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PLASMA FIBRINOLYTIC CAPACITY IN RENAL TRANSPLANT RECIPIENTS: EFFECT OF STEROID-FREE IMMUNOSUPPRESSION THERAPY

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Background. **Cardiovascular disease is the most common cause of death among renal transplant recipients (RTRs). Impaired fibrinolytic capacity caused by an increase in plasminogen activator inhibitor type 1 (PAI-1) levels is involved in the onset of atherosclerosis and thrombotic complications. Long-term steroid treatment may induce arterial hypertension and metabolic and prothrombotic changes (including up-regulation of PAI-1 synthesis), which increase the cardiovascular risk. We evaluated plasma fibrinolytic behavior in two groups of RTRs treated with different immunosuppressive regimens.**

Methods. **Twenty-seven RTRs were randomized to receive long-term (17 patients) or perioperative short-term (10 patients) steroids in addition to immunosuppression with cyclosporine A plus everolimus (Certican; Novartis, Basel, Switzerland) (7 patients) or FK506 plus mycophenolate mofetil (20 patients). In each patient, fibrinolytic capacity was studied with the 20-min venous occlusion test 1 and 6 months after transplantation. The following were assayed: euglobulin lysis time, tissue-type plasminogen activator antigen, and PAI-1 antigen and activity.**

Results. **One month after transplantation, a severe impairment of fibrinolytic capacity, mainly caused by an increase in PAI-1 antigen and activity levels, was seen in patients with and without steroid treatment. Six months after transplantation, an improvement in fibrinolytic potential as the result of a decrease in PAI-1 levels was observed only in patients without steroid therapy. None of the steroid-treated patients demonstrated PAI-1 values correlating with body mass index, blood pressure, and metabolic parameters, thus confirming the effect of exogenous factors on PAI-1 expression. Moreover, all patients revealed a slight impairment of stimulated endothelial tissue-type plasminogen activator release, regardless of any steroid treatment, which was probably attributable to calcineurin inhibitor-induced endothelial dysfunction.**

Conclusions. **Our study suggests that steroid-free immunosuppression is associated with a better fibrinolytic capacity in RTRs. This finding may contribute toward reducing the risk of cardiovascular events.**

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Cardiovascular diseases are a major cause of mortality and morbidity in renal transplant recipients (RTRs), especially in young adult patients (*1*). Cerebrovascular disease, myocardial infarction, and other cardiac events may account for approximately 40% of deaths, followed by infections and malignancy (*2*). Several cardiovascular risk factors, such as hypertension, lipid abnormalities, diabetes mellitus, and obesity may persist or develop after renal transplantation, partly the result of immunosuppressive drugs such as steroids, calcineurin inhibitors, or newer agents (*3–6*). Moreover, a high prevalence of hemostasis-related risk factors for arterial and venous thrombosis, namely, lupus anticoagulant and increased levels of homocysteine, lipoprotein (a), and plasminogen activator inhibitor type 1 (PAI-1), has been described in RTRs, suggesting the need to investigate RTRs for these parameters to improve survival and tailor therapy (*6–9*).

PAI-1 is the main inhibitor of the fibrinolytic system, and its increase in plasma may contribute to both the onset and the thrombotic complications of atherosclerosis, especially in the coronary district (*10–12*). Fibrin clots in the vessels are dissolved because of plasminogen activation into plasmin by the tissue-type plasminogen activator (t-PA), which can be specifically inhibited by PAI-1. The synthesis of PAI-1 may be up-regulated by cytokines, metabolic factors, and hormones, including glucocorticoids (*13–16*); in addition, a few PAI-1 gene polymorphisms can modulate its expression. Patients with hypertriglyceridemia, diabetes mellitus, obesity, and metabolic syndrome often reveal an impaired fibrinolytic capacity as the result of excess PAI-1, which compounds the risk of coronary disease (*17*, *18*).

Plasmin-mediated metalloproteinase activation is physiologically involved in extravascular matrix digestion and tissue remodeling. An up-regulated PAI-1 synthesis has been found in renal transplant specimens with acute rejection, and a worse prognosis for long-term renal graft function has been attributed to glomerular PAI-1 mRNA expression (*19*). It has also been suggested that PAI-1–mediated fibrinolysis inhibition might play a part in chronic renal allograft rejection (*20*, *21*).

The prothrombotic clotting and fibrinolytic abnormalities seen in RTRs often have been associated with immunosuppressive drugs, especially cyclosporine A (CsA) (*22*, *23*), although studies addressing the relationship between thrombotic risk and CsA therapy have been discordant. In both renal and heart transplant patients, long-term steroid treatment was found to affect fibrinolytic capacity, increasing PAI-1 plasma levels (*8*, *9*, *24*). In contrast, a normal fibrinolytic potential was reported in steroid-free immunosuppression after heart transplantation (*24*), and fibrinolysis im-

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proved, with an associated decrease in PAI-1, in RTRs 6 months after steroid withdrawal (*25*).

This study evaluates plasma fibrinolytic behavior in two groups of RTRs randomized to receive different immunosuppressive treatments, that is, long-term or perioperative short-term steroids in addition to CsA plus everolimus (Certican; Novertis, Basel, Switzerland) or FK506 plus mycophenolate mofetil.

MATERIALS AND METHODS

We studied 27 patients (24 males and 3 females; mean age 45.2±12.9 years) who underwent kidney transplantation for terminal renal failure. Following the protocols of two ongoing clinical trials, we randomized the patients after transplantation to receive long-term or perioperative short-term corticosteroids in addition to immunosuppression with CsA plus everolimus or FK506 plus mycophenolate mofetil. More precisely, among seven patients treated with CsA plus everolimus, five received long-term and two received short-term steroids, whereas among 20 patients taking FK506 plus mycophenolate mofetil, 12 received long-term and eight received short-term steroids.

The oral dose of CsA was adjusted to maintain a whole blood trough level of 150 to 250 ng/mL and 50 to 100 ng/mL 1 and 6 months after transplantation, respectively (specific radioimmunoassay). everolimus was administered at a dose of 1.5 mg per day. FK506 oral dose was adjusted to maintain a whole blood trough level of 10 to 15 ng/mL and 5 to 10 ng/mL 1 and 6 months after transplantation, respectively. The mycophenolate mofetil dose was 1 g two times per day for 2 weeks and thereafter 1 g per day.

Patients given CsA plus everolimus were randomized to receive 1 week of prednisone (125 mg methylprednisolone intravenously on day 1, followed by 20 mg/day prednisone for 3 days, 10 mg on day 5, and 5 mg on day 6) or long-term prednisone, starting with 20 mg/day tapered down to a 5 to 10 mg/day maintenance dose within 45 days. Patients treated with FK506 plus mycophenolate mofetil received 500 mg methylprednisolone at the time of surgical intervention and were thereafter randomized to immunosuppression without or with steroids (125 mg methylprednisolone on day 1 followed by 20 mg/day prednisone tapered down to 5 mg/day maintenance dose within 45 days).

Acute renal rejection within 2 weeks of the transplant, treated with steroid boluses (methylprednisolone 500 mg/day for 3 days), occurred in 8 of 17 patients with steroid treatment and 3 of 10 patients without steroid treatment.

Thirty gender- and age-matched healthy subjects were studied as normal controls. None of the patients or controls had received any drugs known to affect coagulation and fibrinolysis or had smoked for at least 15 days before the study. Informed consent was obtained from each subject before their inclusion in this investigation.

All enrolled patients were studied 1 and 6 months after transplantation. In each patient, body mass index (BMI, kg/m^2) and systolic (SBP) and diastolic blood pressure (DBP) were measured, and serum levels of total cholesterol, triglycerides, glucose, and creatinine were assayed using standard procedures. The fibrinolytic study was performed between 8:00 and 10:00 A.M., after an overnight fast and a 15-min period of supine rest to minimize the effects of diurnal variations on the results. The venous occlusion (VO) test was used to evaluate the fibrinolytic potential, as reported elsewhere (*25*). Eighteen milliliters of whole blood were anticoagulated with 2 mL trisodium citrate (0.13 M) and chilled immediately. To determine euglobulin lysis time (ELT), 7 mL of whole blood were anticoagulated with EDTA (1 mg/mL), and the tube was immediately put on ice. After centrifugation at 3000*g* for 15 min, plasma aliquots were stored at −40°C and tested within 1 month.

The following were tested before and 20 min after the VO test: ELT, t-PA antigen (t-PA:Ag), PAI-1 antigen (PAI-1:Ag), and PAI-1 activity (PAI-1:act). ELT (normal range 5–25 hr) was assayed according to our modification (*8*) of the method proposed by Cliffton and Cannamela (*26*). t-PA:Ag (normal range 2–12 ng/mL) and PAI-1:Ag (normal range 4–30 ng/mL) were assayed by enzyme-linked immunosorbent assay methods (TintElize t-PA and TintElize PAI-1, respectively, Biopool, Umeå, Sweden) (*27*, *28*). PAI-1:act (normal range 0–9 IU/mL) was assayed by a chromogenic method (Chromolyze PAI, Biopool, Umeå, Sweden) (*29*).

The criteria for defining a defective fibrinolytic response to the VO test were established from an analysis of data obtained in a large group of healthy subjects and were the following: after VO test, ELT more than 3 hr, residual PAI-1 activity more than 4 IU/mL, t-PA activity increase less than 3.5 times the resting value, and t-PA antigen increase less than three times the resting value.

Statistical analysis was performed using STATISTICA 4.0 for Windows (StatSoft, Tulsa, OK). The results were calculated as means±standard deviation or standard error of the mean. The Student *t* test was used to compare the means, and a logarithmic transformation of not normally distributed data was performed before statistical calculation. Pearson's correlation analysis was used to detect significant univariate associations between the parameters analyzed. A *P* value less than 0.05 was considered statistically significant.

RESULTS

In comparison with healthy controls, both groups of RTRs showed a significant and pathologic impairment in global fibrinolytic capacity 1 month after transplantation, demonstrated by prolonged ELT values before and after VO (Table 1). Six months after transplantation, an improved fibrinolytic capacity, demonstrated by shorter pre- and post-VO ELT values, was only observed in RTRs without steroid immunosuppression (Table 1).

TABLE 1. Statistical analysis (mean±SEM) of euglobulin lysis time and t-PA antigen levels, both before (0′) and 20 min after venous occlusion (20′) test, in RTRs with or without steroids 1 and 6 months after transplantation as compared with normal controls

Test	Controls (n = 30)	RTRs with steroids first month (n = 17)	RTRs without steroids first month (n = 10)	RTRs with steroids sixth month (n = 14)	RTRs without steroids sixth month (n = 9)
ELT 0′ (h)	13.76±1.34	34.82±3.01[a]	31.20±4.91[a]	29.50±3.67[a]	23.60±4.15[b]
ELT 20′ (h)	1.90±1.15	11.94±2.51[a]	18.90±3.97[a]	19.00±4.42[a]	9.87±4.12[a]
t-PA:Ag 0′ (ng/mL)	7.12±0.65	9.60±1.15	11.58±1.50[b]	8.55±1.62	10.75±2.76[c]
t-PA:Ag 20′ (ng/mL)	29.30±2.69	28.88±3.41	17.93±2.04[c]	17.07±3.00[§,b,d]	19.11±3.38

[a] $P<0.0001$.
[b] $P<0.01$.
[c] $P<0.05$ RTRs patients versus controls.
[d] $P<0.05$ steroid-treated RTRs, first month versus sixth month after transplantation.
SEM, standard error of mean; ELT, ehglobulin lysis time; t-PA, tissue-type plasminogen activator; RTR, renal transplant recipient; Ag, antigen.

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One month after transplantation, basal t-PA:Ag levels in RTRs were similar to controls, whereas the stimulated t-PA:Ag release after VO was lower in steroid-free RTRs than in steroid-treated RTRs or normal subjects (Table 1). Six months after transplantation, the t-PA:Ag release after VO remained the same (and lower than in controls) in steroid-free RTRs, and steroid-treated RTRs reached similar levels (Table 1).

One month after transplantation, both RTR groups demonstrated a significant, pathologic rise in mean basal PAI-1:Ag levels (Fig. 1) compared with controls. Six months after transplantation, only the steroid-free RTRs demonstrated a marked decrease to normal values, although not statistically significant, in PAI-1:Ag levels, whereas the mean PAI-1:Ag levels in steroid-treated RTRs remained significantly high (Fig. 1). As a consequence, both basal and post-VO PAI-1:act levels were significantly higher in all RTRs than in controls 1 month after transplantation, whereas after 6 months a considerable reduction in PAI-1:act values was only recorded in RTRs without steroid treatment (Fig. 2). In particular, steroid-treated RTRs demonstrated PAI-1:act levels significantly higher than healthy controls before and after VO, and higher than steroid-free RTRs before VO (Fig. 2).

The mean values of BMI, systolic and diastolic blood pressure, and metabolic parameters evaluated in RTRs 1 and 6 months after transplantation are given in Table 2. All patients demonstrated a satisfactory renal graft function and increased triglyceride levels, whereas only steroid-treated RTRs were hypercholesterolemic 1 month after transplantation. In RTRs treated with steroids, no correlation was found between PAI-1 antigen or activity and BMI, SBP, DBP, or metabolic parameters. In RTRs without steroids, PAI-1:Ag levels correlated significantly with both cholesterol ($r=0.78$, $P=0.007$) and triglyceride levels ($r=0.91$, $P=0.0002$) 1 month after transplantation, whereas after 6 months, a significant correlation persisted between triglyceride levels and PAI-1:act, both before ($r=0.79$, $P=0.009$) and after VO ($r=0.86$, $P=0.003$).

During the first year follow-up after transplantation, 3 of 17 steroid-treated RTRs (17.6%) developed thrombotic events,

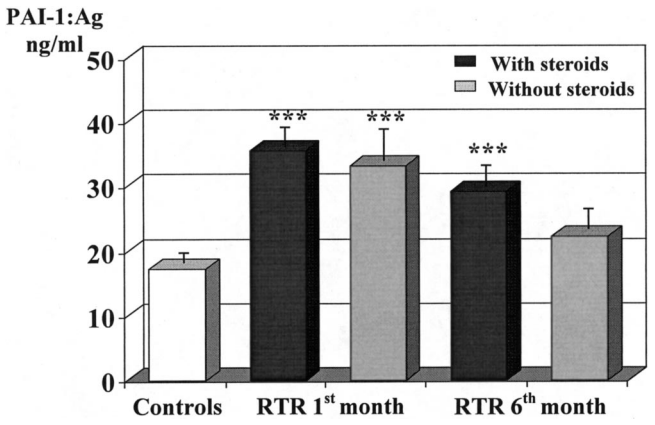

FIGURE 1. Mean (±standard error of mean) plasminogen activator inhibitor (PAI)-1 antigen (PAI-1:Ag) levels in renal transplant recipients (RTRs) with or without steroid immunosuppression 1 and 6 months after transplantation compared with healthy controls. *** $P<0.001$, RTRs versus controls.

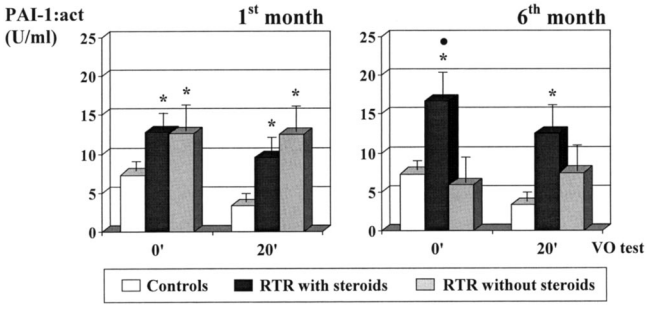

FIGURE 2. Mean (±standard error of mean) PAI-1 activity (PAI-1:act) levels, before and 20' after venous occlusion (VO), in RTRs treated with or without steroid immunosuppression, 1 and 6 months after transplantation, compared with healthy controls. *$P<0.05$, RTRs versus controls. ●$P=0.02$, RTRs with versus without steroids.

namely, one deep vein thrombosis, one central retinal vein occlusion, and one superficial thrombophlebitis; their mean levels of PAI-1:Ag (39.4±8.1 ng/mL) and PAI-1:act (20.1±10.6 U/mL) were increased 6 months after transplantation. One of 10 steroid-free RTRs (10%), showing high PAI-1:act values (56.2 U/mL), suffered from an ischemic stroke.

DISCUSSION

Cardiovascular-related morbidity and mortality in RTRs are associated with a high prevalence and progressive accumulation of risk factors after transplantation (*1–3*). Apart from age, hypertension, diabetes mellitus, hyperlipidemia, obesity, and smoking, other conditions are also considered to affect the risk profile in RTRs. Vascular wall and hemostatic system abnormalities, in particular platelet dysfunction, hyperhomocystinemia, lupus anticoagulant, high lipoprotein (a), clotting factors, and PAI-1 levels, may contribute to atherosclerosis and thrombotic complications (*6–9*). Considerable efforts are now directed toward identifying and controlling known risk factors to ameliorate RTR survival.

Immunosuppressive agents have a pivotal role in increasing the cardiovascular risk. The efficacy of corticosteroids in preventing graft rejection is counterbalanced by well-known morbidities, namely, diabetes mellitus, hypertension, and hyperlipidemia (*4*). Moreover, long-term steroid treatment is associated with prothrombotic changes in clotting and fibrinolytic factors, mainly resulting from a steroid-induced up-regulation of factor VIII, von Willebrand factor, and PAI-1 synthesis, and to a suppression of t-PA production (*8*, *9*). A glucocorticoid-responsive element with enhancer-like properties was found in the promoter region of the PAI-1 gene (*14*).

Impaired endogenous fibrinolysis mainly resulting from excess PAI-1 may contribute to the onset of cardiovascular disease and the progression of atherosclerosis (*10–12*). PAI-1 plasma levels strongly correlate with parameters of metabolic syndrome, a well-known condition carrying a high risk of cardiovascular disease and frequently recognized in RTRs (*17*, *18*, *30*). Moreover, it has been suggested that defective fibrinolysis may have a pathogenic role in graft rejection. In failing transplanted hearts, arteriolar t-PA depletion resulting from PAI-1 inhibition was found to be an early sign of graft failure and associated with a poor prognosis (*31*). In addition, the involvement of deficient fibrinolysis has been

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TABLE 2. Mean values (±SEM) of body mass index, systolic and diastolic blood pressure, serum creatinine, cholesterol, triglycerides, and glucose 1 and 6 months after transplantation in renal transplant recipients treated with and without steroids

Parameter	RTR treatment	First month	Sixth month	Normal range
BMI (kg/m^2)	With steroids	22.81±0.52	23.45±0.69	18–25
	Without steroids	24.79±0.76	24.55±2.75	
SBP (mm Hg)	With steroids	136.76±3.15	132.14±2.75	<140
	Without steroids	132.50±2.26	130.00±3.33	
DBP (mm Hg)	With steroids	86.47±2.40	88.15±2.75	<85
	Without steroids	82.36±3.30	81.11±2.60	
Creatinine (μmol/L)	With steroids	170.23±15.39	153.07±7.95	65–115
	Without steroids	148.10±9.37	134.66±7.14	
Glucose (mmol/L)	With steroids	5.93±0.19	5.77±0.15	3.7–6.1
	Without steroids	7.41±0.57	6.58±0.44	
Cholesterol (mmol/L)	With steroids	6.28±0.32	5.35±0.24	<5.18
	Without steroids	5.06±0.48	4.72±0.37	
Triglycerides (mmol/L)	With steroids	3.45±0.48	2.85±0.43	<2.26
	Without steroids	3.62±0.94	2.70±0.71	

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure.

suggested in the development of transplant-associated coronary artery disease (*32*). An increase in PAI-1 mRNA has been described in transplanted kidneys during both acute and chronic rejection (*19–21*), and a strong predictive value for the occurrence of chronic allograft nephropathy has recently been attributed to glomerular PAI-1 mRNA expression in acute rejection (*21*).

In previous studies, a PAI-1–induced hypofibrinolytic state was observed in patients with Cushing's disease (*33*) and in long-term steroid-treated patients after kidney or heart transplantation, but not in those receiving steroid-free immunosuppression (*24*, *25*). In the present work, a fibrinolytic evaluation was performed both 1 and 6 months after renal transplantation in patients randomized to receive perioperative short-term or long-term steroids in addition to other immunosuppressive agents. Compared with healthy subjects, a pathologic increase in PAI-1 levels was observed in all patients 1 month after transplantation but persisted at 6 months only in the steroid-treated patients. In contrast, a relevant drop in PAI-1 levels was seen in steroid-free RTRs 6 months after transplantation. Therefore, our data further confirm that avoiding long-term steroid therapy is associated with a better fibrinolytic capacity. It is worth adding that the PAI-1 values in RTRs receiving steroids never correlated with BMI, blood pressure, or metabolic parameters known to affect the inhibitor's plasma concentration. This finding supports the role of exogenous factors, namely, steroid use, in determining PAI-1 expression. In steroid-free RTRs, on the other hand, PAI-1 levels correlated with triglycerides and cholesterol levels, suggesting an influence of the latter on PAI-1 behavior. Moreover, although the follow-up period was short, a greater number of thrombotic complications was seen in steroid-treated than in steroid-free RTRs, and in all cases PAI-1 levels were increased.

The prevention of PAI-1–induced hypofibrinolysis may help to reduce cardiovascular risk in RTRs and probably also graft damage resulting from acute or chronic rejection. This aim may be reached by using the new immunosuppressive agents available that enable steroids to be withdrawn and treating metabolic risk factors known to influence PAI-1 levels.

Finally, it should be noted that the fibrinolytic capacity in our RTRs, although better in steroid-free patients, was always impaired compared with controls, a situation attributable to a reduced t-PA release by the endothelium. This suggests an endothelial dysfunction, which might be induced by several factors, including a vascular toxicity mediated by calcineurin inhibitors. In fact, it has been demonstrated in RTRs that both CsA and FK506 can affect endothelial function in a similar manner (*34*), and that fibrinolytic activity can improve after conversion from CsA to azathioprine (*35*). Given the limited number of cases involved, we could not analyze any different effects of single calcineurin inhibitors on fibrinolytic behavior.

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EARLY WITHDRAWAL OF CYCLOSPORINE A IMPROVES 1-YEAR KIDNEY GRAFT STRUCTURE AND FUNCTION IN SIROLIMUS-TREATED PATIENTS

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Background. **Chronic allograft nephropathy (CAN) represents the most common cause of late graft loss. Nephrotoxicity from chronic use of calcineurin inhibitors (CNI) has the potential to contribute to CAN. The present investigation aimed to evaluate the impact of early CNI withdrawal on kidney graft function and structure at 1 year in sirolimus (SRL)-treated patients.**

Methods. **Forty consecutive kidney transplant recipients were initially treated with corticosteroids, cyclosporine A (CsA), and SRL (2 mg/day). After 3 months, patients were randomly assigned to either continue the same treatment (group I) or to withdraw CsA and continue SRL (group II). All patients underwent kidney graft biopsy immediately after graft reperfusion (0-hr biopsy) and 12 months after engraftment.**

Results. **Baseline graft biopsy showed a higher degree of renal damage in group II patients (total score, 4±1.6 vs. 2±0.9; $P<0.05$). Twelve months after engraftment, CAN was diagnosed in 55% of all patients, of**

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