbent assay. Data are represented as pg IL-6 per μg of total cell protein [mean ± SD, PTC, n = 6–13 (n = 4–5), DTC, n = 5–6. *P < 0.01 vs basal, **P < 0.05/0.01 vs IL-1β, ***P < 0.01 vs IL-1 + 50 μM MPA].
a 1.57-fold (PTC) and 2.03-fold (DTC) stimulation by IL-1β compared with unstimulated controls (Figure 2). TNF-α or γ-IFN did not stimulate IL-6 message production (data not shown). Therefore, we used IL-1β alone for all stimulation experiments.

**Effect of MPA on cytokine-stimulated IL-6 protein expression**

As a control, we investigated the effect of dexamethasone on IL-1β-induced IL-6 protein release. While dexamethasone treatment inhibited IL-6 protein release into the supernatant of PTC (P < 0.05) and DTC (not significant), co-incubation of cytokim (combination of γ-IFN, IL-1β and TNF-α) and MPA (2.5–50 μM) induced a significant and dose-dependent decrease in IL-6 release of PTC and DTC (Table 1). Incubation of IL-1β in combination with a low MPA concentration (0.25 μM) induced no inhibitory effect compared with the IL-1β stimulation. These data could be confirmed by IL-6 mRNA analysis (Figure 2). MPA or dexamethasone given alone significantly decreased IL-6 release compared with the constitutive (basal) expression. Significant differences between PTC and DTC could not be detected.

**Effect of MPA on cytokine-stimulated IL-6 mRNA expression**

Increased IL-6 mRNA expression was detected following stimulation with IL-1β (Figure 2). MPA inhibited the IL-1β stimulated IL-6 expression in a dose-dependent manner. Compared with stimulated controls, MPA-treated stimulated cells expressed less IL-6 message (decrease up to ~73%, Figure 2). This inhibition was significant in the experiments with higher MPA doses [P < 0.05 (DTC) and 0.01 (PTC), respectively, for 50, 25 and 2.5 μM MPA]. The lowest MPA concentration (0.25 μM) did not influence stimulated IL-6 mRNA expression.

**Effect of MPA on protein content and cell count of confluent cell monolayers**

In separate experiments, we show the relation of total cell protein to total cell count (Table 2). Therefore, confluent cell monolayers were incubated for 48 h with

**Table 2.** Total cell count and total protein content in DTC cultures after MPA incubation

<table>
<thead>
<tr>
<th></th>
<th>Cell count</th>
<th>Cell protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microscope</td>
<td>DAPI</td>
</tr>
<tr>
<td>Medium 199 (basal)</td>
<td>49.3 ± 7.1</td>
<td>42,082 ± 2404</td>
</tr>
<tr>
<td>IL-1β</td>
<td>60.3 ± 14.4</td>
<td>38,872 ± 8027</td>
</tr>
<tr>
<td>+50 μM MPA</td>
<td>43.3 ± 3.3</td>
<td>39,973 ± 4654</td>
</tr>
<tr>
<td>+25 μM MPA</td>
<td>41.3 ± 7.8</td>
<td>38,063 ± 8121</td>
</tr>
<tr>
<td>+25 μM MPA</td>
<td>45.7 ± 9.5</td>
<td>40,474 ± 6149</td>
</tr>
<tr>
<td>+0.25 μM MPA</td>
<td>46.7 ± 20.0</td>
<td>43,060 ± 3910</td>
</tr>
<tr>
<td>25 μM MPA</td>
<td>55.5 ± 14.5</td>
<td>39,430 ± 9762</td>
</tr>
<tr>
<td>0.25 μM MPA</td>
<td>49.5 ± 12.5</td>
<td>36,600 ± 8558</td>
</tr>
</tbody>
</table>

Relation of cell count to total protein in DTC cultures. Total cell count was determined by microscopic counting or a fluorescence assay. Microscope: cells were counted in a Neubauer haemacytometer (×10^4 cells). DAPI: cell count was determined by a fluorometric assay with the fluorochrome DAPI [10]. Fluorescence was measured in relative fluorescence units in a FluoStar fluorescence reader. Total protein was determined by the BCA protein assay [9]. Cells were lysed using SDS/SSC [0.02% (w/v) SDS, 150 mM NaCl, 15 mM sodium citrate] or Triton X-100 [1% (v/v)] as a lysis buffer. All results were expressed as mean ± SD [n = 6 (microscopic examination: n = 3), cells from two different DTC preparations]. No statistical significance could be detected.
different concentrations of MPA. In contrast to our older data with tubular cells in the proliferation phase, the experiments show that MPA has no effect on confluent cell monolayer cultures. Furthermore, the relation of total cellular protein and cell count is not affected by MPA.

**Discussion**

Only limited data are available about the effects of MPA on renal tubular epithelial cells, a pivotal cell type involved in the rejection process. Renal transplant rejection is characterized by cellular infiltration consisting mostly of monocytes, macrophages and T cells in the graft. It has been shown that the immunosuppressive drug MPA prevents allograft rejection in animal experiments and clinical trials [11]. In general, MPA resulted in a better preservation of graft morphology with less pronounced cellular infiltration and tubular atrophy [11]. The effect of MPA on lymphocytes and the molecular mechanisms underlying this effect are well described [12,13]. In the case of the action mechanism of MPA by specific inhibition of purine de novo synthesis, an antiproliferative effect should be lymphocyte-specific. Nevertheless, in an older study we had been able to demonstrate that MPA inhibits proliferation of human tubular epithelial cells [10]. This effect was abolished by guanosine supplementation [10]. On the other hand, major side effects of MPA therapy such as gastrointestinal disorders with diarrhoea and eosinophilic infiltration indicate the possible effects of MPA on epithelial cells. The concentrations of MPA used in this in vitro study are in the range of blood concentrations obtained in vivo (c_{max} = up to 26 μg/ml [14]). In renal transplant recipients, therapeutically given MPA reaches the tubular epithelium in the graft at the basolateral cell membrane and may affect tubular cell metabolism. Therefore, we investigated the effects of MPA on the stimulated IL-6 expression of renal tubular epithelial cells in vitro.

The tubular epithelium has been recognized both as a target cell in immunologically mediated renal injury and as a possible regulator or effector cell type in immune-mediated renal inflammation [3]. Culture of highly purified human renal proximal and distal tubular epithelial cells offers a unique in vitro system in which their different responses to different stimuli can be studied [15]. The induced expression of cytokines, chemokines, adhesion molecules (i.e. ICAM-1) and major histocompatibility complex molecules (i.e. HLA-DR) by renal tubular cells has further been shown in vivo and in vitro [1,3,16,17]. The characteristic of IL-6 gene regulation is its induction by inflammation-associated cytokines [18]. The cytokine IL-1 is the most potent activator of cells so far described in cell biology [19], and the stimulatory effect on the IL-6 synthesis has been described for renal tubular epithelial cells in vitro [3]. Therefore, we used IL-1β to stimulate IL-6 expression in our experiments. IL-1RA was used to prove the specific cellular response to IL-1. IL-1RA binds to the IL-1 receptor in competition with IL-1 but elicits no detectable response. It is used as a classical antagonist to inhibit the cellular response to IL-1. Others described TNF-α as a potent stimulator of IL-6 synthesis. We were not able to induce a significant stimulation of IL-6 by TNF-α addition to the cell cultures. In agreement with our results, Boswell et al. [3] described no IL-6 stimulation by the addition of TNF-α or γ-IFN.

We found a dose-dependent inhibitory effect of MPA on the stimulated IL-6 expression in renal tubular cells. In contrast, Borger et al. [20] described an upregulation of IL-6 expression by mycophenolate mofetil in the airway-derived cell line A549. Our older studies have shown that cytokine-stimulated RANTES (the chemokine regulated upon activation, normal T-cell expressed and secreted) expression was synergistically increased in the presence of MPA [10]. It has been shown that PTC and DTC express RANTES after stimulation with a cytokine mix (IL-1β, TNF-α and γ-IFN in combination) only [15,21]. The stimulus of IL-1β alone did not increase RANTES expression, but induced the synthesis of IL-6. The results clearly indicate different regulatory mechanisms of the cytokine IL-6 and the chemokine RANTES, which may be due to different responses to the production of transcriptional factors. Recent work on cytokine-mediated intracellular signalling pathways has provided a general paradigm for the molecular mechanisms by which extracellular signals induce transcription of target genes [22]. Although different cytokines independently exert a number of biological activities in a cell type-specific fashion, in many circumstances they have been shown to function cooperatively or antagonistically in controlling gene expression [22].

Like most cytokines, IL-6 gene expression is regulated by both transcriptional and post-transcriptional factors. At the transcriptional level, the regulation is primarily controlled by DNA-binding proteins referred to as transcriptional factors [NF-κB and NF-IL-6 (C-ERBβ)]. Using IL-6 promoter deletion analysis, it was shown that NF-κB and NF-IL-6 binding sites serve as obligatory elements for IL-6 expression in epithelial cells [23]. Due to the fact that the IL-6 gene contains both the NF-IL-6 and the NF-κB binding sites, one may speculate that cooperative interactions play an important role in the gene expression. Many studies have reported functional synergy between cytokines in promoting inflammation and gene expression, some of which could involve an interplay between different transcription factors [22]. The combination of these variables generates high potential for the diversity in the control of gene expression during inflammatory diseases, and may explain the different effects of MPA on the stimulated expression of cytokine IL-6 and chemokine RANTES.

In conclusion, we observed constitutive expression of IL-6 in PTC and DTC (Table 1), and enhanced expression was detected after stimulation with IL-1β.
It has been described further that renal epithelial cells in culture produce low levels of IL-6, suggesting that the IL-6 transcriptional machinery is constitutively activated in these cells [24]. Recent work describes that IL-6 stimulates tubular regeneration in glycerol-induced acute renal failure [25]. IL-6 itself may act in cellular proliferation activating transduction signals in cellular regeneration [25]. In this case, the constitutive expression might be due to ischaemia occurring during nephrectomy, the isolation procedure or other stimuli relating to the cell culture (possibly FCS). Nevertheless, we usually culture tubular cells in medium containing a physiologically normal glucose concentration (5 mM) and we do not use antibiotics in long-term cultures to decrease possible cellular stimulation. The fact that MPA given alone (without IL-1β) significantly decreases the basal IL-6 value shows that IL-6 synthesis of cultured tubular cells is increased due to isolation or culture stimuli.

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Conflict of interest statement. None declared.

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