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Non-invasive monitoring of kidney allograft rejection through IDO metabolism evaluation

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The immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) is activated by interferon-γ (IFN-γ) and via tryptophan depletion, suppresses adaptive T cell-mediated immunity in inflammation, host immune defense, and maternal tolerance. Its role in solid organ transplantation is still unclear. Therefore, we investigated the usefulness of IDO-mediated tryptophan catabolism in the evaluation of kidney allograft rejection. Blood, urine, and tissue samples were collected from 34 renal transplant patients without rejection and from nine patients with biopsy-confirmed episodes of acute rejection ($n = 12$). Concentrations of kynurenine and tryptophan in serum and urine were analyzed by high-pressure liquid chromatography. Kynurenine to tryptophan ratio (kyn/trp) was calculated to estimate IDO activity. Immunostaining for IDO was performed on renal biopsies. Neopterin was assessed using radioimmunoassay. Kyn/trp and neopterin were detectable at low levels in serum of healthy volunteers and were increased in non-rejecting allograft recipients. Serum levels of kyn/trp were higher in recipients with rejection compared to non-rejectors as early as by day 1 post-surgery. Rejection episodes occurring within 13 $\pm$ 5.9 days after transplantation were accompanied by elevated kyn/trp in serum (114 $\pm$ 44.5 μmol/mmol, $P = 0.001$) and urine (126 $\pm$ 65.9 μmol/mmol, $P = 0.02$) compared to levels during stable graft function. Kyn/trp correlated significantly with neopterin suggesting an IFN-γ-induced increase in IDO activity. Immunostaining showed upregulation of IDO in rejection biopsies, localized in tubular-epithelial cells. Non-rejected grafts displayed no IDO expression. Acute rejection is associated with simultaneously increased serum and urinary kyn/trp in patients after kidney transplantation. Thus, IDO activity might offer a novel non-invasive means of immunomonitoring of renal allografts.

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Renal transplantation is currently the treatment of choice for most patients with end-stage kidney disease. However, despite continuous advances in immunosuppressive therapy and prophylaxis of infectious complications, acute rejection still remains a problem following kidney transplantation.[1] To date, needle biopsy of the graft is the most sensitive and specific means of diagnosing acute rejection, although there is a 5–10% risk of biopsy-associated complications, such as hematuria, hematoma, arteriovenous fistulas, and even graft loss.[2] As no tests are available to accurately and consistently predict the risk for allograft rejection, the development of less invasive diagnostic methods that additionally provide insights into the pathophysiology of rejection would be of considerable value. As a consequence, immunosuppressive therapy could be individualized and the adverse effects of inadequate or overimmunosuppression minimized.[3] The ultimate clinical goal, however, remains donor-antigen-specific tolerance induction and thus avoidance of any systemic immunosuppressive treatment.[4]

Tryptophan 2,3-dioxygenase and indoleamine 2,3-dioxygenase (IDO) are rate-limiting enzymes in the degradation of the essential amino acid tryptophan via the kynurenine pathway to form *N*-formyl kynurenine, which in the liver is subsequently converted to niacin. Unlike tryptophan 2,3-dioxygenase, IDO is widely distributed in mammals and is induced in various cell types, particularly by the Th1-type cytokine interferon-γ (IFN-γ).[5] For many years, IDO has been known as an innate defense mechanism limiting growth of viruses, bacteria, intracellular pathogens, or malignant cells by withdrawing tryptophan from the local micro-environment.[6,7] More recently, it has been proposed that activation of IDO is also critically involved in regulating immune responses,[8] establishing immune tolerance in pregnant mice upon their fetuses,[9] or inducing T-cell unresponsiveness.[10] Proliferation of alloreactive T cells is thereby arrested via local tryptophan deprivation and the accumulation of toxic, proapoptotic catabolites such as kynurenine and quinolinic acid.[11] Furthermore, IDO serves as a downstream suppressor mechanism used by T-regulatory cells.[12] Despite growing recognition of the molecular pro-tolerogenic T-cell regulatory mechanisms, the physiologic role of IDO in solid organ

transplantation remains unclear. Available experimental data indicate that IDO is involved in the mechanism of spontaneous donor-specific tolerance of liver grafts.[13] In addition, genetic manipulation by adenovirus-mediated introduction of the IDO gene into pancreatic islet cells is associated with prolonged graft survival.[14] Based on these findings, the concept that cells expressing IDO can inhibit T-cell responses and hence induce tolerance has emerged as a new paradigm in immunology.

The rate of tryptophan degradation expressed by the ratio of product (kynurenine, kyn) and substrate (tryptophan, trp) kyn/trp was seen to be a good estimate of biological IDO-enzyme activity.[15,16] Close correlations exist between markers of immune activation like neopterin and kyn/trp.[16,17] Neopterin, a pteridine derivative secreted by monocyte-derived macrophages upon stimulation with IFN-γ, is a sensitive marker of cellular Th1-type immune responses and has been shown to predict allograft rejection.[18,19]

In this study, serum and urine concentrations of tryptophan, kynurenine, and kyn/trp were analyzed in renal allograft recipients with stable graft function and during rejection episodes to determine the usefulness of peripheral IDO-mediated tryptophan catabolism for non-invasive immunomonitoring. Moreover, IDO expression patterns in renal tissue were also examined by immunohistochemistry.

RESULTS

Patients and clinical follow-up

The clinical characteristics of the patients are summarized in Table 1. According to their postoperative course, patients were divided into two groups: Group I: patients with an immunologically uneventful postoperative course, Group II: patients who experienced at least one acute rejection episode in the first 3 weeks. No statistically significant differences were observed between those groups for age, gender, duration of cold ischemia, number of human lymphocyte antigen mismatches, pretransplant creatinine levels, and cytomegalovirus match. Forty-two of the patients had received a kidney from cadaveric donors; one patient had received a graft from a living related donor. In Group II, we observed 12 acute rejection episodes in nine patients. Acute rejection occurred at a mean of 13 ± 5.9 days after transplantation. Rejection was diagnosed by renal biopsy or on clinical judgement based on the presence of the following criteria: a 0.3 mg/100 ml rise in serum creatinine, oliguria, or an increased resistance index and graft swelling at ultrasound examination. In 10 of 12 cases, the diagnosis of acute rejection was confirmed by histology. Three biopsies were graded type I and seven type II according to the Banff classification.

As shown in Table 1, all patients in Group II received immunosuppressive therapy based on calcineurin inhibitors and prednisone in combination with mycofenolate mofetil. One patient in this group was treated with basiliximab and four patients received induction therapy, either with ATG or Campath-1H. In Group I, 14 patients were treated with basiliximab, seven with azathioprine, three with rapamycin, and nine patients received induction therapy. Six patients experienced infection episodes during the observation period, four had a herpes simplex infection, one patient experienced urinary tract infection, and one suffered from sepsis. However, no complications related to cytomegalovirus infection or disease were observed in this study population.

Serum tryptophan, kynurenine, and neopterin levels following renal transplantation

As early as by day one post-transplantation serum kyn/trp, kynurenine, and neopterin concentrations were significantly elevated in patients who subsequently had an acute rejection episode (Group II) as compared with those of Group I, who had an uncomplicated course after surgery (Figure 1a–d). Furthermore, these differences in kyn/trp, kynurenine, and neopterin concentrations remained significant throughout the entire observation period. Pretransplant levels of

Table 1 | Demographic data and clinical characteristics of patients

	Total	Group I	Group II
Number of patients (M/F)	43 (30/13)	34 (24/10)	9 (6/3)
Age in years (mean ± s.d.)	52.8 ± 13.8	50.8 ± 13.9	47.9 ± 9.7
Cause of end-stage renal disease			
Glomerulonephritis	12	7	5
Diabetes	9	7	2
Shrunken kidney	4	3	1
Polycystic nephropathy	4	2	2
Alport syndrome	2	2	0
Repeated transplantation or graft failure	6	4	2
Other	6	6	0
Drug regimen			
C	16/43	14/34	2/9
T	27/43	20/34	7/9
P	43/43	34/34	9/9
A	7/43	7/34	0/9
MMF	33/43	24/34	9/9
R	3/43	3/34	0/9
B	15/43	14/34	1/9
I	13/43	9/34	4/9
Deceased donor	42	33	9
Mean duration of cold ischemia (h)	14 ± 4.6	14 ± 4.8	14 ± 3.9
⩾1 prior transplantations	6	4	2
>3 HLA mismatches	18	14	4
CMV mismatch	18	16	2
Number of acute rejection episodes	12	NA	12
Time to first episode of rejection (mean days post TX ± s.d.)	13 ± 5.9	NA	13 ± 5.9
Biopsy proven	10	NA	10

A, azathioprine; B, basiliximab; C, cyclosporin A; CMV, cytomegalovirus; F, female; HLA, human leucocyte antigen; I, induction therapy (ATG or campath-1H); M, male; MMF, mycophenolate mofetil; NA, not applicable; P, prednisone; R, rapamycin; T, tacrolimus.
Group I: Patients with an uncomplicated postoperative course; Group II: Patients with at least one acute rejection episode.

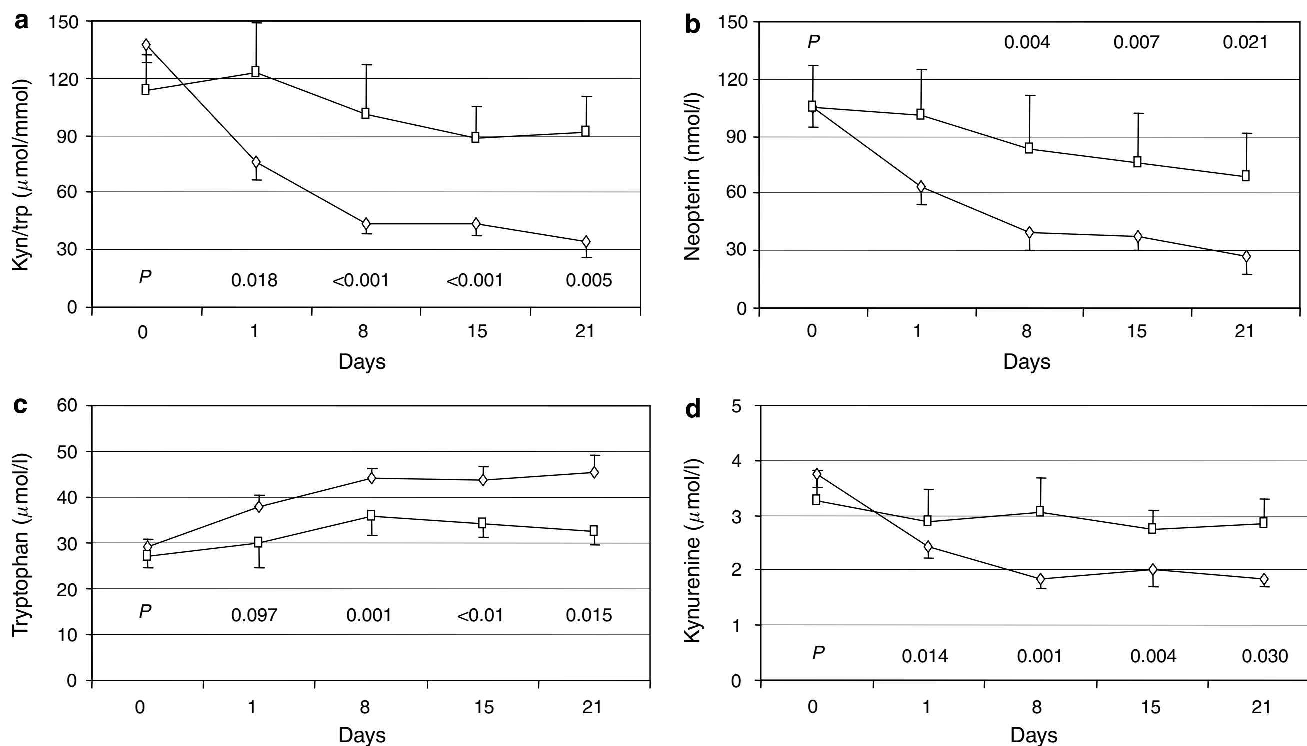

Figure 1 | Changes in serum tryptophan, kynurenine, kyn/trp, and neopterin levels in patients during the early post-transplant course. (**a**) Kyn/trp, (**b**) neopterin, and (**d**) kynurenine levels were higher in the nine patients with acute rejection during the first 21 days after transplantation (Group II, -□-) than in the 34 patients with an uncomplicated postoperative course (Group I, -◇-). (**c**) Tryptophan concentrations were significantly lower in Group II than in Group I after 1-week post transplantation. (*P*-values were determined with use of the Kruskal–Wallis method).

tryptophan, kynurenine, kyn/trp, and neopterin did not differ between groups.

Significant correlations existed between kyn/trp and kynurenine and neopterin concentrations (kyn/trp vs neopterin: rs = 0.675, $P<0.01$; kynurenine vs neopterin: rs = 0.512, $P=0.001$), and tryptophan and neopterin concentrations correlated inversely (rs = −0.541, $P<0.001$).

Serum tryptophan, kynurenine, and neopterin levels during acute rejection

Kyn/trp, neopterin, and kynurenine were detectable at low levels in serum of healthy volunteers (Table 2). The concentrations of tryptophan, kynurenine, and kyn/trp found in the control group thereby agreed well with the concentrations reported earlier.[20] As shown in Table 2, kynurenine, kyn/trp, and neopterin were significantly higher in transplant recipients having an uncomplicated postoperative course than in healthy control individuals. An additional significant increase in all these parameters was measured in serum of patients at the time of an acute rejection episode. Tryptophan concentrations were lower in uncomplicated patients than in healthy controls ($P<0.001$) and lowest concentrations were observed during acute rejection ($P<0.001$; Table 2). These data indicate that IDO enzyme activity is upregulated after renal transplantation and is significantly further elevated during acute rejection episodes.

Six patients in this study had infectious complications (herpes simplex, $n=4$; urinary tract infection, $n=1$; sepsis, $n=1$). However, infection episodes were not associated with significant changes in either kyn/trp, kynurenine, or tryptophan concentrations (data not shown).

Urine kyn/trp and neopterin during acute rejection

In parallel to serum levels, we determined urine concentrations of neopterin and kyn/trp in our patients at the time of acute rejection. As shown in Figure 2, both urine kyn/trp ($126\pm65.9\,\mu$mol/mmol) and neopterin levels (504 ± 303 nmol/l) were significantly higher at the time of acute rejection as compared to samples obtained during stable graft function (kyn/trp $94.6\pm73.9\,\mu$mol/mmol, $P=0.02$; neopterin 279 ± 150 nmol/l, $P<0.01$) from patients who had an uncomplicated course after transplantation.

Serum creatinine, creatinine clearance, and urinary volume

Mean pretransplant serum (±s.d.) creatinine concentrations were not significantly different between patients with an immunologically uneventful postoperative course (7.95 ± 2.32 mg per deciliter) compared to those who subsequently experienced rejection episodes (7.59 ± 2.01 mg per deciliter). However, significant differences in serum creatinine values were found at the time of acute rejection (Figure 3). Urinary output early (week one) and late (week four) post

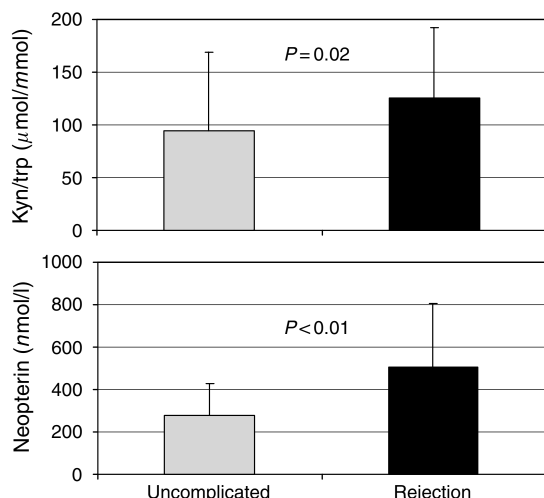

Figure 2 | Urine levels of kyn/trp and neopterin/creatinine at the time of an acute rejection episode (black bars) as compared to levels during an uncomplicated postoperative course (grey bars). Data are expressed as mean ± s.d.

Table 2 | Serum concentrations of tryptophan, kynurenine, kyn/trp, and neopterin

Group	Tryptophan (μmol/l)	Kynurenine (μmol/l)	Kyn/trp (μmol/mmol)	Neopterin (nmol/l)
Uncomplicated	41.6 ± 11.6[a]	2.03 ± 1.04[a]	55.1 ± 39.6[a]	46.1 ± 43.1[a]
Rejection	30. 7 ± 8.29[b]	3.32 ± 1.12[c]	114.1 ± 44.5[c]	90.9 ± 59.1[d]
Controls	57.2 ± 7.54	1.37 ± 0.44	24.9 ± 6.24	4.58 ± 0.99

kyn/trp, kynurenine to tryptophan ratio.
Data are expressed as mean ± s.d. of the individual serum concentrations of tryptophan, kynurenine, kyn/trp, and neopterin in patients with an uncomplicated postoperative course (n=34), pooled patients with acute rejection (n=9), and control individuals (n=30).
[a]$P<0.001$ compared to controls.
[b]P=0.002.
[c]$P<0.001$.
[d]P=0.005 compared to uncomplicated group (Kruskal–Wallis test).

transplantation was similar in both groups (1940 ± 460 ml vs 1870 ± 670 ml and 3280 ± 560 ml vs 3050 ± 880 ml, respectively). Also, the mean creatinine clearance (Cockcroft and Gault) in the subjects with/without acute rejection episodes did not differ significantly (66.4 ± 13.4 ml/min as compared with 52.1 ± 20.5 ml/min, $P = 0.06$).

Immunohistochemistry

In order to localize intragraft expression of IDO, all kidney biopsies ($n = 10$) were stained for IDO by immunohistochemistry. As control, 10 early post-transplant biopsies from patients with poor graft function owing to acute tubular necrosis but showing no histopathologic evidence of acute rejection were selected. Subsequently, all samples were judged by an independent pathologist who quantitated IDO expression as the product of the proportion of positive cells and the staining intensity (see Material and Methods). Representative images are shown in Figure 4. In biopsies from the group of patients without rejection, immunostaining for IDO showed only relatively few distinctly positive areas with a predominantly nuclear staining pattern (Figure 4a). By contrast, biopsies from patients with rejection showed a substantial number of IDO-positive cells in the mononuclear infiltrates. These IDO-positive cells were morphologically classified as antigen-presenting cells such as macrophages and dendritic cells. However, strongest IDO expression was seen in the tubular epithelium with a very high staining intensity (Figure 4b). In these cells, staining was predominantly cytoplasmatic (insert Figure 4b). Interstitial tissue and the vasculature stained negative in all samples.

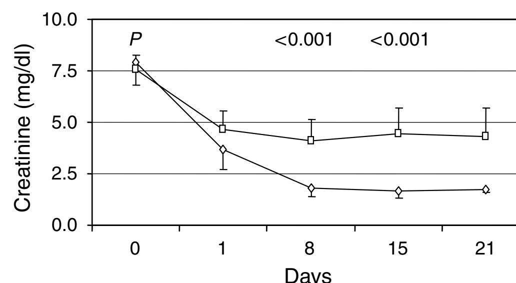

Figure 3 | Time course of changes in serum creatinine concentrations in patients with an uncomplicated postoperative course (Group I, -◇-) and patients with acute rejection (Group II, -□-). Data are expressed as mean ± s.d.

IDO expression and enzyme activity in renal epithelial cells

Next, immunostaining for IDO was performed on renal epithelial cells (A498) following *in vitro* stimulation with IFN-γ to confirm the main cellular source of IDO during acute rejection. All cytokine-stimulated cells showed strong positive cytoplasmic staining for IDO (Figure 4d), whereas cells without IFN-γ-stimulation were negative for IDO (Figure 4c). These data suggest that during acute rejection, tubular cells can be induced to express and activate IDO.

DISCUSSION

This study demonstrates that systemic changes in tryptophan catabolism are associated with local expression and activation of IDO in the kidney graft of patients during acute rejection episodes. In addition, the rate of IDO-mediated tryptophan degradation as expressed by kyn/trp correlates with neopterin levels. Quantitative changes in kyn/trp and neopterin in serum and urine thus permit accurate and non-invasive diagnosis of acute renal allograft rejection.

High serum levels of kyn/trp have been reported in patients with chronic infection (HIV),[21] malignant disease,[22] coronary heart disease,[23] and neuropsychiatric disorders.[24] Furthermore, associations between accelerated tryptophan catabolism and concentrations of immune stimulation markers like neopterin, soluble cytokine receptors, or IFN-γ have been described previously.[25,26] Although a correlation does not necessarily confirm a causal relationship, our data are in line with the assumption that enhanced degradation of tryptophan and thus lowered tryptophan concentrations are associated with immune activation pathways. We could

demonstrate that as early as by day one post-transplantation serum kyn/trp, kynurenine, and neopterin were significantly elevated in patients who subsequently had an acute rejection episode as compared with those who had an uncomplicated course after surgery. In addition, kyn/trp and neopterin were also significantly increased in urine during renal rejection and, as shown by immunostaining, IDO was strongly expressed in the graft during rejection. Accurate diagnosis of acute rejection still relies on the invasive procedure of needle biopsy, which can entail various complications and sampling errors.[27,28] Since during acute rejection, lymphocytes and macrophages are rapidly activated and release large amounts of cytokines and other inflammatory mediators upon activation, an alternative approach to graft biopsy could be to measure such molecules or their soluble receptors in biological fluids.[29,30] However, despite a multitude of studies on virtually all cytokines, there are still almost no convincing data so far on which of the many potential factors to focus on.[31–34]

Data of our study suggest that changes of kyn/trp develop earlier than renal impairment. In the whole dataset, kyn/trp correlated significantly with creatinine and neopterin concentrations. However, kyn/trp already was significantly different between patients with/without rejections from post-transplant day one on, whereas the difference of creatinine levels became significant only around the exact day of rejection. No difference in creatinine values was observed between the two groups of patients from the beginning. The similar chemical nature of amino acids tryptophan and kynurenine suggests that renal impairment may influence the two compounds in a similar way. Thus, one may expect that the direct influence of renal function on kyn/trp would be minor.

In previous studies neopterin measurement in serum and urine has been shown to be of clinical value in predicting immunological complications such as acute rejection in patients following kidney transplantation.[35,36] The current study shows that the reliability and the advantages of such a non-invasive approach could be further increased by simultaneously measuring neopterin and kyn/trp. These initial observations allow us to hypothesize that changes in tryptophan metabolism hold the potential for developing a novel prognostic test for acute rejection of renal allografts. Analyzing IDO activity immediately after transplantation could help define the subgroup of patients more likely to experience acute rejection with additional implications for immediate implementation of graft-saving therapy. However, in this regard, further prospective studies with larger patient numbers, long-term follow-up, and day-to-day analysis of tryptophan metabolism in conditions that may mimic acute rejection such as ATN, calcineurin toxicity, and infection will be necessary to validate our findings.

Holmes *et al.*[37] in a previous report suggested that the serum abnormalities in oxidative tryptophan metabolism observed in renal allograft recipients during acute rejection, infection, and OKT3 therapy reflect a biological response to

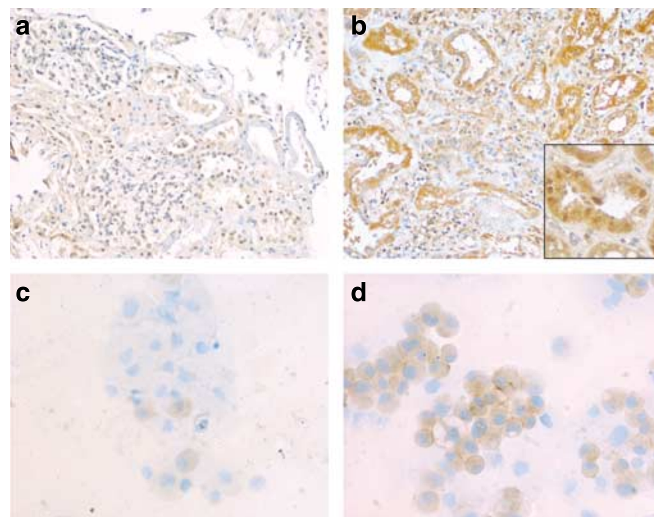

Figure 4 | Immunostaining for IDO. (**a**) Non-rejected grafts show almost no IDO expression. Immunostaining revealed relatively few distinctly positive areas with a predominantly nuclear staining pattern (score 1.67 ± 0.56; see Materials and Methods). (**b**) In biopsies from patients with rejection, IDO-positive cells were found in the mononuclear infiltrates mainly classified as antigen-presenting cells. Strongest IDO expression was seen in tubular epithelial cells with a very high staining intensity (score 8.63 ± 1.94). In these cells, staining was predominantly cytoplasmatic (inset in (**b**)). (**c**) Unstimulated renal epithelial cells did not stain for IDO. (**d**) Following *in vitro* stimulation with IFN-γ, these cells showed strong positive cytoplasmic IDO expression. (Original magnifications $\times 250$ and $\times 400$).

IFN-γ release in that patient population. However, as far as the systemic changes in tryptophan and kynurenine owing to IDO activation are concerned, the cellular source of excessive IDO production in the graft during renal transplant rejection has not yet been precisely defined. Beside monocytes and macrophages, dendritic cells are likely candidates. Indeed, Munn *et al.* showed that IDO is expressed in a certain population of splenic dendritic cells which acquire potent T-cell regulatory functions as a consequence.[38] Upregulation of IDO has also been reported in monocytes and peripheral blood mononuclear cells upon mitogen activation or with IFN-γ.[39] However, despite a limited number of dendritic cells and macrophages, our data show intense immunostaining for IDO mainly in proximal and distal tubuli in patients with acute rejection episodes, whereas tubular epithelial injury in the absence of rejection was not associated with increased IDO expression. The cytoplasmatic and paranuclear pattern of IDO immunostaining further suggests that during acute rejection, tubular epithelial cells can be induced to generate IDO. In addition, our *in vitro* experiments stimulating renal epithelial cells with IFN-γ also revealed a massive induction of IDO in these cells, giving further support to the hypothesis that renal tubuli express functional IDO upon IFN-γ stimulation during acute rejection *in vivo*. Whether increased tubular IDO expression downregulates interstitial T-cell activation via tryptophan deprivation and by proapoptotic activity of tryptophan metabolites, is open to speculation.

Furthermore, the question whether IDO enzyme activation exerts detrimental or beneficial effects during renal allograft rejection remains to be elucidated. Various experimental studies indicate that the IDO system might be able to some extent to protect an allograft from immunological injury. Using a mouse heart transplantation model, we showed that cardiac allografts are able to express and activate IDO. Pharmacological inhibition of IDO activity with 1-methyl tryptophan (1-MT) in recipient animals, however, resulted in massive and accelerated rejection and graft survival in the 1-MT-treated animals was significantly reduced (Brandacher *et al.* unpublished observation). IDO inhibition also led to accelerated rejection of murine major histocompatibility complex class I disparate skin grafts, and 1-MT treatment abrogated tolerance induction of otherwise spontaneously accepted murine liver grafts.[13,40] Recently, Grohmann *et al.*[41] reported that tolerance induction via costimulatory blockade was IDO-dependent, as administration of 1-MT also abrogated the tolerogenic properties of CTLA4-immunoglobulin in an islet cell transplant model. Such interactions are of particular interest, since costimulatory blockers such as CTLA4-Ig and LEA29Y (belatacept) represent a new class of primary immunosuppressants that have shown promising results in human kidney transplantation.[42] By contrast, overexpression of IDO results in immunosuppression and tolerance. Adenoviral-mediated IDO gene transfer into pancreatic islet cells prolonged their survival in allogeneic hosts, and transfection with IDO protected allogeneic lung transplants from rejection.[14,43] These data strongly indicate that IDO activity might have implications for transplantation biology that reach far beyond their utilization as a possible diagnostic tool for acute allograft rejection.

In conclusion, the present study shows that in renal allograft recipients IDO-mediated tryptophan degradation is increased before and during allograft rejection and that measurement of kyn/trp in serum and urine might permit non-invasive monitoring of renal allograft rejection. Activation of IDO takes place at highest rates during acute rejection episodes, but IDO activity, despite otherwise profound tolerogenic functions, is obviously insufficient to prevent or counterbalance this alloresponse. Future studies in animal models are needed to determine the pathophysiologic role of IDO in solid organ transplantation before it can serve as a target for therapeutic interventions during acute renal rejection or potentially for tolerance induction.

MATERIALS AND METHODS

Patients

A total of 43 renal transplant recipients (13 female, 30 male; mean age $\pm$s.d., 52.8 ± 13.8 years) were followed prospectively during the first three postoperative weeks. Immunosuppression consisted of calcineurin inhibitor-based triple drug therapy with corticosteroids and mycofenolate mofetil in the majority of cases. Patients were divided into two groups according to their postoperative course. Group I ($n=34$) consisted of patients with an immunologically uneventful postoperative course. Patients in Group II ($n=9$) experienced at least one acute rejection episode within the first 3 weeks; Thirty healthy non-transplanted volunteers served as controls.

The study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki Principles and according to the institutional guidelines at the Department of General and Transplant Surgery of Innsbruck Medical University. Informed consent was obtained from all patients and volunteers participating in this study.

Serum and urine samples from these patients were collected before, and on days 1, 8, 15, and 21 post-transplantation as well as at the time of acute rejection. Patients' basic demographic data and clinical characteristics are summarized in Table 1.

Blood sampling

Blood samples were collected after an overnight fast and immediately centrifuged at 4500 *g*. Serum and urine were stored at -20°C until further analysis.

Tryptophan and kynurenine measurement

Tryptophan and kynurenine concentrations in serum and urine were determined by reversed-phase high-pressure liquid chromatography as described earlier.[20] Briefly, specimens were deproteinized with trichloroacetic acid and were separated on reversed phase C18 material using 0.015 M potassium phosphate buffer (pH = 6.4). Tryptophan was monitored by means of its native fluorescence at 285 nm excitation and 360 nm emission wavelengths; kynurenine was detected by UV-absorption at 365 nm wavelength in the same chromatographic run. Finally, kyn/trp was calculated as an indirect estimate of IDO activity by dividing kynurenine concentrations (μmol/l) by tryptophan concentrations (mmol/l). Tryptophan and kynurenine concentrations were compared to levels determined earlier in 30 healthy blood donors (15 females and 15 males, aged 20–63 (42.1 ± 10.4) years).

Neopterin measurement

Serum and urine neopterin concentrations were analyzed using an enzyme-linked immunosorbent assay, (BRAHMS Diagnostica GmbH, Berlin, Germany). The sensitivity was 2 nmol/l neopterin and the interassay variation coefficient ranged from 4.7 to 8.5%. Normal serum value provided by the supplier was: mean 5.4 nmol/l ($\pm$2.3) with an upper normal limit of 10 nmol/l.

Immunohistochemistry

Immunostaining was performed on paraffin-embedded sections (4–6 μm) fixed in 10% formalin in phosphate-buffered saline: after deparaffinization and rehydration, the sections were treated with 0.3%. hydrogen peroxide (and incubated with 10% BSA) to block nonspecific staining. Incubation with proteinase for 15 min at 37°C was used for antigen-retrieval on the IDO tissue sections. The primary antibodies were rabbit anti-IDO polyclonal antibodies (AB5968, Chemicon, Hampshire, UK) and were used at a dilution of 1:300. The sections were incubated with the antibody at 4°C overnight. After washing in Tris-buffered saline, they were incubated with biotinylated swine anti-rabbit IgG (Dako, Copenhagen, Denmark) at a dilution of 1:500, and detected with an ABC-peroxidase-Kit (Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine as a substrate.

For quantification, the product of proportion of positive cells in quartiles (0, 1, 2, 3, 4), and the staining intensity (0 no staining;

1 weak; 2 moderate; 3 strong) was calculated, yielding a total immunostaining score ranging from 0 to 12.[44]

Cytospins

A total of 10^6 A-498 kidney epithelial cells (American Type Culture Collection, Rockville, MD, USA) were cultured in 3 ml Dulbecco's minimal essential medium containing 2 mM L-glutamine, 1 mmol/l pyruvate, and 10% heat-inactivated fetal calf serum in a humidified atmosphere containing 5% carbon dioxide at 37°C with or without 250 U/ml human recombinant IFN-γ (a kind gift of Bioferon Ges.m. b.H., Laupheim, Germany) for 24 h, detached by trypsinization, and resuspended in 1 ml of culture medium. Cells were then spun onto glass slides in a cytospin centrifuge and subsequently immunostained for IDO.

Statistical analysis

Group comparisons were performed with analysis of variance and Student's *t*-test; Spearman rank correlation coefficients were calculated because distribution of data was non-Gaussian. $P<0.05$ was considered to indicate significant differences. All statistical analyses and tests were performed with the SPSS statistical package (SPSS 11.0 for windows, Chicago, IL, USA) on a personal computer.

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