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Determinants of enhanced thromboxane biosynthesis in renal transplantation

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Determinants of enhanced thromboxane biosynthesis in renal transplantation.

Background. Despite great improvement in patient and graft survival, the long-term morbidity and mortality in renal transplant recipients (RTRs) are still significant, with a high incidence of cardiovascular disease-related deaths.

Methods. We investigated thromboxane (TXA_2) biosynthesis and endothelial and coagulative activation in 65 patients who received a renal transplant.

Results. The rate of TXA_2 biosynthesis (urinary 11-dehydro-TXB_2 excretion largely reflects platelet TXA_2 production in vivo) was significantly ($P < 0.0001$) higher in RTRs than in healthy subjects. Plasma von Willebrand factor (vWF) and thrombin-antithrombin (TAT) complexes were significantly higher ($P < 0.001$) in RTRs compared with controls. Urinary 11-dehydro-TXB_2 directly correlated with plasma vWF and cholesterol. We next examined the relative influence of cyclosporine A (CsA) on TXA_2 biosynthesis and endothelial activation, comparing a group of RTRs not receiving CsA with an age- and sex-matched group of patients treated with CsA. Urinary excretion of 11-dehydro-TXB_2 and plasma levels of vWF were significantly increased in RTRs who received CsA compared with those who did not. After an overall follow-up of 120 months, RTRs who experienced cardiovascular events had a higher frequency of abnormal plasma levels of vWF than patients who remained event free.

Conclusion. Renal transplantation is associated with in vivo platelet activation highly related to endothelial activation. This is particularly evident in CsA-treated patients. Administration of drugs that are able to reduce or eliminate thromboxane-dependent platelet activation in vivo may be beneficial to reduce the risk of cardiovascular events in RTRs.

Accelerated arterial disease is a leading cause of mortality in long-term survivors of renal transplantation [1].

Key words: von Willebrand factor, kidney transplantation, graft survival, cardiovascular disease, immunosuppression, nephrotoxicity.

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Evidence supports the notion that the urinary excretion of unmetabolized thromboxane (TXB_2) reflects intrarenal production [2, 3], in contrast to the enzymatic metabolites (11-dehydro-TXB_2 and 2,3-dinor-TXB_2), which reflect mainly systemic extrarenal production [3, 4]. Studies in patients with lupus nephritis, measuring the urinary excretion of TXB_2 and 2,3-dinor-TXB_2 have demonstrated a dissociation of their excretory pattern in the setting of renal disease [5]. Moreover, urinary excretion of enzymatic thromboxane metabolites is increased in patients with in vivo platelet activation [6–8].

Increased urinary excretion of 2,3-dinor-TXB_2 has been reported in cyclosporine A nephrotoxicity (CsA) in rats [9] and in humans [10]. Because in vitro CsA did not interfere with platelet arachidonic acid metabolism in rats [9], it has been suggested that the cause of the increased urinary excretion of 2,3-dinor-TXB_2 is the consequence of intrarenal platelet activation, triggered by endothelial damage.

Plasma measurements of von Willebrand factor (vWF) may give a good indication of the degree of endothelial perturbation [11]. It is synthesized by endothelial cells and secreted via constitutive and inducible pathways triggered by exposure to a wide array of inflammatory mediators [11]. In particular, vWF is an adhesive multimeric glycoprotein, also contained in platelets and megakaryocytes, that regulates platelet adhesion to the subendothelium [11].

The aims of this study were as follows: (*1*) to evaluate the rate of thromboxane biosynthesis in a relatively large group of renal transplant recipients (RTRs) and controls by measuring the urinary excretion of its major enzymatic metabolite 11-dehydro-TXB_2; (*2*) to characterize the determinants of altered thromboxane biosynthesis in this setting; (*3*) to explore the interplay between markers of endothelial damage, thrombin generation, and

Table 1. Characteristics of renal transplant recipients

	All ($N = 65$)	Males ($N = 41$)	Females ($N = 24$)
Age *years*	40.5 ± 10.6	42.5 ± 10.9	37.2 ± 9.4
BMI	24.1 ± 3.8	24.1 ± 3.6	23.9 ± 4.2
Dialysis time *months*	54.6 ± 36.3	55.6 ± 36.7	52.9 ± 36.3
Post-transplantation time *months*	56.9 ± 35.2	56.9 ± 37.7	57.0 ± 31.3
Fasting glucose levels *mg/dL*	88.8 ± 12.7	91.1 ± 13.9	84.8 ± 9.4
Creatinine levels *mg/dL*	1.5 ± 0.7	1.6 ± 0.8	1.4 ± 0.6
Prednisone dose ($N = 64$) *mg/day*	10.3 ± 4.8	9.7 ± 3.2	11.2 ± 6.6
Cyclosporine dose ($N = 55$) *mg/kg/day*	3.6 ± 1.2	3.5 ± 1.2	3.6 ± 1.2
Azathioprine dose ($N = 56$) *mg/kg*	71.2 ± 25.3	69.2 ± 31.6	49.9 ± 33.9
Hypertension *N* (%)	44 (67.7)	29 (70.7)	15 (62.5)
β-blocker users *N* (%)	30 (46.1)	22 (53.6)	8 (33.3)
Diuretic users *N* (%)	10 (15.4)	7 (17.1)	3 (12.5)
Cholesterol *mg/dL*	213.1 ± 42.1	205.3 ± 39.1	226.1 ± 44.7
Triglycerides *mg/dL*	149.4 ± 55.2	155.2 ± 59.7	139.4 ± 46.1
HDL-cholesterol *mg/dL*	52.5 ± 15.8	40.0 ± 14.9	60.1 ± 14.6
LDL cholesterol *mg/dL*	130.7 ± 35.2	126.2 ± 33.9	138.3 ± 39.3
Apolipoprotein A-I *mg/dL*	159.4 ± 33.9	149.5 ± 32.6	176.5 ± 29.4
Apolipoprotein B *mg/dL*	120.5 ± 26.2	120.6 ± 25.2	120.3 ± 28.3

Abbreviations are: BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

platelet activation; and (*4*) to examine the relative influence of cyclosporine treatment on these parameters.

METHODS

Subjects

Sixty-five consecutive patients (24 women and 41 men, aged 41 ± 11 years, range 22 to 60 years) who had undergone cadaveric renal transplantation within the previous 57 ± 35 months (range 2 to 145 months) and 20 age-matched healthy subjects (7 women and 13 men, aged 42.3 ± 11.3 years, range 21 to 57 years) were asked to participate in the study (Table 1). Informed consent was obtained from each participating subject. The internal medicine review board of our institutions approved the study protocol.

Specific exclusion criteria included the following: clinical evidence of acute or ongoing allograft rejection, the presence of serious systemic disease unrelated to the kidney, thrombocytopenia or a history of any disorder associated with bleeding, a history of coronary heart disease, diabetes mellitus, elevated serum liver enzymes, and inability to refrain from the use of nonsteroidal anti-inflammatory agents (NSAIDs) for two weeks prior the study.

Table 2. Characteristics of the patients who never received cyclosporine compared with the patients who received cyclosporine

	Patients treated with cyclosporine ($N = 10$)	Patients who never received cyclosporine ($N = 10$)
Age *years*	42.3 ± 10.4	42.2 ± 10.3
BMI	24.8 ± 6.5	24.4 ± 3.1
Dialysis time *months*	56.6 ± 42.9	64.9 ± 49.3
Post-transplantation time *months*	54.6 ± 30.6	79.9 ± 34.8
Fasting glucose levels *mg/dL*	89.9 ± 13.0	86.9 ± 10.7
Creatinine levels *mg/dL*	1.4 ± 0.2	1.5 ± 0.6
Prednisone dose *mg/day*	12.5 ± 9.8	12.0 ± 3.5
Cyclosporine dose *mg/kg/day*	3.1 ± 1.2	
Azathioprine dose *mg/kg*	62.3 ± 14.4	100.0 ± 23.6
Hypertension *N* (%)	7 (70)	5 (50)
β-blocker users *N* (%)	4 (40)	4 (40)
Diuretic users *N* (%)	0	2 (20)

All patients studied were on hemodialysis (54 ± 37 months; range 7 to 164) because of end-stage renal disease prior to surgery.

Patients were on an American Heart Association step I diet. They were not taking lipid-lowering drugs or any drug known to interfere with the coagulation system or platelet function during the previous month. Most of them were hypertensive ($N = 44$) and were treated with β blockers or diuretics. All patients were taking prednisone (5 to 20 mg/day) or azathioprine (25 to 125 mg/day). All but 10 were taking cyclosporine A (1.6 to 6.1 mg/kg/day). This group of 10 RTRs (Table 2) underwent renal transplantation before CsA was widely employed, and they were available for study only 31 to 145 months after surgery.

Follow-up

The vital status of the study patients was reviewed annually for 10 years to verify the occurrence of major endpoints. For the survival analysis, myocardial infarction, cardiac death, and need of hemodialysis were considered major end points. Nonfatal myocardial infarction (MI) was defined as rapid onset of typical symptoms consistent with acute MI plus either typical electrocardiographic changes (including new Q waves) or a significant serum enzyme increase. Fatal MI was confirmed by a death certificate diagnosis plus preterminal hospitalization, with a definite or suspected diagnosis of MI within four weeks of death. Cardiac death was defined as a death within 24 hours of the onset of severe cardiac symptoms, unrelated to other known causes.

Analyses

Urine was collected from each subject during the 12-hour period preceding blood sampling; the samples were frozen immediately and kept at −20°C until extraction.

Fig. 1. Urinary excretion rates of 11-dehydro-TXB_2 in 65 renal transplant recipients (RTRs) and 20 healthy subjects of equivalent age. The dots represent individual measurements; horizontal bars represent mean value for each group ($P < 0.0001$).

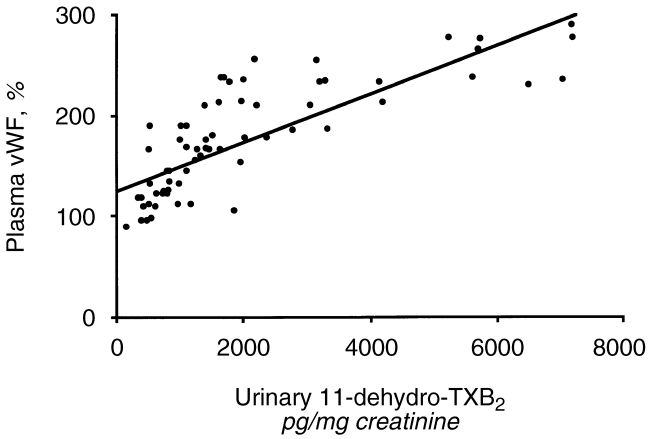

Fig. 2. Correlation between plasma von Willebrand factor (vWF) and urinary excretion of 11-dehydro-TXB_2 in 65 renal transplant recipients (RTRs). Spearman's rank correlation test yielded a statistically significant coefficient of correlation ($\rho = 0.838$, $P = 0.0001$).

Immunoreactive 11-dehydro-TXB_2 was extracted from 20 mL urine aliquots and measured by a previously validated radioimmunoassay technique [7, 8].

Blood samples were obtained by standard venopuncture after a 12-hour fast. Whole blood was immediately anticoagulated with ethylenediaminetetraacetic acid (EDTA; 1 mg/mL) and centrifuged at 3000 $\times$ *g* at 4°C for 10 minutes to obtain plasma. Samples were frozen at −20°C until assayed. Plasma concentrations of thrombin-antithrombin complexes (TAT) were measured with an enzyme immunoassay (Behringwerke AG, Marburg, Germany), as previously described [12]. Plasma vWF antigen was determined with an enzyme immunoassay (Asserachrom vWF-Ag; Boehringer Mannheim, Mannheim, Germany) as previously described [13]. For all assays, interassay and intra-assay coefficients of variation were <8%.

All blood samples for the lipid studies were collected in tubes containing EDTA 1 mg/mL and separated within one hour after sampling. Total cholesterol (TC) and triglycerides were determined by an enzymatic method. High-density lipoprotein-cholesterol (HDL-C) was measured after phosphotungstic acid/$MgCl_2$ precipitation on fresh plasma. Low-density lipoprotein-cholesterol (LDL-C) was calculated by the Friedewald's formula. Apolipoprotein (Apo) B and A1 were measured by nephelometric method. These procedures have been described in detail elsewhere [7, 13].

Statistical analysis

The data were analyzed by nonparametric methods to avoid assumptions about the distribution of the measured variables. An analysis of variance (ANOVA) was performed with the Kruskal–Wallis method. Subsequent pair-wise comparisons were made with the Mann–Whitney *U* test. Moreover, the association of eicosanoid measurements with other biochemical parameters was assessed by the Spearman rank correlation test. Prognostic values of the variables under study were assessed by Kaplan–Meier method and were compared by long-rank test. All values are reported as means ± 1 SD. Statistical significance was considered to be indicated by a value of $P < 0.05$. All calculations were made with the computer program Stat View (Abacus Concepts, Berkeley, CA, USA).

RESULTS

Baseline characteristics of RTRs are shown in Table 1.

The rate of TXA_2 biosynthesis, as reflected by the excretion of its major enzymatic metabolite, 11-dehydro-TXB_2, was significantly ($P < 0.0001$) higher in RTRs (2055 ± 1498 pg/mg creatinine; median, 1408; range, 161 to 7200) than in healthy subjects (329 ± 132 pg/mg creatinine; median, 284; range, 165 to 582). In 54 (83%) of the 65 patients, metabolite excretion was more than two standard deviations above the control mean (Fig. 1).

Plasma vWF was significantly ($P < 0.001$) increased in RTRs compared with those in healthy subjects (178 ± 55 vs. 110 ± 13%). In 45 (69%) of the 65 patients, plasma vWF was more than two standard deviations above the control mean. Plasma TAT complexes were significantly higher in RTRs (4.6 mg/L; range, 1.7 to 28; $P < 0.001$) compared with healthy individuals (2.4 mg/L; 1.7 to 4.12).

Urinary 11-dehydro-TXB_2 directly correlated with plasma vWF ($\rho = 0.84$, $P = 0.0001$; Fig. 2). No correlation was found between thromboxane metabolite excretion and TAT levels. However, a weak direct correlation was found between vWF and TAT levels ($\rho = 0.32$, $P = 0.009$).

Urinary 11-dehydro-TXB_2 directly correlated with plasma TC ($\rho = 0.39$, $P = 0.001$), LDL-C ($\rho = 0.39$, $P = 0.001$), and Apo B levels ($\rho = 0.32$, $P = 0.0091$).

Because cyclosporine A has been associated with in-

creased urinary excretion of TXB_2 and 2,3-dinor-TXB_2, we next examined the relative influence of immunosuppressant therapy on TXA_2 biosynthesis and endothelial activation. Ten RTRs (3 women and 7 men, aged 42 ± 10 years) who never received CsA were matched for sex, age, and months after surgery with 10 RTRs who received CsA (Table 2). The two groups of patients were comparable for lipid parameters and dialysis time. The RTRs who did not receive CsA (group I) were studied 80 ± 35 months after surgery in comparison to 55 ± 31 months of the group II that received CsA ($P = 0.0506$).

Urinary excretion of 11-dehydro-TXB_2 was significantly increased in group II (3067 ± 2003 pg/mg creatinine) compared with both controls (344 ± 142, $P = 0.0004$) and group I (979 ± 477 pg/mg creatinine, $P = 0.0047$). Patients who received CsA also had higher levels of vWF compared with controls and those of patients who did not receive CsA (213 ± 51 vs. 107 ± 10, $P = 0.0064$, and vs. 151 ± 36%, $P = 0.0011$, respectively; Fig. 3).

Plasma levels of TAT were not significantly increased in group II (3.8 mg/L, range 2.3 to 15) compared with those in group I (4.0 mg/L, 2 to 28); meanwhile, control subjects had significantly lower plasma levels (2.3 mg/L, 1.7 to 4.1; $P = 0.013$) than the CsA-treated group.

Follow-up

The overall duration of follow-up was of 120 (109 ± 23; range 14 to 120) months. One patient died from lung cancer, and three patients were lost to follow-up because they refused to undergo the scheduled visits. These four patients were excluded from the survival analysis.

During a median follow-up of 90 (86 ± 29; range 14 to 120) months, 16 patients experienced major endpoints. There were five cardiac deaths, six acute myocardial infarctions (1 fatal and 5 nonfatal), and five patients who needed hemodialysis. These patients had a similar pattern of all variables investigated in the study compared with patients who remained event free. Nevertheless, patients who experienced major endpoints during follow-up had a higher frequency of abnormal vWF plasma levels (94 vs. 67%, $P = 0.03$) at the baseline than patients who remained event free. In particular, all patients who experienced acute myocardial infarction or cardiac death during the follow-up had abnormal vWF plasma levels at the baseline. Among patients who needed hemodialysis, all but one showed baseline vWF values more than two standard deviations above the control mean.

Univariate analysis of survival showed that plasma values of vWF in excess of the mean ± 2 SD of control subjects was significantly related to cumulative endpoints (log rank = 3.8, $P < 0.05$) as well as to cardiovascular events (log rank = 4.2, $P < 0.04$).

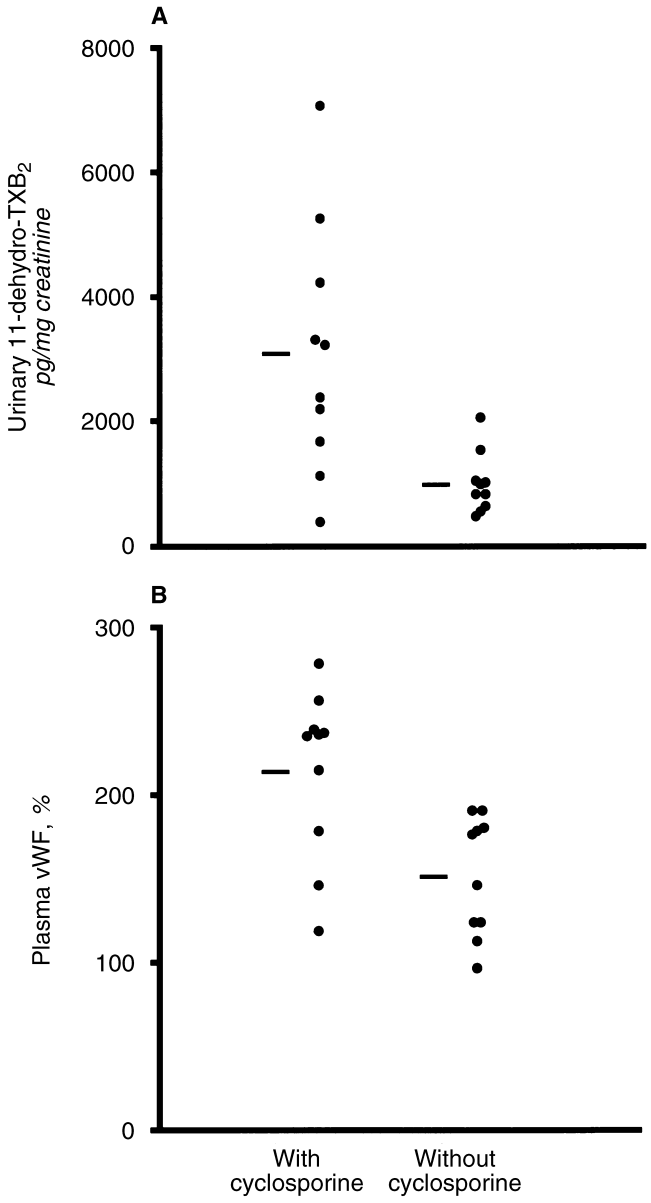

Fig. 3. Urinary excretion rates of 11-dehydro-TXB_2 (*A*; $P = 0.0069$) and plasma vWF (*B*; $P = 0.0076$) in 10 RTRs taking cyclosporine and in 10 RTRs who never received cyclosporine. The dots represent individual measurements; horizontal bars represent mean value for each group.

DISCUSSION

Cardiovascular disease is the major cause of mortality in RTRs [14–17]. The high rate of cardiovascular disease has been due to a cluster of risk factors, including dyslipidemia and hypertension [18, 19].

Because thromboxane-dependent platelet activation has been considered responsible for, or at least a contributor of, acute thromboembolic complications such as myocardial infarction and ischemic stroke [17], we sought to determine whether the systemic formation of

thromboxane A_2 is altered in vivo through measurement of a major metabolite (11-dehydro-TXB_2) in the urine of RTRs. In fact, native unmetabolized TXB_2 in urine reflects thromboxane production by the kidney, while 11-dehydro-TXB_2 or 2,3-dinor-TXB_2 primarily may reflect systemic thromboxane biosynthesis [2–8, 20, 21]. Endothelial cell activity was evaluated in vivo by determination of circulating levels of vWF, synthesized and secreted by the endothelial cells. vWF is an adhesive multimeric glycoprotein, also contained in platelets and megakaryocytes, that regulates platelet adhesion to the subendothelium [11]. The circulating vWF pool is derived mainly from endothelial cells [11], and high plasma vWF levels have been proposed as a marker of endothelial damage [11, 22].

Sixty-nine percent of our patients showed plasma levels of vWF more than two standard deviations above the control mean, strongly indicating that vascular endothelium is altered in RTRs. Elevated levels of vWF have been described earlier in CsA-treated RTRs [23]. In this report, we provide biochemical evidence for the occurrence in RTRs of thromboxane-dependent platelet activation in vivo, and demonstrate that this activation is highly related to peripheral signs of altered endothelial cell function. Moreover, the follow-up data suggest that endothelial perturbation may represent a link between in vivo platelet activation and the occurrence of cardiovascular events.

This process may be exacerbated by the frequent occurrence of hyperlipidemia after transplantation [24]. Hyperlipidemia may be due to alterations in metabolism and nutritional status that occur after successful transplantation, with reversal of chronic renal failure. Moreover, drugs used for immunosuppression and the frequent use of diuretics and β blockers to treat hypertension induced by cyclosporine may also affect lipoprotein metabolism [25]. Because abnormal cholesterol levels are related to persistent thromboxane-dependent platelet activation [26], we correlated urinary 11-dehydro-TXB_2 levels to cholesterol or LDL-C levels in RTRs. The weak correlation between the two suggests that thromboxane-dependent platelet activation is only partly a consequence of abnormal cholesterol levels.

Renal transplant recipients show increased plasma TAT complexes, consistent with coagulative activation in vivo. In fact, TAT complexes result from the neutralization of thrombin by its principal naturally-occurring inhibitor [27]. However, the lack of correlation between urinary excretion of 11-dehydro-TXB_2 and plasma TAT complexes excludes that increased thrombin generation induces platelet activation. Platelet and coagulative activation may be related to the presence of endothelial activation, as demonstrated by the correlation between plasma vWF levels and both urinary 11-dehydro-TXB_2 and plasma TAT levels. Therefore, our finding of abnormal levels of vWF and TAT, an index of thrombin generation, in RTRs with enhanced thromboxane biosynthesis provides in vivo evidence for the concomitant occurrence of these events, and suggests that platelet activation and thrombin generation may be a consequence of endothelial damage or dysfunction.

Based on current evidence, vascular complications might be a consequence of long-standing endothelial injury and the accompanying platelet activation and thrombin generation.

We also report the first evidence, to our knowledge, that RTRs who received cyclosporine had significantly higher in vivo platelet and endothelial activation than RTRs who were not on CsA immunosuppression. Increased urinary excretion of 2,3-dinor-TXB_2 has been previously demonstrated in rat [9] and human [10] CsA nephrotoxicity. In vitro CsA did not interfere with platelet thromboxane generation when rat platelet-rich plasma was exposed to CsA and then challenged with adenosine diphosphate (ADP) or arachidonate [9]. However, in humans, Jorkasky et al reported increased production of thromboxane by platelets exposed to cyclosporine [28], and cyclosporine may activate platelet arachidonate metabolism in the systemic circulation directly [29–31]. Moreover, by increasing the production of reactive oxygen species, CsA may induce endothelial damage [32–34], at the renal circulation activating platelets and macrophages during their passage through the renal circulation. On the other hand, human platelets exposed to cyclosporine have an impaired ability to mediate vasodilation. This appears to be caused by a compound released by cyclosporine-exposed platelets that interferes with nitric oxide-dependent vasodilation [35].

In conclusion, we have characterized an exquisitely sensitive marker of platelet activation, which is abnormal in approximately 80% of RTRs even several months after surgery. Although the mechanisms responsible for platelet activation are likely to be multifactorial in this disease (for example, endothelial injury, disease-or treatment-related metabolic disorders), we show that renal transplantation is associated with in vivo platelet activation and highly related to endothelial activation. This is particularly evident in the CsA-treated patients. Moreover, the follow-up study showed that plasma vWF levels are significantly related to cardiovascular endpoints.

The analytical approach we have used may help identify those patients at increased thrombotic risk as potential candidates for antiplatelet therapy [20]. Of course, long-term, prospective clinical studies are needed to verify the validity of the proposed sequence of events and to determine the prognostic value of abnormal thromboxane biosynthesis vis-à-vis other indexes of vascular injury, including vWF levels. Moreover, controlled clinical trials of low-dose aspirin may help define the pathophysio-

logic significance of thromboxane-dependent platelet activation in this setting.

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