Original Articles

Protein A immunoadsorption cannot significantly remove the soluble receptor of urokinase from sera of patients with recurrent focal segmental glomerulosclerosis

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ABSTRACT

Background. Focal segmental glomerulosclerosis (FSGS) is a serious disease, the pathogenesis of which is unknown. Its recurrence after transplantation (Tx) and its partial remission after treatment with immunoadsorption (IA) on a protein A column indicate the existence of a circulating factor responsible for the disease that is able to bind to a protein A column. Recently, the soluble receptor of urokinase (suPAR) was described as the factor responsible for FSGS. We tested the capacity of suPAR to bind to protein A and to be eliminated by IA.

Methods. We measured suPAR in eluates of protein A columns from seven patients with recurrent FSGS after Tx (rFSGS) treated with IA, and in the serum of 13 patients with rFSGS and 11 healthy donors (HDs). Additionally, the plasma of these patients was immunoadsorbed in vitro on a protein A Sepharose column, and we quantified suPAR in the eluates and in pre- and post-column samples.

Results. The concentration of suPAR was higher in the plasma of patients with rFSGS than that of HD patients. However, the concentration of suPAR was similar before and after IA on protein A for the rFSGS and HD samples. The suPAR concentration was very low in the eluates from protein A columns incubated with plasma from HD or rFSGS patients. However, 85% of rFSGS patients showed a decrease in immunoglobulin G and proteinuria.

Conclusions. Thus, suPAR does not significantly bind to protein A in vitro or in vivo.

Keywords: focal and segmental glomerulosclerosis, immunoadsorption, protein A Sepharose column, soluble urokinase plasminogen activator receptor

INTRODUCTION

Focal segmental glomerulosclerosis (FSGS) is the most common glomerular cause of chronic kidney disease [1]. FSGS is associated with the effacement of the foot processes and the destruction of the slit diaphragm of podocytes, which correlate with nephrotic syndrome [2]. Genetic mutation of proteins involved in the formation of the slit diaphragm and/or the organization of the actin cytoskeleton is associated with steroid-resistant nephrotic syndrome [3]. FSGS has also been reported to be promoted by viruses (HIV, parvovirus B19 and cytomegalovirus), by drugs (intravenous heroin and interferon) or by glomerular capillary pressure elevation, as observed in obesity or cyanotic congenital heart disease [1]. However, in most cases, the origin of primary FSGS is not known. However, the presence of a soluble factor of glomerular permeability (SFP) involved in the disease has been suggested. This concept is supported by the recurrence of FSGS after transplantation (Tx) [4], by a decrease in nephrotic syndrome after plasma exchange (PE) or immunoadsorption (IA) [5, 6],
by a case of transient nephrotic syndrome in a newborn whose mother had FSGS [7] and by a case where proteinuria was absent in renal Tx with an FSGS donor [8].

Although the SFP and the cells or tissues implicated in the production of the SFP remain unknown, some evidence suggests that it may be produced by immune cells, since vaccination or viral infections can promote the recurrence of FSGS, and FSGS can be controlled by high doses of steroids and/or cyclosporin A [9]. Zhang et al. demonstrated modifications in gene regulation in T lymphocytes from patients with recurrent FSGS (rFSGS), suggesting the involvement of T lymphocytes in the ontogeny of the disease [10]. On the other hand, the control of nephrotic syndrome by IA columns containing protein A Sepharose suggests that immunoglobulins or their fractions can be an SFP, implicating B cells as a source of the SFP [11]. Additionally, several recent observations showing a decrease of nephrotic syndrome after anti-CD20 therapy reinforced the role of B cells [12]. However, and despite decades of efforts to identify the true nature of this enigmatic circulating factor, it still remains unknown [13].

Recently, Wei et al. [14] proposed the soluble receptor of urokinase (suPAR) as a cause of FSGS. suPAR is physiologically present at low concentrations in human blood [15]. Its overexpression in mice can cause podocyte foot process effacement and proteinuria [16]. In humans, elevated levels of suPAR have been found in two-thirds of subjects with primary FSGS, in contrast with other glomerulopathies. Although a non-negligible number of patients with FSGS did not exhibit high levels of suPAR, a threshold value of 3000 pg/mL of suPAR was an important risk factor for primary FSGS [14]. However, based on the quantification of suPAR by enzyme-linked immunosorbent assay (ELISA), Maas et al. [17] observed that suPAR is also elevated in secondary FSGS, suggesting that suPAR may be associated with glomerular lesions, rather than being an initiating factor for FSGS. Moreover, high concentrations of suPAR have been reported in cases of inflammation and infectious [18] or tumour disease [19], suggesting that suPAR is not a specific marker of FSGS.

Both PE and IA on protein A Sepharose have been demonstrated to be effective in reducing proteinuria very rapidly before the occurrence of FSGS lesions and after the recurrence of the disease [6, 9, 11]. Both techniques efficiently remove the SFP, and PE has been demonstrated to reduce the level of suPAR as well as many other proteins [14]. In contrast, the effect of IA on protein A Sepharose is restricted to proteins that can bind to protein A, such as immunoglobulin G (IgG) [11]. The ability of suPAR to bind to protein A columns and to be removed by IA on protein A columns is not known. In this study, to determine whether suPAR is a soluble factor removed by IA, we analysed whether protein A Sepharose, as used for the treatment of rFSGS, can bind to suPAR.

**Materials and Methods**

**Patients**

Patients with rFSGS after renal Tx were retrospectively included in the study. All of them had initial FSGS before dialysis. They were transplanted and were treated with an induction therapy with thymoglobulin (Merieux, France), cyclosporin A (Sandimmun or Neoral, Novartis, Switzerland), steroids and azathioprine or mycophenolate mofetil. All patients underwent a renal biopsy when they suffered a relapse of nephrotic syndrome, to exclude acute rejection. They were separated into two groups: those treated with IA (IA group), and those treated with PE (PE group).

In the IA group, patients received treatment with an IA procedure. IA was performed with a plasma separation device (plasma filter PF2000, Gambro, Mechingen, Germany) or by centrifugation (BT798, Didecco, Mirandola, Italy) to deliver plasma to two pyrogen-free adsorption cartridges (Immunoadsorb, Excorim, Lund, Sweden) at a maximal continuous flow of 35 mL per min. An adsorption device (Citem 10, Excorim) was used to monitor plasma flow, to elute proteins bound to protein A covalently linked to Sepharose with 0.13 M sodium citrate, pH 2.2, and to equilibrate columns. After use, columns were preserved with 0.1% merthiolate and stored at 4°C. The columns were used a maximum of 10 times (on alternate days). One day of procedure allowed the treatment of 2.5 volumes of plasma. Eluted proteins bound to protein A Sepharose were collected to measure the suPAR concentration.

In the PE group, patients were treated only with high-dose immunosuppressive treatment (tacrolimus, corticosteroids and mycophenolate mofetil) and PE, but not with the in vivo IA procedure.

The PE group was compared with healthy volunteers during IA in vitro. All patients provided informed consent.

**Methods**

*In vitro assay of IA on protein A Sepharose with the plasma of healthy subjects and rFSGS patients from the PE group and with recombinant uPAR.* Plasma from healthy donors (HDs) and FSGS patients were incubated (150 µL) with protein A Sepharose (50 µL) (Sigma) (pre-equilibrated in phosphate-buffered saline, PBS) for 2 h at room temperature. The efficiency of protein A Sepharose to bind IgG was previously confirmed by ELISA and dot blot (data not shown). Unbound fractions were recovered by centrifugation (800 g) and, after washing (3× with PBS, the protein A-bound proteins were eluted with 100 mM glycine, pH 2.5, 100 mM NaCl and immediately equilibrated to pH 7.4 (by adding 1 M Tris pH 8.5, 1/10 of final volume). The suPAR concentration was determined in: (i) the initial plasma (input), (ii) the supernatant after pull down of the beads and (iii) in the eluted fluid.

Recombinant urokinase type plasminogen activator receptor (uPAR) and antibody anti-uPAR came from R&D Systems.

**ELISA**

All samples (sera before and after IA, eluates) were aliquoted and then stored at −20°C in order to prevent eventual damages to the proteins from successive freeze–thaw events. The presence of suPAR in the collected fractions was determined with an ELISA kit against human suPAR (Quantikine ELISA, human suPAR, R&D Systems), as reported by Wei et al. [14].
Quantitative values were compared by a non-parametric, Mann–Whitney U-test. A P-value of <0.05 was considered significant.

**RESULTS**

**Patients**

A total of 20 patients with rFSGS were studied. Seven patients were treated with IA (IA group) and 13 with PE (PE group) in addition to a high dose of steroids and cyclosporin A.

In the IA group, the sex ratio of patients was 2 males/5 females and their mean age was 26 (±9) years. The time until the recurrence of FSGS after Tx was 29.7 (±37.7) days and the number of rounds of IA was 7.1 (±3). Their serum median creatinine was 122 ± 9 µmol/L at the time of recurrence. Their median proteinuria was 11 (5–25) g/day.

In the PE group, the sex ratio of the patients was 9 males/4 females and their mean age was 40 (±5) years. The time until recurrence after Tx was 35 (±32) days and the number of rounds of PE was 12 (±4). The serum creatinine was 150 ± 20 µmol/L at the time of recurrence and the median proteinuria was 7 (3–12) g/day.

In the HDs group, the sex ratio of the patients was 5 males/6 females and their mean age was 42.6 (±8) years.

**Analysis of suPAR concentration from eluted proteins bound to protein A Sepharose after in vivo IA in the IA group**

After one IA procedure, the median IgG value decreased from 2.5 (range 1.4–8) to 0.4 (0.35–1.2) g/L (Figure 1A). After three IA procedures, proteinuria decreased from 11 (5–25) to 2 (0–20) g/day. All patients except one showed a significant decrease in their proteinuria (Figure 1B).

We collected fluids eluted from protein A columns by acidic elution after the IA procedure. After elution, the soluble fractions were neutralized and had a pH value between 7 and 7.4. The concentration of suPAR in the fluid eluted from the protein A columns of treated patients was 34.1 (±15.5) pg/mL (Figure 2A).

**Analysis of suPAR concentration after in vitro IA in the PE group and healthy subjects**

We tested the reduction in the concentration of suPAR in the plasma that was incubated in vitro with protein A beads (Figure 2B). We used plasma from patients with rFSGS and plasma from HDs. As previously reported, the concentration of suPAR was higher in the plasma of patients with FSGS (3206.6 ± 1530.3 pg/mL) than that of HD (1715.7 ± 621 pg/mL) (P < 0.007) (Figure 2B). The concentration of suPAR collected after incubation with protein A beads was quite similar to its concentration in the plasma before passing through the protein A beads, for both: patients with rFSGS (3121 ± 621 pg/mL) (P = NS) and HD (1627 ± 577) (P = NS). Hence, the level of suPAR in the protein fraction bound to protein A Sepharose was very low (Figure 2A). The concentrations of suPAR from FSGS patients and from control patients eluted from the column were 81.4 ± 52.3 and 74.3 ± 60.0 pg/mL, respectively.

These results suggested that suPAR cannot significantly bind to protein A Sepharose columns, while excluding the possibility of an acidic denaturation of the molecule by the elution buffer (data not shown). To confirm this, we used recombinant uPAR. We incubated uPAR alone with protein A beads or in the presence of anti-uPAR antibody. In addition, to immunocapture uPAR from the sera of HDs or PE group, we incubated samples with protein A beads and anti-uPAR antibody. uPAR cannot bind protein A beads without an anti-uPAR antibody. The presence of anti-uPAR antibody is necessary to elute uPAR from protein A beads (Figure 3).

**DISCUSSION**

The risk of recurrence of primary FSGS may occur very early after the recovery of renal filtration following renal Tx [4]. The
common maintenance treatment is to use calcineurin inhibitors and, for some groups, to use intravenous cyclosporin A in combination with high doses of steroids [9]. However, the most rapid and significant intervention in case of non-response to the above treatments is to control proteinuria with PE or with IA on protein A Sepharose columns, suggesting the presence of a circulating factor acting as SFP that can interact with protein A Sepharose [6, 11]. In this study, most of our patients had severe nephrotic syndrome due to the recurrence of FSGS and were not controlled by steroids and cyclosporin A. Most of the patients treated with IA showed a strong decrease in the plasma concentration of immunoglobulins after one IA procedure, indicating the strong ability of these columns to bind immunoglobulins, as well as a rapid decrease in proteinuria after three consecutive IA procedures, suggesting that the factor involved in this disease is able to bind to protein A Sepharose. The SFP has been reported to have a hydrophobic domain, as evidenced by its affinity in a hydrophobic interaction column [11]. Additionally, it has been previously demonstrated that injecting rats with the eluted material from a protein A column increases urinary albumin excretion 2.9- to 4.6-fold [11], indicating that the SFP binds to protein A columns and has a molecular weight of <100 kDa [11]. Other studies, using affinity chromatography and fractionation by membrane sieving, indicate that the SFP activity is present in a fraction with an apparent molecular weight of ≤30 kDa [20].

Recently, suPAR (15–55 kDa), the soluble form of the uPAR, was reported to be associated with rFSGS [14]. It is a glycosylphosphatidylinositol-anchored molecule with three domains that can bind several ligands, including uPA, vitronectin or integrins [21]. A uPAR is expressed by active haematological cells, including monocytes, neutrophils, activated T lymphocytes and macrophages, and on endothelial cells, keratinocytes, fibroblasts, smooth muscle cells, megakaryocytes, podocytes and certain tumour cells [15]. suPAR is produced by cleavage and release of the membrane-bound uPAR and can be detected in plasma, urine, blood, serum, cerebrospinal fluid and saliva [21]. suPAR is increased in several pathological
conditions, such as HIV-1 [18], bacterial infections, malaria and various types of cancer [19] not necessarily associated with nephrotic syndrome. uPAR and suPAR exert various functions in cell migration and are linked to adhesion and chemotaxis [15]. suPAR can bind to integrins and thus, is responsible for cellular activation [18]. The reduced motility of podocytes during injury in uPAR knock-out mice can result in stable glomerular filtration, thus preventing proteinuria [16]. The pathological elevation of suPAR concentration mediates an increase in the activation of β3 integrin, shown to be a mechanism for podocyte foot process alteration during the onset of native and post-transplant FSGS [16]. suPAR concentration was found to be significantly elevated in primary FSGS, but not in other glomerulopathies, such as minimal change disease, membranous nephropathy and pre- eclampsia [14]. High pre-Tx and post-Tx serum suPAR levels were described in ~70% of FSGS that would later relapse, and associated with a heightened risk of FSGS recurrence, suggesting that suPAR contributes to both native and rFSGS [14]. Overexpression of wild-type suPAR but not of a β3 integrin-binding-deficient point mutant of suPAR in mice causes a glomerulopathy characteristic of FSGS [16]. However, 30% of primary or rFSGS cases are not associated with elevated suPAR levels [14]. On the other hand, non-specific and increased glomerular uPAR protein expression are found in proteinuric diseases, such as FSGS and diabetic nephropathy [16]. Maas et al. [17] observed that suPAR is also elevated in secondary FSGS. In addition, suPAR levels correlated with glomerular filtration rate, male sex and treatment with mycophenolate mofetil. However, patients with a mutation in the podocin (nephrosis 2, idiopathic, steroid-resistant, NPHS2) gene had higher suPAR levels than those without a mutation, suggesting that suPAR could be an independent biomarker of FSGS disease progression, but not a circulating factor of glomerular permeability [22].

In our study, we demonstrate that suPAR is detected in the plasma of patients with rFSGS, but is not significantly retained by protein A Sepharose columns. These columns are currently employed for IA in order to treat and control the proteinuria in these patients.

We analysed several samples from patients with FSGS and from HDs before and after IA on protein A columns. Our results show that the concentration of suPAR before and after IA was similar and, accordingly, suPAR was not significantly associated with protein A beads. Likewise, we have verified that an acidic treatment of the samples did not affect the sensitivity of the suPAR ELISA test (data not shown).

In our in vitro assays, only a very small amount of suPAR was recovered from beads, so it could be considered as a residual contamination. Other hypothesis would be that a minor portion of suPAR could exert some biological effects. It is also possible that a small fragment of suPAR, not detectable by the commercial antibodies, was responsible of the disease. However, the recombinant uPAR (detectable by Ab), which included all structural domains, did not bind protein A beads.

In conclusion, our data demonstrated that suPAR is not able to significantly bind to protein A Sepharose columns, whereas IA efficiently reduces proteinuria. However, we cannot exclude the presence of an aberrant/minor form, undetectable, but indeed responsible of the disease. The question is not totally closed. In addition, we cannot exclude that suPAR may act locally to amplify the effect of an alternative serum factor of glomerular permeability.

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CONFLICT OF INTEREST STATEMENT

None declared.

REFERENCES

Ticlopidine and clopidogrel, sometimes combined with aspirin, only minimally increase the surgical risk in renal transplantation: a case–control study

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ABSTRACT

Background. Patients undergoing kidney transplantation are sometimes being treated with antiplatelet agents such as ticlopidine or clopidogrel. Some teams refuse to wait-list these patients for fear of bleeding during transplant surgery.

Methods. We retrospectively reviewed the records of 702 adult patients with a kidney transplant alone between 2000 and 2010. Nineteen (2.7%) patients were taking clopidogrel or ticlopidine when called in for transplantation. Furthermore, 10 of these 19 patients were also taking low-dose aspirin (ASA). We compared the risk of bleeding peri- and postoperatively, and the occurrence of cardiovascular complications within 30 days after renal transplantation between 19 cases and 39 controls randomly selected within the cohort.

Results. Platelets were administered to 7 cases (37%) versus 0 controls (P < 0.001). A single case (5.3%) presented with significant bleeding during surgery following an implantation biopsy, and required 4 red blood cell (RBC) units. During the first day, 3 of the 19 cases (16%) and 1 of the 39 controls required RBC (P = 0.1). No reoperation was performed for bleeding. After the transplant, clopidogrel or ticlopidine was resumed in only two patients. The platelet count and haemoglobin were similar between cases and controls at Day 30. No cardiovascular event occurred in cases or controls during the first month post-transplantation. At 5 years, graft and patient survival was similar in cases and controls.

Conclusions. Clopidogrel and ticlopidine, sometimes in combination with ASA, are associated with a low risk of bleeding during renal transplantation and does not seem to be a contraindication for renal transplant surgery.