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Non-Invasive Monitoring of Renal Transplant Recipients: Urinary Excretion of Soluble Adhesion Molecules and of the Complement-Split Product C4d

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Key Words
Graft rejection · sICAM-1 · sVCAM-1 · sC4d · Transplantation · Urinary excretion

Abstract

Background: The number of inducible adhesion molecules known to be involved in cell-mediated allograft rejection is still increasing. In addition, recent data describe complement activation during acute humoral allograft rejection. The aim of this study was to assess whether specific molecules from either pathway are excreted into urine and whether they can provide useful diagnostic tools for the monitoring of renal transplant recipients. ***Methods:*** Urinary concentrations of soluble adhesion molecules (sICAM-1, sVCAM-1) and of the complement degradation product C4d were determined by standardized ELISA technique in 75 recipients of renal allografts and 29 healthy controls. Patient samples were assigned to four categories according to clinical criteria: *group 1:* acute steroid-sensitive rejection (ASSR, n = 14), *group 2:* acute steroid-resistant rejection (ASRR, n = 12), *group 3:* chronic allograft dysfunction (CAD, n = 20) and *group 4:* stable graft function (SGF, n = 29). ***Results:*** All patients with rejection episodes (groups 1–3) had significantly higher values of urinary sC4d compared with healthy controls and patients with stable graft function ($p < 0.05$). The urinary levels of sVCAM-1 were significantly higher in group 2 (ASRR) compared with all other groups ($p < 0.001$). Uniformly low amounts of s-VCAM-1 and complement-split product C4d were excreted by healthy controls (group 0). In contrast, urinary sICAM-1 concentration in healthy controls was almost as high as in group 2 (ASRR) whereas patients with a stable functioning graft (group 4) excreted significantly less sICAM-1 ($p < 0.05$). ***Conclusion:*** The evaluation of sVCAM-1 and sC4d excretion in urine can provide a valuable tool with regard to the severity and type of allograft rejection. With respect to long-term allograft survival, serial measurements of these markers should have the potential to detect rejection episodes and prompt immediate treatment.

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Introduction

Inducible adhesion molecules are well-recognized players in cell-mediated allograft rejection. There is accumulating evidence that specific binding of T-lymphocyte receptors to antigen as well as adhesive and invasive interactions between circulating leukocytes and vascular endothelial cells during non-specific inflammatory reactions are operative during allograft rejection [1]. With respect to this, intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule-1 (VCAM-1, CD106) have attracted special interest.

ICAM-1, a 76- to 114-kDa type I transmembrane molecule (507 amino acids), is the major ligand for CD11a/CD18 (LFA-1) on leukocytes and CD11b/CD18 (Mac-1) on monocytes and granulocytes. ICAM-1 mediates leukocyte migration and adhesion to target structures by binding to LFA-1 and Mac-1 [2]. Although these receptors are only expressed on leukocytes, ICAM-1 can be expressed weakly on resting endothelium by lymphocytes and some other leukocytes. The expression on other cell types such as mesangial cells and epithelial cells is rapidly increased in vitro upon stimulation by cytokines [3]. Four hours after stimulation ICAM-1 is initially expressed on the cell surface, reaches a peak level expression after 24 h and is sustained for at least 72 h [4]. Its expression is increased on capillary endothelium during acute and chronic allograft rejection, and de novo synthesis in tubular epithelial cells has been reported correlating with the severity of rejection [5–7].

VCAM-1 is a 110-kDa glycoprotein of the immunoglobulin superfamily which is absent on resting endothelium. It is one of the major ligands for the very late antigen-4 (VLA-4) on T lymphocytes [8] and supports adhesion of eosinophils, basophils, monocytes and lymphocytes, but not neutrophils. In contrast to ICAM-1, VCAM-1 is minimally expressed in normal kidneys, but is up-regulated in transplanted allografts. It is highly expressed on capillary endothelium in areas of mononuclear cell infiltration in human cardiac, pancreatic and liver allograft rejection [9–11]. During renal allograft rejection, up-regulated expression of VCAM-1 on both renal tubular and peritubular vascular endothelial cells has been demonstrated [12, 13]. Usually prominent induction of VCAM-1 is observed on the endothelium of peritubular capillaries, venules and arterioles in areas of leukocyte infiltration [14].

In acute humoral allograft rejection, several studies have recently re-introduced the complement system [15]. Complement activation within the graft might potentiate antibody-mediated graft injury. Deposition of the complement-split product C4d along peritubular capillaries has been suggested to be a valuable marker for activation of the antibody-dependent classical pathway in humoral rejection. Furthermore, the incidence of capillary C4d was associated with accelerated graft loss [16, 17]. C4d in PTC walls was reported to be more specific and sensitive than traditional criteria in distinguishing acute humoral from acute cellular rejection [18]. A recent study implicated deposition of C4d in peritubular capillaries with development of chronic humoral graft rejection [19].

The aim of our study was to assess to what amount specific adhesion molecules and the complement-split product C4d derived from the classical pathway are excreted into urine during renal allograft rejection. This would add a non-invasive method to the diagnostic armament of renal allograft dysfunction.

Patients and Methods

Patients

This study included 75 recipients of cadaveric renal allografts (mean age 49.5 ± SD 10.6 years). Patients received a standard immunosuppressive regime consisting of ciclosporine, azathioprine and prednisolone. By the time of urine sampling, kidney transplantation dated back for an average of 65.4 months (± SD 43.4; range 1–204). All patients with signs of bacterial or viral infections were excluded from the study. Thus only those patients with normal values for C-reactive protein (<50.0 mg/l) and negative serologic tests for CMV, EBV and HHV-6 were considered. All patients had been transplanted at the Department of Transplant Surgery, Klinikum Grosshadern, University of Munich, Germany.

In order to prove the clinical relevance of differences in urinary excretion, patients were subdivided into four categories according to the severity of graft rejection classified primarily by clinical criteria. 26 patients undergoing acute rejection episodes were treated with prednisolone (250 mg i.v. daily for 3 consecutive days) and/or antilymphocyte antibodies. Thus, 14 patients with reversible creatinine increases were classified as steroid-sensitive acute rejection (group 1). 12 patients showing no reaction to steroids were classified to group 2 (steroid-resistant acute rejection). Acute rejections (ASSR and ASRR) were observed within the first year after transplantation. 20 patients with ongoing, biopsy confirmed chronic kidney allograft dysfunction (CAD) were classified as group 3. Urine samples of this group were obtained between 12 and 204 months after transplantation. Serum creatinine in this subgroup was ≥2.5 mg/dl (191.3 μmol/l). The remaining 29 patients with stable graft function, defined as change in serum creatinine <0.2 mg/dl (15.3 μmol/l) within the last 2 months, were assigned to group 4.

Healthy Controls

29 subjects without clinical or laboratory signs of kidney disease (group 0) served as normal controls (mean age 27 ± SD 13 years).

Urine Samples

Urine samples from patients with clinical suspicion of acute rejection episodes were collected in a range of 0–5 days before antirejection therapy was started. Samples from all other patients were

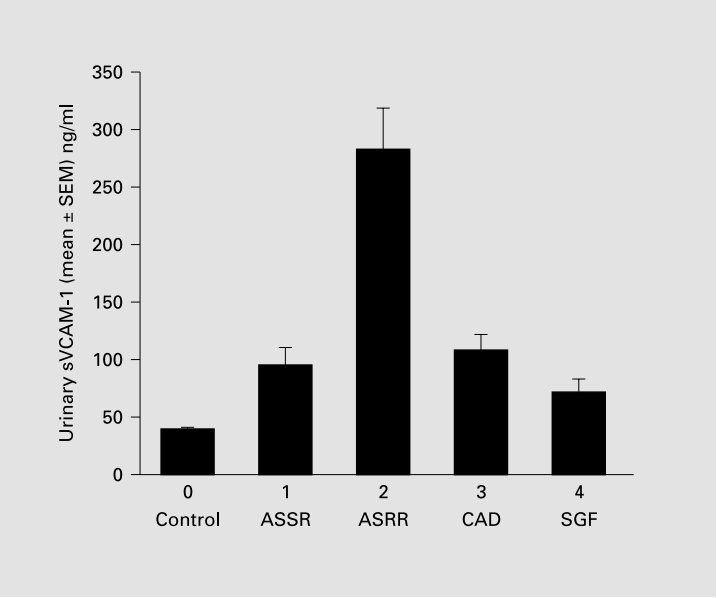

Fig. 1. Urinary excretion of sVCAM-1 of healthy controls and renal transplant recipients with stable graft function (SGF) and recipients with rejection episodes (ASSR, acute steroid-sensitive rejection; ASRR, acute steroid-resistant rejection; CAD chronic allograft dysfunction). p values: 0 vs. 1, 0 vs. 2, 0 vs. 3, 2 vs. 4 (<0.0001); 1 vs. 2, 2 vs. 3 (0.001); 3 vs. 4 (0.05).

tested every 6 months. In the case of worsening graft function and histologic signs of chronic allograft dysfunction, we collected urine on the day of biopsy. 10 ml of urine, taken as a second morning sample, was centrifuged for 10 min at 2,000 min^{-1} at 8 °C. After determination of urinary creatinine and total urine protein in supernatant, the urine was stored at −20 °C until sICAM-1, sVCAM-1 and sC4d concentrations were tested by an ELISA technique.

ELISAs

Commercially available ELISA kits were used to determine urinary concentrations of soluble adhesion molecules sICAM-1 (Serva, Heidelberg, Germany), sVCAM-1 (Hermann Biermann, Bad Nauheim, Germany) and of complement-split product sC4d (Quidel, San Diego, Calif., USA). The C4d fragment enzyme immunoassay measures the amount of C4d-containing activation fragments of C4 (C4b, iC4b and C4d) present in human plasma or serum specimens. The limit of detection of C4d ELISA is 0.007 μg/ml.

All samples were tested in duplicate by a standardized ELISA technique. For sICAM-1 and sC4d ELISA, undiluted urine was used. sVCAM-1 assays were performed with 1:10 diluted urine. Extinctions were measured at 405 nm (against the blank) and documented by a computerized ELISA reader (Multiscan Plus MKII, Flow Laboratories, Meckenheim, Germany).

Statistical Analysis

Data were expressed as mean ± SEM. Urine samples were grouped by clinical diagnosis. In order to test for significant differences in urinary adhesion molecule and complement component C4d levels between the groups, analysis of variance was performed. A p value of <0.05 was considered to be significant. Possible correlations between serum creatinine, total urine protein and the levels of adhesion molecules and C4d in urine were analyzed by linear regression. For data management and computations, the BMDP statistical software (BioMeDical Programs, Cork, Ireland) was used.

Results

sVCAM-1

Patients with acute steroid-resistant rejection (group 2) excreted urinary sVCAM-1 at a significantly higher level (282.94 ± 35.91 ng/ml; $p < 0.001$) than all other groups (fig. 1). The lowest amounts of sVCAM-1 (7 times less) were excreted by healthy controls (group 0, 39.47 ± 1.18 ng/ml) reaching statistical significance compared to groups 1, 2 and 3 ($p < 0.0001$). Measurements of urinary sVCAM-1 concentrations in patients with ASSR (group 1, 95.74 ± 13.97 ng/ml) and those with CAD (group 3, 108.24 ± 12.27 ng/ml) resembled each other. Patients with stable graft function (group 4) showed a lower sVCAM-1 excretion (71.32 ± 10.29 ng/ml).

sC4d

As shown in figure 2, urinary excretion of sC4d was similar to that of sVCAM-1, albeit at a lower quantitative level. Again, patients with acute steroid-resistant rejection (group 2) showed highest levels of urinary sC4d (72.72 ± 18.49 ng/ml) reaching significance compared to group 0 (healthy controls) and group 4 (stable graft function; $p < 0.001$) but not to the other groups. Patients with chronic allograft dysfunction (group 3; 54.20 ± 17.98 ng/ml) and patients with acute steroid-sensitive rejection (group 1; 31.38 ± 7.85 ng/ml) displayed significant differences in urinary C4d excretion compared to group 0 (healthy controls) and group 4 (stable graft function; $p < 0.0001$ and

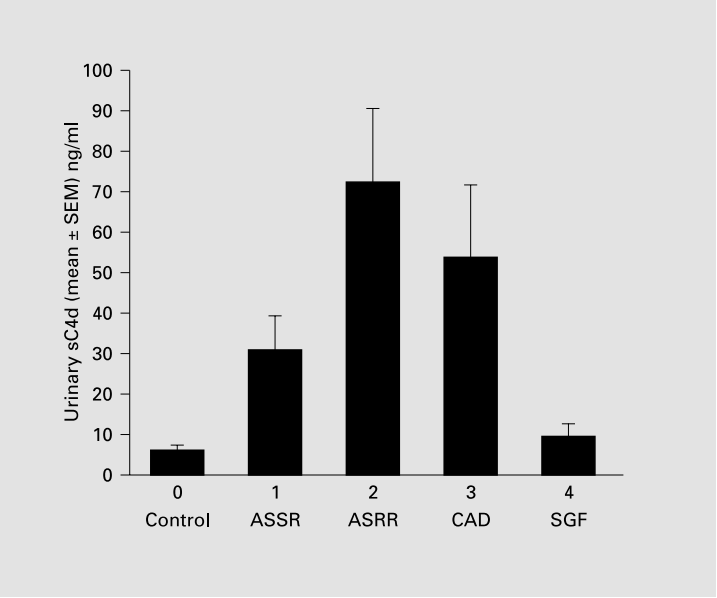

Fig. 2. Urinary excretion of sC4d of healthy controls and renal transplant recipients with stable graft function (SGF) and recipients with rejection episodes (ASSR, acute steroid-sensitive rejection; ASRR, acute steroid-resistant rejection; CAD, chronic allograft dysfunction). p values: 0 vs. 2, 0 vs. 3, 2 vs. 4, 3 vs. 4 (0.0001); 1 vs. 4 (0.01); 0 vs. 1 (0.05).

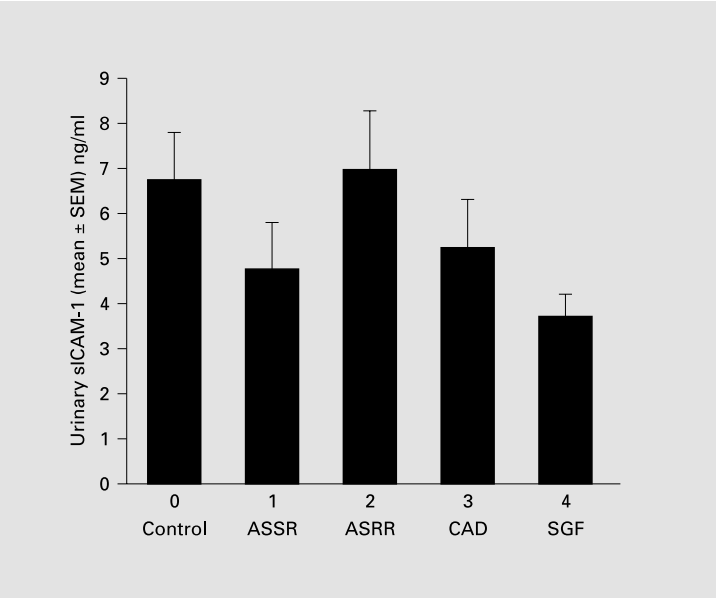

Fig. 3. Urinary excretion of sICAM-1 of healthy controls and renal transplant recipients with stable graft function (SGF) and recipients with rejection episodes (ASSR, acute steroid-sensitive rejection; ASRR, acute steroid-resistant rejection; CAD, chronic allograft dysfunction). p values: 0 vs. 4 (<0.01); 2 vs. 4 (<0.05).

$p < 0.01$ respectively). Patients with stable graft function (group 4) and healthy controls (group 0) excreted low amounts of sC4d: 9.48 ± 2.90 and 5.81 ± 1.16 ng/ml (fig. 2).

sICAM-1

Surprisingly, patients with stable graft function (group 4) excreted lower levels of sICAM-1 as healthy controls (3.74 ± 0.48 vs. 6.72 ± 1.04 ng/ml). Patients with rejection episodes showed the following urinary sICAM-1 excretions: group 1 (acute steroid-sensitive rejection) 4.75 ± 1.01 ng/ml, group 2 (acute steroid-resistant rejection) 6.79 ± 1.28 ng/ml and group 3 (chronic allograft dysfunction) 5.24 ± 1.09 ng/ml. These differences reached no statistical significance. Only group 2 (ASSR) patients revealed significantly higher sICAM-1 values than patients with stable functioning renal transplants (group 4, p = 0.0153) (fig. 3). However, no statistical difference was observed between patients with acute steroid-resistant rejection (group 2) and normal controls (group 0).

In order to determine the kinetics of sC4d and sVCAM-1 concentrations in urine, serial measurements were performed in various patients. Over a period of 3 years, we collected urine samples every 6 months. Uniformly elevated levels of sC4d and sVCAM-1 were observed within the first half year after transplantation in all patient groups. After 12 months, concentrations of sC4d and sVCAM-1 in group 4 (stable graft function) fell to levels comparable to normal controls. In patients with acute rejection episodes, a rapid increase in sVCAM-1 and sC4d excretion within the first year after transplantation could be observed preceding a rise in serum creatinine and the initiation of antirejection therapy for several weeks. In the case of successful treatment the urinary concentrations of both molecules returned to the initial values after a time period of some months. In the CAD group the values did not return to the initial levels.

To exclude that a raised urinary concentration of sVCAM-1 and sC4d was merely the function of an increased loss of this molecule, the excretion of urinary protein and creatinine was compared with that of urinary sVCAM-1 and sC4d in the healthy control group. By the means of regression analysis, no correlation was found between urinary creatinine or protein concentration and excretion of adhesion molecule sVCAM-1 or complement-split product sC4d. Since no correlation was documented for the normal controls, we did not perform further studies in the patient groups.

Discussion

The present study investigates the clinical relevance of soluble adhesion molecules and complement-split product C4d in urine for diagnosis of renal allograft rejections and monitoring after kidney transplantation. Under most circumstances, serum levels of adhesion molecules are unspecific markers for ongoing immune reactions like allograft rejection. Yet in the situation of renal transplantation the determination of such molecules in urine offers a specific tool for the detection of a state of immunological responsiveness. Nevertheless, ongoing infections should be excluded in order to avoid false positive results.

We could demonstrate significant differences in urinary excretion of sVCAM-1 and complement-split product sC4d between patients with stable graft function and those with acute and chronic rejection episodes. In contrast, differences between urinary sICAM-1 excretion between the four transplant groups were less distinctive. Normal controls displayed similar sICAM-1 concentrations in urine than acutely rejecting allograft recipients. The observation that healthy controls excrete more sICAM-1 than patients with stable graft function is surprising and may be an unspecific effect of immunosuppression limiting the usefulness of this parameter. sICAM-1 is shed from ICAM-1-expressing cells, most likely by proteolytic cleavage, and is released in the surrounding fluid [20]. Increased serum sICAM-1 levels were found in patients with acute rejections 3–4 days before diagnosis of acute rejection [21], and high urinary levels in steroid-resistant acute kidney allograft rejection [22]. By urine flow cytometry, Roberti et al. [23] determined active urine sediments whereby HLA-DR turned out to be the most sensitive and ICAM-1 the most specific marker for acute rejection. The presence of $CD14^+$ cells was highly suggestive of chronic rejection. By using urine immunocytology, Chan et al. [24] could distinguish between the heightened ICAM-1 expression on renal tubular epithelial cells in acute rejection and the lower expression in acute tubular necrosis. They found a strong correlation with immunohistology and the clinical diagnosis. Furthermore, they demonstrated that tubular ICAM-1 expression in graft biopsies was proportional to the expression on urinary tubular cells.

In contrast, other studies indicated that both sICAM-1 in urine and in serum are not useful to estimate ICAM-1 expression in a transplant. Lhotta et al. [25] reported urinary sICAM-1 as a marker of proteinuria that did not reflect ICAM-1 expression on proximal tubular epithelial cells [26]. Our similar results in all patient groups indicate that measurement of urinary sICAM-1 is unsuitable to determine the specific pathology and severity of allograft rejection. This could be a result of the use of miscellaneous ELISA kits, differing in specificity and sensitivity. Furthermore, it is not known whether these kits distinguish between active or inactive material. On the other hand, it is unclear whether molecules are altered during excretion. In summary, the measurement of urinary sICAM-1 appears to be imprecise because different isoforms of the molecule may be detected by different ELISA kits, and the results cannot be interpreted adequately [27].

Measuring urinary sVCAM-1 concentration, we observed substantially elevated levels of this molecule in patients with ASRR. Patients with ASSR and those with CAD excreted sVCAM-1 at a lower but still significantly higher level than patients with stable graft function and normal subjects. Patients with ASSR could not be distinguished from those with CAD. Stable graft function was

not associated with a significant increase in sVCAM-1 excretion as compared to normal controls. Thus the highly significant differences in urinary sVCAM-1 concentrations between patients with acute ASRR, CAD and stable graft function were predictive for the type and severity of allograft rejection. A single previous study detecting VCAM-1 in urine reported a correlation between urinary sVCAM-1 and up-regulation of VCAM-1 expression at vascular and non-vascular sites [28]. Although the expression of VCAM-1 in renal kidney biopsies was found to be raised during allograft rejection [29–31], no consistent data exist concerning serum levels of this molecule during allograft rejection. Several studies have reported elevated levels of cVCAM-1 in patients with acute and/or chronic renal allograft rejection [32], but contradictory results failed to confirm these data [21, 33, 34]. In summary, serum concentration measurements of cVCAM-1 add little to the monitoring of kidney allograft rejection episodes.

Deposition of complement-split product C4d in peritubular capillary (PTC) walls was strongly associated with inferior kidney allograft outcome [17, 35]. Being more specific and sensitive than traditional criteria [18, 19, 36], it has been suggested that C4d is a valuable marker for acute and chronic humoral rejection. The de novo production of donor-specific antibodies (DSA), another clinicopathological factor carrying a poor prognosis, was detected in one third of biopsies in which C4d was present [17, 36].

Our results demonstrated strongly elevated levels of sC4d in urine of patients with ASRR and CAD. Nonetheless, because of relatively high standard errors, no clear distinction between these two subgroups could be made. To a lesser, but still significant extent, levels of sC4d in urine were elevated in ASSR as compared to patients with stable graft function and healthy controls.

Vallhonrat et al. [37] identified C4d and the pore-forming, cytolytically inactive, fluid-phase SC5b-9 complex (terminal pathway) in plasma of human heart allograft recipients. No correlation between plasma levels of complement-split products and biopsy-proven acute rejection or accelerated graft atherosclerosis was found. Levels were significantly elevated in the immediate post-transplantation period followed by a sharp decrease during the first 4–6 weeks after transplantation. Determination of C5b-9 in urine and plasma of patients after renal transplantation resulted in no correlation with regard to the severity of graft rejection [38]. Measurements of C5a (the most stable anaphylatoxin) in kidney transplant recipients showed a strong correlation of this urinary complement component and the occurrence of acute rejection episodes. More interestingly, the urinary C5a elevation preceded the clinical diagnosis by 1–2 days [38, 39].

In conclusion, renal ASRR is characterized by massively elevated levels of sVCAM-1 and the complement-split product sC4d in urine. In our view, the high urinary sC4d levels in ASRR episodes and in CAD point to a humoral component in the pathogenesis of this rejection type. This might explain the insufficient efficacy of conventional immunosuppressive treatment and the high proportion of graft losses in this subgroup of kidney allograft rejections [37, 40, 41]. Preliminary data exist concerning the effect of plasmapheresis in combination with tacrolimus-mycophenolate mofetil rescue therapy or the use of intravenous immune globulin application to suppress alloantibody production and modulate immune responses. Serum creatinine levels and circulating DSA could be diminished to almost pretransplant levels [42, 43]. Protein A immunoadsorption may eventually be a more advanced technique than plasmapheresis for therapy of C4d-positive acute humoral rejection. In two small series this possible treatment modality for AHR resulted in the restoration of excellent renal allograft function over a prolonged time period [44, 45]. Tacrolimus-mycophenolate mofetil treatment of patients with chronic humoral rejection has also provided evidence for a sustained decrease of DSA and reduction of covalent bound C4d in PTC over a 12-month time period [46].

There is an absolute need for non-invasive screening tests which monitor allograft dysfunction and are highly sensitive, reliable and safe. Measurements of immunological activity in serum or plasma do not reflect the situation in the transplanted organs themselves [26, 33, 34, 38]. Our data indicate that the determination of sVCAM-1 and sC4d in urine might provide a valuable method to determine the severity and type of renal allograft rejection. Investigation of these molecules with respect to long-term allograft survival represents a simple, inexpensive and non-invasive method for the routine clinical monitoring after kidney transplantation.

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