Interleukin-6 expression after renal transplantation

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Abstract

Background. Interleukin-6 (IL-6) is an inflammatory cytokine that plays a role in transplant rejection. We tested the hypothesis that IL-6 levels in serum or urine could be of value in predicting acute and chronic allograft rejection. Furthermore, we examined whether not such levels reflected IL-6 expression in the kidney.

Methods. We measured IL-6 and IL-6 soluble receptor (IL-6sR) in serum and urine of 145 transplant patients and 20 normal controls. In parallel, we studied 108 renal biopsies. IL-6 was measured with a bioassay system using an IL-6 dependent cell line. IL-6sR was measured with enzyme-linked immunosorbent assay. The biopsies were examined for IL-6 and IL-6 receptor (IL-6R) expression with immunohistochemistry.

Results. Rejection episodes occurring within 2 months of transplantation were accompanied by elevated IL-6 concentrations in serum (17 ± 4.8 pg/ml, P < 0.05) and urine (114 ± 27 pg/ml, P < 0.005), compared to controls. These values returned towards baseline (0–5 pg/ml) after successful rejection treatment. The sensitivity of urine measurements was much higher (93%) than serum (54%). The specificity in serum (70%) and urine (60%) was reduced by infection, acute tubular necrosis, and antithymocyte globulin treatment. Serum and urine IL-6sR values did not correlate with rejection. In biopsy tissue, IL-6 and IL-6R were both elevated during rejection. Especially, mono-nuclear cells within the interstitial infiltrate stained positive. However, the amount of IL-6 positive cells did not correlate with peripheral IL-6 concentrations.

Conclusions. Urine but not serum IL-6 values are sensitive indicators of rejection; however, they are confounded by infection, acute tubular necrosis, and certain antirejection treatments. These features limit their usefulness.

Key words: renal transplantation; kidney transplantation; rejection; IL-6; IL-6 soluble receptor

Introduction

The 5-year graft survival after allogenic human renal transplantation ranges between 40 and 65% [1]. Acute and chronic rejection remain the major causes of graft failure [2,3]. Regardless of the immunosuppressive regimen, successful antirejection treatment is mainly dependent on an early and distinct diagnosis.

After transplantation, the presence of non-self antigens induces the activation of antigen-specific T- and B-lymphocytes. A cascade of soluble mediators released by graft infiltrating and local cells further promotes the inflammatory process. Interleukin-6 (IL-6) is a pleiotropic 26-kDa glycoprotein released by T- and B-lymphocytes, macrophages, fibroblasts, and endothelial and epithelial cells [4]. Within the renal parenchyma IL-6 is produced by mesangial [5] and tubular epithelial cells [6]. Despite the fact that glucocorticosteroids [7] and cyclosporin A (CsA) [8] inhibit the production of IL-6, increased IL-6 expression during renal allograft rejection has been shown by in situ hybridization [9–12], immunohistochemistry [13,14], and measurement of peripheral cytokine concentrations in serum and urine [15–21].

To determine whether or not IL-6 and its 50-kDa soluble receptor (IL-6sR) [22] might be of diagnostic value, we measured IL-6 and IL-6sR in serum and urine simultaneously in transplant patients with clinical rejection. Additionally we examined IL-6 and IL-6 receptor (IL-6R) expression in renal tissue. We analysed our data according to the histological diagnosis and also examined the clinical course including graft survival at 1 year.

Subjects and methods

Patients and samples

Altogether, 145 patients after cadaveric kidney transplantation were investigated. 116 patients (≤2 months after transplantation) were followed from the day of transplantation until the day of discharge; 29 outpatients (>2 months after transplantation: 288 ± 52 days after transplantation, maximum: 762 days after transplantation), who entered the
hospital because of graft dysfunction of unknown origin were followed from the day of admission until the day of discharge. Graft dysfunction was defined as an increase in serum creatinine >0.5 mg/dl within 2 days. Samples from 37 patients with stable graft function (stable serum creatinine <2 mg/dl, no adverse event), as well as samples from 20 healthy volunteers, were used as controls.

Standard immunosuppressive therapy consisted of CsA and methylprednisolone (MP). MP was given as a bolus of 250 mg intravenously before transplantation, 100 mg on day 1, 60 mg for the next 3 days, 40 mg within the first week. Thereafter, the drug was reduced in a stepwise fashion to 4 mg/day, 6 months after transplantation. CsA was adjusted according to whole-blood trough levels to between 100 and 150 ng/ml (TDX, Abbott Co., Wiesbaden, Germany). Rejection episodes were routinely treated with intravenous MP boluses (250 mg/day) for 3 days. Steroid-resistant rejections were treated with antithymocyte globulin (ATG, Fresenius, Bad Homburg, Germany: 4–6 mg/kg) for 10 days.

Serum and urine samples from all 145 patients were collected sequentially, at least three times per week between 8:00 and 9:00 in the morning. Samples from outpatients with unknown graft dysfunction were collected before performing renal biopsy. Within 1 h, all samples were centrifuged for 10 min (serum 600 g, urine 1000 g). The supernatants were stored at −20 °C. From all patients 24-h urine were collected in order to calculate the daily amount of IL-6 excretion.

From 104 episodes of rejection, 80 occurred within the first 2 months after transplantation, while 24 occurred more than 2 months after transplantation. Rejection was confirmed on histopathological grounds according to the Banff working classification [23]. In 108 biopsies, we found 86 acute rejections (mild: n = 49; moderate: n = 15; severe: n = 22) and 7 chronic rejections. Acute tubular necrosis (ATN) was diagnosed in 23 cases, of which 14 were in combination with mild acute rejection and three were combined with moderate acute rejection. CsA nephrotoxicity was diagnosed in only one case. Eight biopsies showed borderline or non-specific changes requiring no rejection treatment. In 11 patients with clinically diagnosed rejection (unexplained increase in serum creatinine concentration >0.5 mg/dl within 2 days), biopsy tissue was not sufficient or no biopsy was performed. Bacterial infections (n = 55) were diagnosed according to standard culture criteria including a positive control (Serotec, Oxford, UK, 25 μg/ml). As a negative control, we used a mouse monoclonal antibody (IgG1) directed towards Aspergillus niger glucose-oxidase, an enzyme that is neither present nor inducible in mammalian tissues, (Dako, 50 μg/ml). The healthy part of human kidneys nephrectomized because of hypernephroma was used as an additional negative control. Cytokine expression was quantified by calculating the mean number of positive cells in four randomly chosen vision fields per biopsy.

Two primary mouse monoclonal antibodies against IL-6 (IgG1) were applied (Chemicon International Inc., Temecula, CA, USA, 50 μg/ml; Genzyme Diagnostics, Cambridge, MA, USA, 100 μg/ml). For IL-6R staining, three mouse monoclonal antibodies (IgG1) against gp80 were used (Biosource International, Camarillo, CA, USA, 50 μg/ml).

Statistics

All data are expressed as mean ± standard error of the mean (SEM). Statistical significance for dependent variables was evaluated by the Mann–Whitney U-test and Kruskal–Wallis test. Transplant survival and patient survival were evaluated according to Kaplan–Meier with a log-rank test. A probability of less than 0.05 was considered as statistically significant.

Results

IL-6 expression in serum

The mean IL-6 concentration in serum was 3.5 ± 1.1 pg/ml in healthy volunteers, 3.3 ± 2.3 pg/ml in
transplanted patients with stable graft function, and 
8.6 ± 2.7 pg/ml in patients with minimal graft dysfunction. This designation was based on histopathological investigations, showing no, non-specific, or only very borderline changes (Figure 1). Within the first 2 months after transplantation, the serum concentrations (<3 days before diagnosis) were significantly elevated during rejection (17 ± 4.8 pg/ml; P < 0.05) compared to normal values (Figures 1, 2). After successful rejection treatment, IL-6 concentrations returned to normal (0–5 pg/ml; P < 0.05). In case of therapy resistant rejection (n = 11), elevated IL-6 concentrations persisted (>5 pg/ml; P < 0.05). Subdivision according to the Banff classification revealed a trend (P = n.s.) towards higher IL-6 concentrations during mild (21 ± 7.9 pg/ml) and moderate acute rejections (17 ± 11 pg/ml) compared to values measured during severe acute rejections (9.7 ± 4.4 pg/ml) (Figure 1). Rejection episodes were detected with a sensitivity of 54% and a specificity of 70% (threshold IL-6 concentration: 5 pg/ml). More than 2 months after transplantation, IL-6 serum concentrations during rejection (<3 days before diagnosis) were not significantly elevated (9.6 ± 2.6 pg/ml; P = n.s.) (Figure 1). Infections (19 ± 4.4 pg/ml; P < 0.05) also induced significantly elevated IL-6 serum concentrations (Figure 1). Histologically defined chronic rejection (7.8 ± 2.7 pg/ml; P = n.s.), ATN (9.4 ± 1.9 pg/ml; P = n.s.), and CsA nephrotoxicity (4.7 pg/ml) had no significant effect (Figure 1).

### IL-6 expression in urine

In urine the mean IL-6 concentration was 2.1 ± 0.7 pg/ml in healthy volunteers and 3.9 ± 0.7 pg/ml in transplanted patients with stable graft function (Figure 3). In patients with borderline graft dysfunction (above criteria), the mean IL-6 concentration was 2.7 ± 1.3 pg/ml (Figure 3). During rejection episodes within the first 2 months after trans-
plantation, urinary IL-6 concentration (<3 days before diagnosis) was significantly elevated (115 ± 27 pg/ml; \( P < 0.005 \)) (Figures 2, 3). After successful rejection treatment, IL-6 concentrations returned to normal (0–5 pg/ml; \( P < 0.001 \)). In case of therapy-resistant rejection (\( n = 11 \)), elevated IL-6 concentrations persisted (>5 pg/ml; \( P < 0.05 \)). Subdivision according to the Banff classification revealed a trend (\( P = \text{n.s.} \)) towards higher IL-6 concentrations during mild rejection (155 ± 49 pg/ml) and moderate acute rejection (129 ± 51 pg/ml) compared to severe acute rejection (25 ± 6.1 pg/ml) (Figure 3). Rejection episodes were detected with a sensitivity of 93% and a specificity of 60% (threshold IL-6 concentration: 5 pg/ml). Using a threshold IL-6 concentration of 10 pg/ml, specificity was 71% and sensitivity 87%. During rejection episodes more than 2 months after transplantation, IL-6 concentrations were only slightly elevated (17 ± 6.6 pg/ml; \( P = \text{n.s.} \)) (Figure 3). During infection (74 ± 15 pg/ml) and ATN (111 ± 49 pg/ml) elevated urinary IL-6 concentrations (\( P < 0.05 \)) were also found (Figure 3). In patients with acute rejection (115 ± 27 pg/ml), the presence of ATN seemed to have an additional effect (226 ± 76 pg/ml; \( P = \text{n.s.} \)) further increasing IL-6 concentrations (Figure 3). Histologically defined chronic rejection (16 ± 12 pg/ml; \( P = \text{n.s.} \)) and CsA nephrotoxicity (2.4 pg/ml) did not cause significant changes (Figure 3).

The finding that rejection episodes during the first 2 months after transplantation reduced urinary volume from 1.8 ± 0.2 l/day to 1.4 ± 0.1 l/day (\( P < 0.05 \)) raised the question whether or not elevated IL-6 concentrations were related to urinary volume. Therefore, we infiltrate (Figure 4a) and in the periglomerular area from 1.8 months after transplantation reduced urinary volume different histopathological diagnoses. IL-6 staining was (Figure 3). unable to explain the negative staining of five negative patients with acute rejection (115 ± 27 pg/ml), the presence of ATN seemed to have an additional effect (226 ± 76 pg/ml; \( P = \text{n.s.} \)) further increasing IL-6 concentrations (Figure 3). Histologically defined chronic rejection (16 ± 12 pg/ml; \( P = \text{n.s.} \)) and CsA nephrotoxicity (2.4 pg/ml) did not cause significant changes (Figure 3).

\[ \text{Mean allograft survival was mainly influenced by the frequency of rejection episodes (no rejection: 1152 ± 88 days; I rejection: 982 ± 62 days; > 1 rejection: 526 ± 137 days; } P < 0.01). \] Therefore, we also investigated whether or not repeated rejections could be predicted by the urinary IL-6 concentration. However, urinary IL-6 concentration had no predictive value on rejection frequency, 1-year graft function and 1-year graft survival (\( P = \text{n.s.} \)).

\[ \text{IL-6sR measurements} \]

In serum, the concentration of IL-6sR was 35 ± 1.8 ng/ml in healthy volunteers and 49 ± 5.9 ng/ml in transplanted patients with stable graft function. No significant increase (<3 days before diagnosis) was found during rejection episodes (<2 months after transplantation: 55 ± 5.8 ng/ml; \( P = \text{n.s.} \); >2 months after transplantation: 47 ± 8.6 ng/ml; \( P = \text{n.s.} \)) and during infection (57 ± 3.9 ng/ml; \( P = \text{n.s.} \)). During ATN there was a trend towards elevated serum IL-6sR concentrations (74 ± 18 ng/ml; \( P = \text{n.s.} \)). In urine, the IL-6sR concentrations of patients with stable graft function were significantly reduced compared to healthy volunteers (285 ± 180 vs 1315 ± 144 pg/ml; \( P < 0.05 \)). A significant increase was found during urinary tract infections (1066 ± 176 pg/ml; \( P < 0.05 \)). During rejection episodes occurring less than 2 months after transplantation (835 ± 156 pg/ml) and during those occurring more than 2 months after transplantation (965 ± 329 pg/ml), there was only a trend (\( P = \text{n.s.} \)) towards higher IL-6sR concentrations. ATN (441 ± 51 pg/ml) did not cause significant changes (\( P = \text{n.s.} \)).

\[ \text{Immunohistochemistry} \]

None of the negative controls showed significant staining. Of 108 transplant biopsies, 103 stained positive for IL-6 and 101 stained positive for IL-6R. We are unable to explain the negative staining of five negative IL-6 and 7 negative IL-6R biopsies; each biopsy had different histopathological diagnoses. IL-6 staining was located in the cytoplasm of cells. Most of the IL-6 positivity was seen on the edges of the interstitial infiltrate (Figure 4a) and in the periglomerular area (Figure 4b). The number of IL-6 positive cells per field ranged from 5 to 300 with a mean value of 52 ± 7.3. IL-6 expression during acute rejection episodes within the first 2 months after transplantation (33 ± 6.4) was not different from rejection events >2 months after transplantation (49 ± 4.2; \( P = \text{n.s.} \)). In several cases, tubular epithelial cells (24/108; Figure 4c) and cells within the glomerular area (12/108; Figure 4b) also stained positive. Many CD45 positive cells were found within these glomeruli. Thus, the intraglomerular IL-6 expression was likely caused by infiltrating mononuclear cells. Interstitial tissue and the vasculature invariably stained negative. Regression analysis of the number of IL-6 positive cells in tissue and the concentration of IL-6 in serum/urine did not show any correlation (\( r = 0.05/0.23 \)). Furthermore, the number of IL-6 positive cells did not correlate with the histological diagnosis according to the Banff classification (\( r = 0.16 \)). The distribution pattern of IL-6R resembled the IL-6 pattern. Most staining was found in mononuclear cells around the interstitial infiltrate (Figure 4d). The number of positive cells ranged from 4 to 200 with a mean value of 59 ± 23. The positive cell number neither correlated with IL-6sR concentrations in serum or urine, nor with the histopathological diagnosis. The vasculature, the tubular cells and the glomeruli were always negative.
Discussion

Our findings support earlier work [15–21] showing that IL-6 concentrations in serum and in urine are elevated during episodes of acute graft rejection. We observed significant changes only within the first 2 months after transplantation. This finding suggests that IL-6 is mainly involved in early acute rejection. Persisting elevations indicated therapy-resistant rejection, while successful rejection treatment reduced IL-6 concentrations to normal. Several investigations [15–17,19] have concluded that sequential IL-6 serum measurements might be a useful parameter for the diagnosis of allograft rejection; however, their numbers of subjects were small. Newstead et al. [21] could not confirm these results. We monitored a large number of patients (n = 145) with sequential IL-6 serum measurements (≥3 per week) and compared these results with histopathological diagnosis according to Banff classification [23]. The clinical course of our patients was statistically analysed. In agreement with Newstead et al. [21] our results clearly indicate that sequential IL-6 serum measurements are of no value in predicting allograft rejection due to their low sensitivity (54%).

In contrast, elevated urinary IL-6 values during the first 2 months after transplantation proved to be a sensitive parameter of allograft rejection (93%). Elevated IL-6 concentrations were found 0–3 days before the diagnosis of rejection was made, i.e. 0–3 days before a significant rise in serum creatinine (>0.5 mg/dl) was detectable. Extending previous reports [18–21], we were able to show that this elevation was not due to changes in renal function. In the peripheral circulation, IL-6 exists in the form of high-molecular-weight complexes with other proteins, including fragments of the C-reactive protein, the soluble IL-6 receptor, and components of the complement C4B and C3C [26–28]. The greater success in predicting rejection using urine as opposed to serum could be due to the possibility that the former does not exist in multimeric forms and therefore might be more reflective of biologically relevant changes.

Surprisingly, subdivision of rejection episodes according to the Banff classification [23] revealed a trend towards a negative correlation between the peripheral IL-6 concentration and rejection severity. Vasculitis is the main feature of severe rejection [23]. As already reported by Raasveld et al. [14], endothelium always stained negative for IL-6, even in cases of severe rejection. The recruitment of different cell types during different types of rejection might be the reason for this phenomenon. No correlation was found...
between urinary IL-6 concentrations and 1-year graft survival.

Sequential urinary IL-6 measurements are unfortunately not very specific (60%). Bacterial and viral infections are known to induce an increased expression of IL-6 in serum and urine [15,16,19,29]. In the present study sepsis, urinary tract infection, and CMV infection proved to be the main causes of elevated IL-6 in urine besides rejection. CMV infection is no longer a diagnostic problem since a PCR assay has been established. Bacterial infections can be ruled out by urinalysis and culture. With these precautions, sequential urinary IL-6 measurements within the first 2 months after transplantation could conceivably be useful in diagnosing renal allograft rejection.

ATG also induced IL-6 elevations in serum and in urine. The effect was difficult to delineate, as ATG was only given in case of an ongoing steroid-resistant rejection. Most probably the ATG activated T-lymphocytes via antibodies within the panel of polyclonal immunoglobulins. The same phenomenon was previously found in patients treated with the murine anti-CD3 monoclonal antibody OKT3 [20]. Such antibodies are allegedly the cause of side-effects such as fever, chills, nausea, vomiting, diarrhea and pulmonary oedema.

As described earlier [19,21], ATN also induced significant elevations in urinary IL-6 concentrations. Our immunohistochemical investigations indicated that this increase was due to IL-6 release from damaged tubular epithelial cells. In a primarily well-functioning graft, ATN occurred very rarely. However, in case of delayed graft function the differentiation between ATN and rejection was not possible. In this situation a renal biopsy remains inevitable.

Peripheral cytokine action can be influenced by soluble receptors [22]. IL-6sR was shown to bind IL-6 in vitro and to augment its biological activity by binding of the IL-6/IL-6sR complex to membrane-bound gp130 [30,31]. The regulation and function of this complex in biological fluids is currently not understood [32]. It has been suggested that pathological states involving elevated levels of IL-6 might be associated with increased production of soluble IL-6R. Thus far, IL-6sR has been found in the urine of healthy adult humans [33] and in the serum of HIV-positive patients [34]. The mean urinary IL-6sR concentrations from stable transplant patients were significantly reduced compared to healthy volunteers. However, the serum IL-6sR concentrations did not differ. This finding might be caused by a reduced glomerular filtration rate and by binding of the IL-6/IL-6sR complex to potential target cells within the kidney. Significantly elevated IL-6sR concentrations were only found in urine during urinary tract infection, suggesting local production of IL-6sR. No correlation was found between rejection and the expression of IL-6sR in serum or urine. Since the expression of IL-6sR during rejection did not change significantly and since the IL-6/IL-6sR complex is biologically active, the sensitivity of urinary IL-6 measurements by bioassay should not be influenced by IL-6sR.

IL-6 staining was always located in the cytoplasm of cells. In agreement with the findings reported by Raasveld et al. [14], single mononuclear cells within the interstitial infiltrate stained positive. However, in contrast to their observations, interstitial IL-6 expression far exceeded tubular IL-6 expression. By isolating infiltrating cells, Merville et al. [35] were able to show that renal grafts removed for irreversible rejection contain predominantly T-lymphocytes (CD3+) and monocytes-macrophages (CD14+), spontaneously producing IL-6. The lack of quantitative correlation between the positive cell count and the peripheral IL-6 concentrations might be due to additional cytokine production in circulating cells [36]. As shown by Raasveld et al. [14], tubular epithelial cells also stained positive. Thus the release of intracellular IL-6 from the damaged tubules might explain the occurrence of elevated IL-6 concentrations during ATN and rejection in urine. In contrast to Hancock et al., who described a persistent and dense intraglomerular IL-6 expression in case of chronic rejection in a rat renal allograft model [12], we only found weak glomerular IL-6 expression, even in case of chronic rejection. This discrepancy might be related to species-specific differences. IL-6R positive cells, the target cells of IL-6 action, were found in mononuclear cells within the interstitial infiltrate. Tubular cells, glomerular cells and vasculature stained negative. We conclude that interstitial mononuclear cells are the main targets of IL-6 during allograft rejection.

In conclusion, IL-6 expression in serum, in urine and in biopsy tissue is elevated during renal allograft rejection. Sequential urinary but not serum IL-6 measurements are a sensitive parameter of rejection within the first 2 months following renal transplantation. However, such determinations are confounded by ATN, infection and ATG therapy, which also induce IL-6 production. A cytokine profile may be helpful to further increase the specificity.

Acknowledgements. We are indebted to Friedrich C. Luft MD for his valuable advice and critical review of the paper.

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Received for publication: 19.8.96
Accepted in revised form: 3.12.96