Normalization of Multiple Hemostatic Abnormalities in Uremic Type 1 Diabetic Patients After Kidney-Pancreas Transplantation

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To evaluate the effects of kidney-pancreas transplantation on hemostatic abnormalities in uremic type 1 diabetic patients, we conducted a cross-sectional study involving 12 type 1 diabetic patients, 30 uremic type 1 diabetic patients, 27 uremic type 1 diabetic patients who had a kidney-pancreas transplant, 12 uremic type 1 diabetic patients who had a kidney-alone transplant, and 13 healthy control subjects. We evaluated platelet and clotting system. Platelets in the group of uremic type 1 diabetic patients were significantly larger than platelets in the other groups. Resting calcium levels were significantly higher in the uremic type 1 diabetic patients and uremic type 1 diabetic patients who had a kidney-alone transplant than in the type 1 diabetic patients who had a kidney-pancreas transplant and control subjects. CD41 expression was significantly reduced in platelets from the uremic type 1 diabetic patients compared with the other groups. Levels of hypercoagulability markers in the type 1 diabetic patients who had a kidney-pancreas transplant and, to a lesser extent, the uremic type 1 diabetic patients who had a kidney-alone transplant but not the uremic type 1 diabetic patients were similar to those of the control subjects. A reduction in natural anticoagulants was evident in the uremic type 1 diabetic patients, whereas near-normal values were observed in the type 1 diabetic patients who had a kidney-pancreas transplant and uremic type 1 diabetic patients who had a kidney-alone transplant. Hemostatic abnormalities were not observed in type 1 diabetic patients who had a kidney-pancreas transplant. This finding might explain the lower cardiovascular death rate observed in type 1 diabetic patients who had a kidney-pancreas transplant compared with uremic type 1 diabetic patients who had a kidney-alone transplant or uremic type 1 diabetic patients. *Diabetes* 53:2291–2300, 2004

Hemostatic abnormalities are commonly observed in patients who are affected by type 1 diabetes and in uremic patients (1–10). Enhanced activation of the clotting system has been implicated in the pathogenesis of atherosclerosis and vascular complications in patients with diabetes and uremia (11–15), even after kidney transplantation (16). It is interesting that bleeding tendencies coexist with a prothrombic state in uremia (10).

Abnormal thrombocytopoiesis has been found in diabetic patients (17). Changes in platelet morphology may play a role in cardiovascular events in type 1 diabetic patients and uremic patients (18). Previous studies have shown increased platelet size in patients with cardiovascular disease and also in patients who are affected by uremia and diabetes (17,19–22). An altered thrombocytopoiesis could lead to the release of platelets with an increased expression of constitutive adhesion receptors, such as the GPIIb/IIIa (CD41) and P-selectin (17,23). CD41, expressed on normal platelets, reacts with fibrinogen (Fg)/von Willebrand factor (vWF) to mediate platelet adhesion and aggregation (17). P-selectin, a marker of platelet function, mediates platelet adhesion to neutrophils and allows neutrophil rolling at the vessel wall (17,23). Finally, protease activated receptor-1 (PAR-1) and PAR-3 are thrombin receptors expressed constitutively on platelets (24,25).

A primary role in platelet signaling, impaired in diabetes, has been attributed to intracellular calcium ($[Ca^{2+}]_i$), which is involved in the signal transduction pathway that leads to platelet activation (2,3). An increase in resting $[Ca^{2+}]_i$, as shown in uremic type 1 diabetic patients, could lower the threshold for cellular activation and therefore could be considered a sign of latent activation of circulating platelets (26).

Although the risk of life-threatening cardiovascular events is particularly high in uremic type 1 diabetic patients, kidney transplant (27–32) and, to a greater extent, kidney-pancreas and kidney-islet transplant play a protective role in the risk of cardiovascular events in uremic type 1 diabetic patients (28,29,31–34). In this study, we examined the effects of kidney-alone or combined kidney-pancreas transplantation on platelets and hemo-

static abnormalities that are present in uremic type 1 diabetic patients.

RESEARCH DESIGN AND METHODS

This cross-sectional study compared hemostatic abnormalities in five groups of patients: patients who had type 1 diabetes and were not undergoing dialysis, type 1 diabetic patients who had uremia and were undergoing dialysis, type 1 diabetic patients who had uremia and received kidney-pancreas transplants, type 1 diabetic patients who had uremia and received kidney transplants, and healthy control subjects. The group of uremic type 1 diabetic patients who had a kidney-alone transplant consisted of diabetic patients who either lost their pancreatic graft early in the postoperative period or received only a renal transplantation because of macroscopic damage of the donor pancreas at harvesting.

The study was conducted from June 2000 to June 2002, and all of the patients who received a transplant and were admitted consecutively to San Raffaele Hospital, Milan, for regular check-up were included in the study when they met the inclusion/exclusion criteria. Of the transplant patients, only those with transplant follow-up >1 year and good graft function were included in the study. Patients with clear signs of systemic infection, lymphoproliferative disease, urinary infection, enhanced erythrocyte sedimentation velocity, or C-reactive protein were excluded from the study. Patients who were taking an oral anticoagulant agent were not included. Diabetic patients in the four groups were matched for age, sex, diabetes, and dialysis duration (when performed). All type 1 diabetic patients who had a kidney-pancreas transplant and were considered for the study were insulin independent, whereas the uremic type 1 diabetic patients who had a kidney-alone transplant, type 1 diabetic patients, and uremic type 1 diabetic patients were on intensive subcutaneous insulin therapy. All of the patients included in the uremic type 1 diabetic group, type 1 diabetic patients who had a kidney-pancreas transplant, and uremic type 1 diabetic patients who had a kidney-alone transplant were on antiplatelet therapy (80% aspirin and 20% ticlopidine) to prevent graft or fistula thrombosis. Healthy volunteers were enrolled as control subjects. In hemodialyzed patients, blood samples were collected before dialysis treatment to avoid the confounding effect mediated by heparin administration and by the contact with hemodialysis membrane, which can activate coagulation pathway. To evaluate the effect of immunosuppression, we compared in a subanalysis uremic type 1 diabetic patients who had a kidney-alone transplant and type 1 diabetic patients.

All subjects provided informed consent before study enrollment. The study did not require institutional review board approval at our institution.

Transplantation and immunosuppression. Organs for transplantation were obtained from cadaver donors through Nord Italia Transplant (NITp, Milan, Italy). After induction with ATG (thymoglobulin, IMTIX, SANGSTAT), immunosuppression was maintained using cyclosporine (circulating blood levels between 100 and 250 ng/ml), mycophenolate mofetil (500–2000 mg/day), and methylprednisolone (10 mg/day). Steroids were withdrawn within 3–6 months after transplantation. Episodes of renal rejection were treated with pulses of 500 mg of methylprednisolone. Cases of "steroid-resistant" rejection were treated with OKT3 or a course of ATG.

Intracellular calcium in platelets. Blood was drawn by clean puncture from an antecubital vein and collected into plastic tubes that contained 1 ml of ACD solution: 0.065 mol of citric acid (Riedle, Hannover, German), 0.085 mol of sodium citrate (Farmitalia, Milan, Italy), and 2% glucose monohydrate (Riedle). Platelet-rich plasma (PRP) was obtained by centrifugation at 220g for 15 min at room temperature, and the supernatant was collected. Platelet $[Ca^{2+}]_i$ was evaluated according to the method previously described (2).

$[Ca^{2+}]_i$ was evaluated fluorometrically under resting conditions and after the addition of human thrombin at 0.05, 0.1, and 0.5 units/ml (final concentration). The three doses of thrombin were added in a randomized manner as described previously (24). The parameters analyzed were resting calcium $[Ca^{2+}]_i$, $[Ca^{2+}]_i$ curve, $[Ca^{2+}]_i$ plateau, and $[Ca^{2+}]_i$ peak.

Flow cytometry analysis. Expression of GpII/IIIa (CD41), P-selectin (CD63), and PAR-1 and PAR-3 receptors was examined by flow-activated cell sorting (FACS) analysis of PRP aliquots as previously described (35), using the following antibodies: anti–PAR-1 and anti–PAR-3 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), anti–P-selectin (CTB201) monoclonal antibody (Santa Cruz Biotechnology), and anti-human CD-41a (BD Biosciences, San Diego, CA). Results are expressed as mean log fluorescence intensity versus number of cells.

Electron microscopy. Aliquots of PRP were fixed in 2% Karnovsky solution for electron microscopy of platelets to evaluate platelet size, morphology, and granule content (36). Ultrastructural analyses were carried out on four cases for each group. Measurements were performed using the "measure arbitrary area" tool of AnalySIS Image Processing 3.0 software (Soft Imaging System,

Münster, Germany), which permits measurement of the areas of platelets and granules.

Laboratory analysis. Pancreas and renal function (glycated hemoglobin and serum creatinine) were tested at enrollment and every 6 months thereafter. Platelet-poor plasma was obtained within 2 h by centrifugation at room temperature for 10 min at 1,500g. Determinations of prothrombin time (PT), activated partial thromboplastin time (aPTT), Fg, antithrombin (AT), D-dimer fragments (D-dimer), protein C, and protein S (PS) were carried out on fresh plasma samples as previously reported (37–39). For monitoring changes in in vivo thrombin generation, plasma levels of prothrombin fragments $1 + 2$ ($F_{1 + 2}$) were measured with a commercial enzyme-linked immunosorbent assay (Enzygnost by $F_{1 + 2}$; Dade-Behring, Milan, Italy). Fasting homocysteine was obtained after cleavage and reduction with sodium borohydride followed by derivatization with SBD-F (32). Plasminogen activator inhibitor-1 (PAI-1) was measured with an in-house two-site immunoassay. This assay measures free PAI-1 but not PAI-1 complexed with tissue plasminogen activator (40,41).

Statistical analyses. Data were analyzed using SPSS statistical package for Windows, 10.1 (SPSS, Chicago, IL). Quantitative data were expressed as mean ± SE and were tested for normal distribution with the Kolmogorov-Smirnov test and for homogeneity of variances with Levene's test. When more than two groups were compared cross-sectionally, ANOVA (for parametric data) or Kruskal-Wallis (for nonparametric data) was used according to distribution. When ANOVA was used, multiple post hoc comparison analysis was performed with Tukey test. $P < 0.05$ (by two-tailed testing) was considered an indicator of statistical significance.

RESULTS

Patient characteristics. This cross-sectional study included 12 type 1 diabetic patients, 30 dialyzed uremic type 1 diabetic patients, 27 uremic type 1 diabetic patients who had a kidney-pancreas transplant, 12 uremic type 1 diabetic patients who had a kidney-alone transplant, and 13 healthy control subjects. Table 1 displays the demographics and clinical characteristics of the study population. Transplant and uremic patients were similar regarding the most important clinical characteristics, although the type 1 diabetic patients and the control subjects were younger than patients in the other groups (Table 1). Complicated diabetic patients showed a longer diabetes duration than other patients, but there were no major discrepancies in sex distribution or in dialysis duration (Table 1). Type 1 diabetic patients who had a kidney-pancreas transplant and uremic type 1 diabetic patients who had a kidney-alone transplant had a similar duration of transplant follow-up and similar creatinine levels, but type 1 diabetic patients who had a kidney-pancreas transplant showed lower HbA_{1c} levels than uremic type 1 diabetic patients who had a kidney-alone transplant, as expected (Table 1). Peripheral insulin delivery by the transplanted pancreas (30) resulted in mild hyperinsulinemia in type 1 diabetic patients who had a kidney-pancreas transplant. Total cholesterol, triglycerides, HDL, BMI, and smoking habits were similar in type 1 diabetic patients who had a kidney-pancreas transplant, uremic type 1 diabetic patients who had a kidney-alone transplant, and uremic type 1 diabetic patients (Table 1). Uremic type 1 diabetic patients and uremic type 1 diabetic patients who had a kidney-alone transplant showed higher systolic blood pressure than the control group. Six acute cardiovascular events (acute myocardial infarction or unstable angina) were observed in uremic type 1 diabetic patients, four in uremic type 1 diabetic patients who had a kidney-alone transplant, one in type 1 diabetic patients who had a kidney-pancreas transplant, and one in type 1 diabetic patients.

Platelet morphology. In each case, we analyzed an average of 10 platelets to evaluate platelet area, 10 platelets to evaluate number of granules, and 10 granules to

TABLE 1
General characteristics of type 1 diabetic patients, uremic type 1 diabetic patients, patients who received a kidney-alone transplant, patients who received a kidney-pancreas transplant, and control subjects

	Type 1 diabetic patients	Uremic type 1 diabetic patients	Patients who received kidney-alone transplant	Patients who received a kidney-pancreas transplant	Control subjects
n	12	30	12	27	13
Age (years)	33.3 ± 2.3	48.5 ± 1.9	45.6 ± 2.2	40.5 ± 1.3	26.1 ± 0.8
Sex (M/F)	9/3	20/10	6/6	16/11	7/6
Dialysis and type 1 diabetes duration	$\surd$	3.0 ± 0.6	3.9 ± 0.8	3.3 ± 0.3	$\surd$
(years)	15.7 ± 1.7	31.1 ± 2.7	30.9 ± 3.6	25.7 ± 1.4	$\surd$
Transplant follow-up (months)	$\surd$	$\surd$	62.5 ± 23.3	51.1 ± 6.8	$\surd$
Creatinine (mg/dl)	0.8 ± 0.3	$7.7 \pm 0.6*$	1.5 ± 0.1	1.4 ± 0.1	0.8 ± 0.3
Glycated hemoglobin (%)	8.8 ± 0.4	8.7 ± 0.3	9.2 ± 0.6	$5.6 \pm 0.1†$	$5.2 \pm 0†$
PT	1.00 ± 0.01	1.03 ± 0.04	1.00 ± 0.01	1.01 ± 0.01	0.96 ± 0.01
aPTT	1.01 ± 0.02	0.97 ± 0.02	0.97 ± 0.03	0.98 ± 0.01	1.02 ± 0.02
Fasting homocysteine (μmol/l)	7.0 ± 0.6	$24.8 \pm 2.6*$	14.3 ± 2.1	11.9 ± 1.6	9.9 ± 1.1
PAT-1 (ng/ml)	17.5 ± 1.8	20.0 ± 3.6	17.5 ± 2.3	10.5 ± 1.7	14.0 ± 0.7
Serum-free insulin (μU/ml)	19.8 ± 3.9	29.1 ± 5.7	28.2 ± 8.9	13.9 ± 1.2	$7.8 \pm 1.1‡$
Serum C-peptide (ng/ml)	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	$3.6 \pm 0.7§$	$2.1 \pm 0.3§$
Total cholesterol (mg/dl)	180.7 ± 13.3	206.2 ± 14.9	205.0 ± 9.9	176.9 ± 8.6	186.0 ± 12.2
Triglycerides (mg/dl)	81.0 ± 13.0	$141.1 \pm 17.7\|$	$152.0 \pm 15.4\|$	92.4 ± 7.3	87.9 ± 9.5
HDL (mg/dl)	56.1 ± 4.2	64.5 ± 9.8	53.8 ± 4.6	56.9 ± 2.9	57.2 ± 5.1
Smoking patients	2/12	9/30	3/12	6/27	3/13
Systolic blood pressure (mmHg)	123.6 ± 2.8	$139.0 \pm 5.6¶$	$134.5 \pm 4.7¶$	124.4 ± 4.0	117.1 ± 3.3
Diastolic blood pressure (mmHg)	78.8 ± 1.5	78.0 ± 3.8	81.8 ± 2.2	80.0 ± 2.9	75.3 ± 2.4
BMI (kg/m^2)	23.1 ± 0.9	22.4 ± 1.4	26.1 ± 1.6	25.1 ± 0.8	22.3 ± 1.2

Data are means $\pm$ SE. *$P < 0.01$ for uremic type 1 diabetic patients vs. other groups; †$P < 0.01$ for patients who received a kidney-pancreas transplant and control subjects vs. other groups; ‡$P < 0.05$ for control subjects vs. other groups; §$P < 0.01$ for patients who received a kidney-pancreas transplant and control subjects vs. other groups; $\|P < 0.05$ for patients who received a kidney-alone transplant and uremic type 1 diabetic patients vs. other groups; ¶$P < 0.05$ for uremic type 1 diabetic patients and patients who received a kidney-alone transplant vs. control subjects. $\surd$, not applicable.

evaluate granule area. Increased platelet size was evident in the uremic type 1 diabetic patients compared with the other groups ($P < 0.05$; Figs. 1 and 2A) and compared with the uremic but not the diabetic group (P.F. et al., unpublished observations). Platelet size almost doubled in the uremic type 1 diabetic patients compared with the control group (Figs. 1A and B and 2A). The size of platelet granules was higher in the uremic type 1 diabetic patients than in all other groups ($P < 0.05$; Figs. 1 and 2B). In type 1 diabetic patients and uremic type 1 diabetic patients who had a kidney-alone transplant, a nonsignificant increase in platelet volume, granule area, and granule number was observed (Figs. 1D and E and 2). Platelet features in the type 1 diabetic patients who had a kidney-pancreas transplant were similar to the control group (Figs. 1A and C and 2).

Platelet signaling. Resting $[Ca^{2+}]_i$ was significantly higher in the uremic type 1 diabetic patients and uremic type 1 diabetic patients who had a kidney-alone transplant than in each of the other three groups (uremic type 1 diabetic patients = 137.6 ± 11.2, uremic type 1 diabetic patients who had a kidney-alone transplant = 133.3 ± 16.6, type 1 diabetic patients who had a kidney-pancreas transplant = 97.2 ± 9.2, control subjects = 72.0 ± 11.0, type 1 diabetic patients = 60.9 ± 7.7 nmol/l; $P < 0.01$; Fig. 3A and B). The high resting calcium levels found in uremic type 1 diabetic patients were also partially evident in the uremic type 1 diabetic patients who had a kidney-alone transplant (Fig. 3A and B).

After thrombin stimulus, a different calcium response

was observed in the transplant patients, with significantly higher peak platelet calcium levels in the groups that received transplant than in the uremic type 1 diabetic patients, control subjects, and type 1 diabetic patients (Fig. 3A and C). Platelets from type 1 diabetic patients who had a kidney-pancreas transplant and uremic type 1 diabetic patients who had a kidney-alone transplant were able to restore calcium levels in a near-normal manner, whereas platelets from uremic type 1 diabetic patients retained high calcium levels at the plateau (Fig. 3A and C). Overall, platelets from the uremic type 1 diabetic patients had the highest plateau calcium levels after thrombin stimulus of all groups, even compared with the uremic but not the diabetic group (P.F. et al., unpublished observations). At all thrombin concentrations tested, plateau calcium levels in the uremic type 1 diabetic patients were higher than those in the type 1 diabetic or control groups, and at the highest thrombin concentration tested, plateau calcium levels were higher in the uremic type 1 diabetic patients than in the type 1 diabetic patients who had a kidney-pancreas transplant/uremic type 1 diabetic patients who had a kidney-alone transplant (Fig. 3A and C).

Platelet receptors. Platelets from the four groups of patients and the control subjects exhibited a different percentage of CD41 expression ($P < 0.01$), PAR-1 ($P < 0.01$), and PAR-3 ($P < 0.05$) but not P-selectin (Fig. 4). Platelets from uremic type 1 diabetic patients showed a significant reduction in CD41 expression compared with platelets from all other patient groups, with a platelet expression percentage of 53.2 ± 7.7 for the uremic type 1
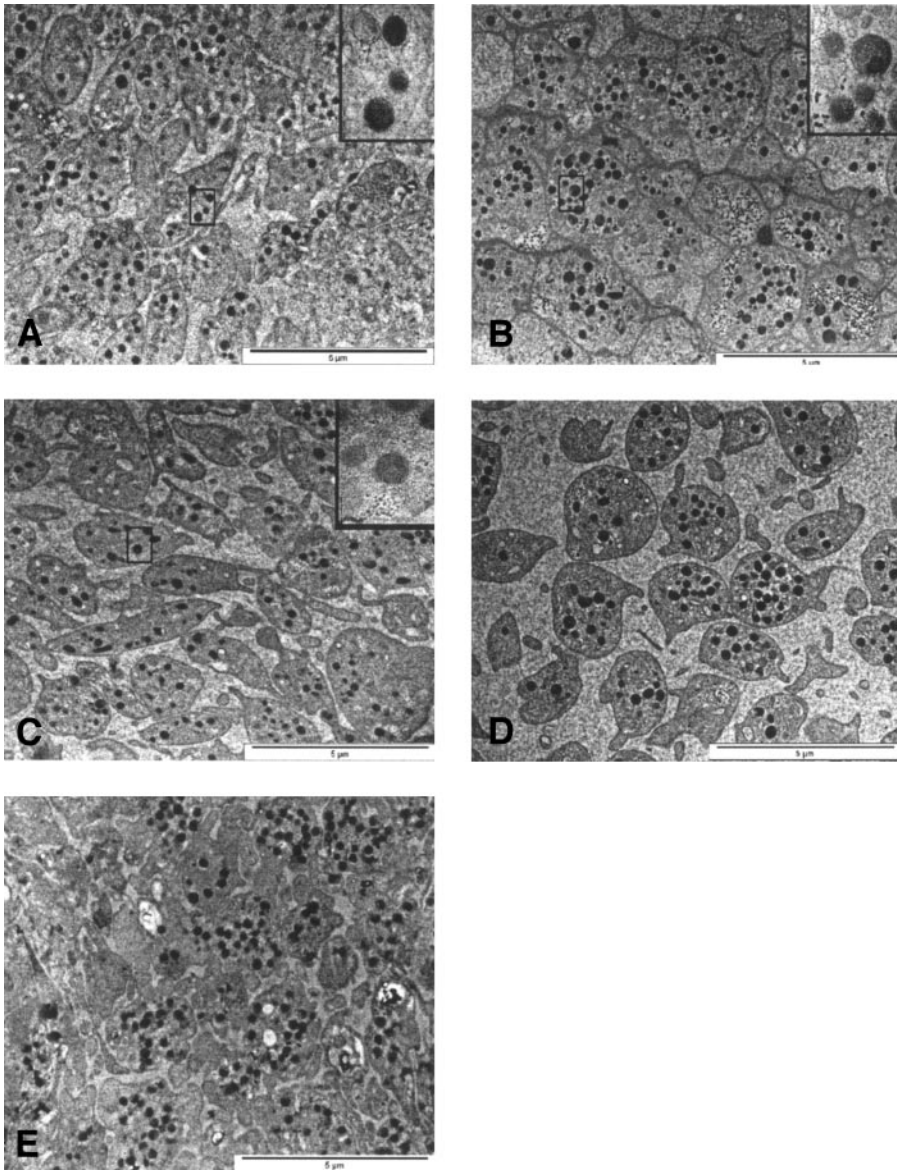
FIG. 1. Electron microscopic view of human platelets of a healthy subject (*A*), a uremic type 1 diabetic patient (*B*), a patient who received a kidney-pancreas transplant (*C*), a patient who received a kidney-alone transplant (*D*), and a type 1 diabetic patient (*E*). Platelets show irregular shape, numerous granules (mainly α type), glycogen β particles, and vesicles. Magnification: ×7,100; insert ×89,000.

diabetic patients compared with percentages of 82.6 ± 3.4, 81.6 ± 2.2, 80.4 ± 4.3, and 75.7 ± 6.2 for the type 1 diabetic patients, control subjects, type 1 diabetic patients who had a kidney-pancreas transplant, and uremic type 1 diabetic patients who had a kidney-alone transplant, respectively ($P < 0.05$; Fig. 4*A*). Platelets from diabetic patients (type 1 diabetic, uremic type 1 diabetic patients, and uremic type 1 diabetic patients who had a kidney-alone transplant) overexpressed PAR-1, compared with platelets from the nondiabetic patients (type 1 diabetic patients who had a kidney-pancreas transplant and control subjects), with mean PAR-1 percentages of 2.86 ± 0.50 and 1.05 ± 0.27, respectively ($P < 0.01$). The same pattern was observed for PAR-3 expression, with mean PAR-3 percentages of 9.80 ± 1.77 for diabetic patients and 3.23 ± 1.12 for nondiabetic patients ($P < 0.01$). Notably, CD41, PAR-1, and PAR-3 platelet expression were similar in type 1 diabetic patients who had a kidney-pancreas transplant and in control subjects.

Hypercoagulability markers. Plasma levels of Fg, $F_{1 + 2}$, and D-dimer were similar in type 1 diabetic patients and control subjects (Fig. 5*A*–*C*). Fg levels were significantly

higher in the uremic type 1 diabetic patients, uremic type 1 diabetic patients who had a kidney-alone transplant, and type 1 diabetic patients who had a kidney-pancreas transplant groups than in the control group ($P < 0.01$). Type 1 diabetic patients who had a kidney-pancreas transplant showed lower levels of Fg compared with uremic type 1 diabetic patients ($P < 0.05$). $F_{1 + 2}$ levels were higher in uremic type 1 diabetic patients compared with type 1 diabetic patients and control subjects ($P < 0.05$). Overall, a similar pattern was observed for the three markers, with the highest levels detected in uremic type 1 diabetic patients, intermediate levels detected in uremic type 1 diabetic patients who had a kidney-alone transplant and, to a lesser degree, in type 1 diabetic patients who had a kidney-pancreas transplant, and the lowest levels detected in type 1 diabetic patients and control subjects (Fig. 5*A*–*C*). The uremic but not diabetic group showed reduced levels of hypercoagulability markers compared with uremic type 1 diabetic patients (P.F. et al., unpublished observations). Fasting homocysteine was statistically higher in uremic type 1 diabetic patients as compared with the other groups (uremic type 1 diabetic patients vs. all other
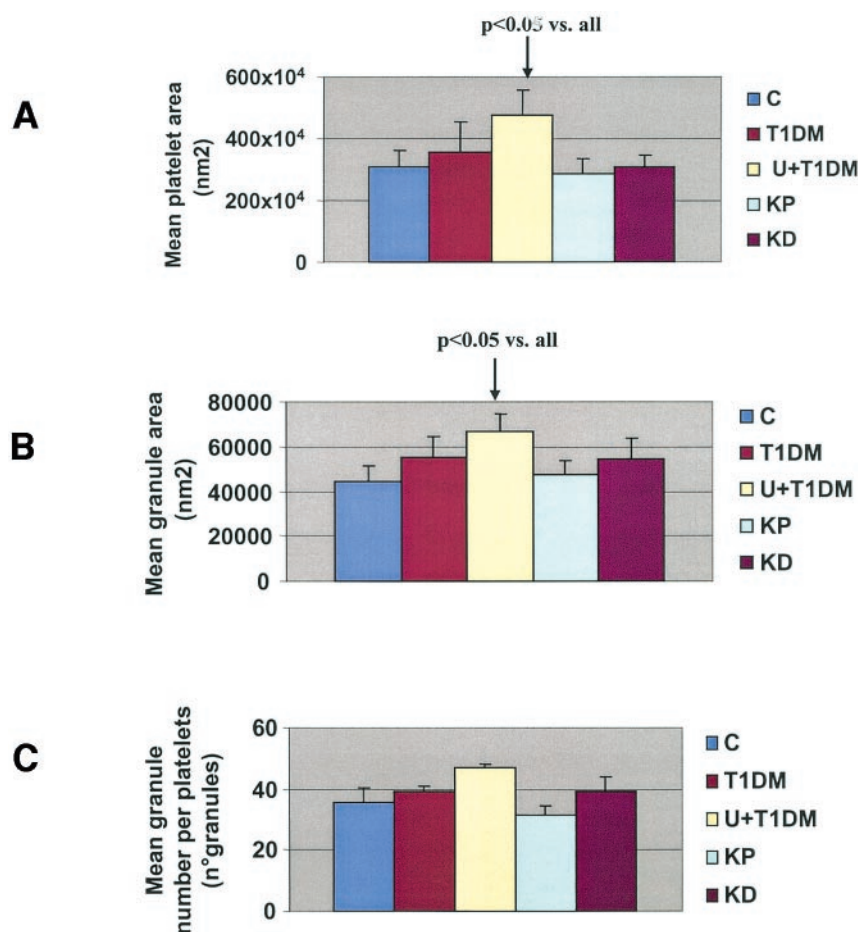
FIG. 2. Score of electron microscopic characteristics of human platelets of healthy subjects (C), type 1 diabetic patients (T1DM), uremic type 1 diabetic patients (U+T1DM), patients who received a kidney-pancreas transplant (KP), and patients who received a kidney-alone transplant (KD). Increased platelet size was evident in the U+T1DM group compared with the other groups (*A*). Platelet size almost doubled in the U+T1DM group compared with the C group (*A*). The size of platelet granules was higher in the U+T1DM than in all other groups (*B*). Platelet features in the KP group were similar to the C group (*A–C*).

groups, $P < 0.01$; Table 1). PAI-1 was lower, although not statistically, in type 1 diabetic patients who had a kidney-pancreas transplant and control subjects compared with uremic type 1 diabetic patients ($P = 0.06$; Table 1).

PT, aPTT, and natural anticoagulants. PT and aPTT were similar in the different groups (Table 1). AT activity was significantly reduced in uremic type 1 diabetic patients compared with control subjects, type 1 diabetic patients, and uremic type 1 diabetic patients who had a kidney-alone transplant ($P < 0.05$; Fig. 5*D*). Uremic type 1 diabetic patients showed lower levels of protein C activity compared with control subjects ($P < 0.05$; Fig. 5*E* and *F*) and lower levels of PS antigen compared with type 1 diabetic patients (data not shown, $P < 0.05$) and type 1 diabetic patients who had a kidney-pancreas transplant (data not shown, $P < 0.01$).

Subanalysis of uremic type 1 diabetic patients who had a kidney-alone transplant versus type 1 diabetic patients. No statistical differences were evident in platelet area, platelet granule area, and number among the uremic type 1 diabetic patients who had a kidney-alone transplant, type 1 diabetic patients, and control subjects (Fig. 2). Uremic type 1 diabetic patients who had a kidney-alone transplant showed higher levels of resting calcium than type 1 diabetic patients and control subjects (uremic type 1 diabetic patients who had a kidney-alone transplant vs. control and type 1 diabetes, both $P < 0.01$; Fig. 3*B*). No differences were evident for CD41 and P-selectin platelet expression, whereas PAR-1 was higher in type 1 diabetic patients compared with control subjects (PAR-1: uremic

type 1 diabetic patients who had a kidney-alone transplant $= 2.26 \pm 0.67\%$, control subjects $= 0.65 \pm 0.18\%$, type 1 diabetic patients $= 4.49 \pm 1.09\%$; type 1 diabetic patients vs. control subjects, $P < 0.05$). PAR-3 seemed to be upregulated in uremic type 1 diabetic patients who had a kidney-alone transplant (PAR-3: uremic type 1 diabetic patients who had a kidney-alone transplant $= 13.54 \pm 3.69\%$, control subjects $= 2.09 \pm 0.75\%$, type 1 diabetic patients $= 6.55 \pm 1.56\%$; uremic type 1 diabetic patients who had a kidney-alone transplant vs. control subjects, $P < 0.05$). Uremic type 1 diabetic patients who had a kidney-alone transplant showed higher levels of Fg and F_{1+2} than both control subjects and type 1 diabetic patients (Fg: uremic type 1 diabetic patients who had a kidney-alone transplant $= 403.5 \pm 17.3$ mg/dl, control subjects $= 262.5 \pm 15.5$ mg/dl, type 1 diabetic patients $= 304.3 \pm 19.0$ mg/dl; uremic type 1 diabetic patients who had a kidney-alone transplant vs. control subjects and type 1 diabetic patients, both $P < 0.01$; F_{1+2}: uremic type 1 diabetic patients who had a kidney-alone transplant $= 137.6 \pm 17.2$ mmol/l, control subjects $= 63.3 \pm 5.9$ mmol/l, type 1 diabetic patients $= 69.1 \pm 12.1$ mmol/l; uremic type 1 diabetic patients who had a kidney-alone transplant vs. control subjects and type 1 diabetic patients, both $P < 0.01$).

DISCUSSION

Several alterations in platelet structure, function, and hemostatic profiles were observed in uremic type 1 diabetic patients. Platelets in uremic type 1 diabetic patients
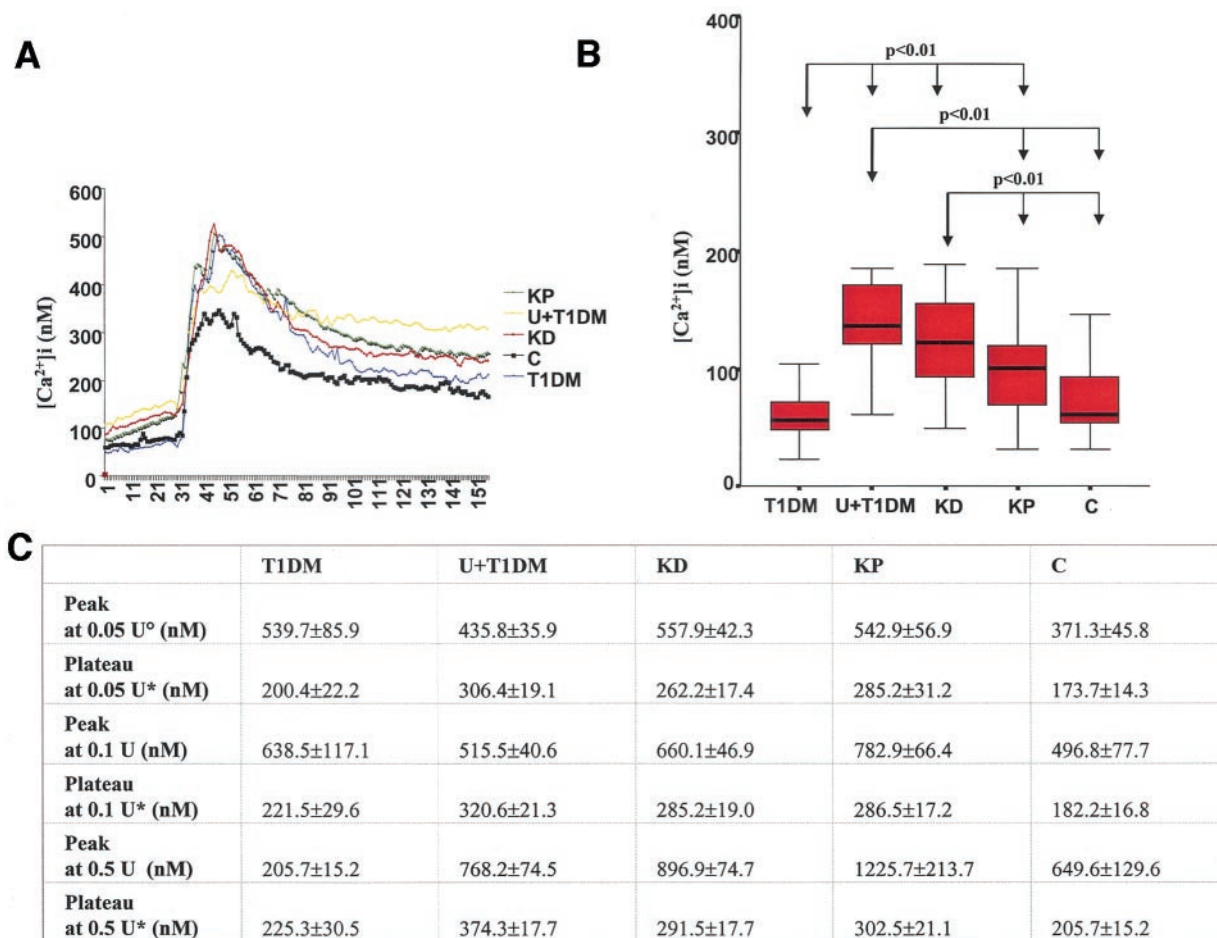
C

	T1DM	U+T1DM	KD	KP	C
Peak at 0.05 U° (nM)	539.7±85.9	435.8±35.9	557.9±42.3	542.9±56.9	371.3±45.8
Plateau at 0.05 U* (nM)	200.4±22.2	306.4±19.1	262.2±17.4	285.2±31.2	173.7±14.3
Peak at 0.1 U (nM)	638.5±117.1	515.5±40.6	660.1±46.9	782.9±66.4	496.8±77.7
Plateau at 0.1 U* (nM)	221.5±29.6	320.6±21.3	285.2±19.0	286.5±17.2	182.2±16.8
Peak at 0.5 U (nM)	205.7±15.2	768.2±74.5	896.9±74.7	1225.7±213.7	649.6±129.6
Plateau at 0.5 U* (nM)	225.3±30.5	374.3±17.7	291.5±17.7	302.5±21.1	205.7±15.2

FIG. 3. Platelet calcium curve profile after thrombin stimulus (0.05 units; A) and resting calcium ($P < 0.01$; B) in control subjects (C), type 1 diabetic patients (T1DM), uremic type 1 diabetic patients (U+T1DM), patients who received a kidney-alone transplant (KD), and patients who received a kidney-pancreas transplant (KP). Resting $[Ca^{2+}]_i$ was significantly higher in the U+T1DM and KD groups than in each of the other three groups (B). After thrombin stimulus, a different calcium response was observed in the transplant patients, with significantly higher peak platelet calcium levels in the transplanted groups than in the U+T1DM, C, and T1DM groups (*$P < 0.01$, °$P = 0.05$; C). Overall, platelets from the U+T1DM group had the highest plateau calcium levels after thrombin stimulus of all groups (C).

were larger and had a greater granule size and number compared with platelets in other patients. Increased platelet size and higher platelet activation could lead to reduced platelet function in uremic type 1 diabetic patients, as shown by reduced CD41 expression. Moreover, the clotting system seemed to be activated, leading to a prothrombic state associated with bleeding tendencies in uremic type 1 diabetic patients. Type 1 diabetic patients did not show all of the hemostatic abnormalities evidenced in uremic type 1 diabetic patients.

Normalization of uremia in type 1 diabetic patients who had a kidney-alone transplant and particularly in kidney-pancreas transplant patients, when it is combined with restoration of euglycemia, too, is associated with near-normal hemostasis, despite immunosuppressive therapy. The prothrombic state observed in uremic type 1 diabetic patients is not observed in type 1 diabetic patients who had a kidney-pancreas transplant. The higher levels of D-dimer fragments, Fg, and partially of PAI-1 in uremic type 1 diabetic patients who had a kidney-alone transplant compared with type 1 diabetic patients who had a kidney-pancreas transplant confirmed an adjunctive benefit of kidney-pancreas transplantation on hemostasis. Patients in both transplant groups showed reductions in platelet

volume, compared with uremic type 1 diabetic patients; moreover, type 1 diabetic patients who had a kidney-pancreas transplant had normal platelets in terms of platelet volume and granule number. Under both resting and stimulated conditions, calcium homeostasis was normal in platelets from type 1 diabetic patients who had a kidney-pancreas transplant but not completely in platelets from uremic type 1 diabetic patients who had a kidney-alone transplant. The presence of a better platelet function and morphology, together with a better clotting system profile and lower levels of hypercoagulability markers in uremic but not diabetic patients (P.F. et al., unpublished observations), confirmed that the association of diabetes and uremia—not only uremia per se—could be responsible for the many profound abnormalities evident in these patients (42). Regarding the effects of immunosuppression, PAR-1 seems to be downmodulated by these drugs, given its low levels in uremic type 1 diabetic patients who had a kidney-alone transplant. However, the subanalysis revealed that kidney-alone transplantation has higher prothrombic tendencies than type 1 diabetes.

Cardiovascular and cerebrovascular events account for most of the excess death rate seen in diabetic patients on hemodialysis (43). The finding that platelets in uremic type 1
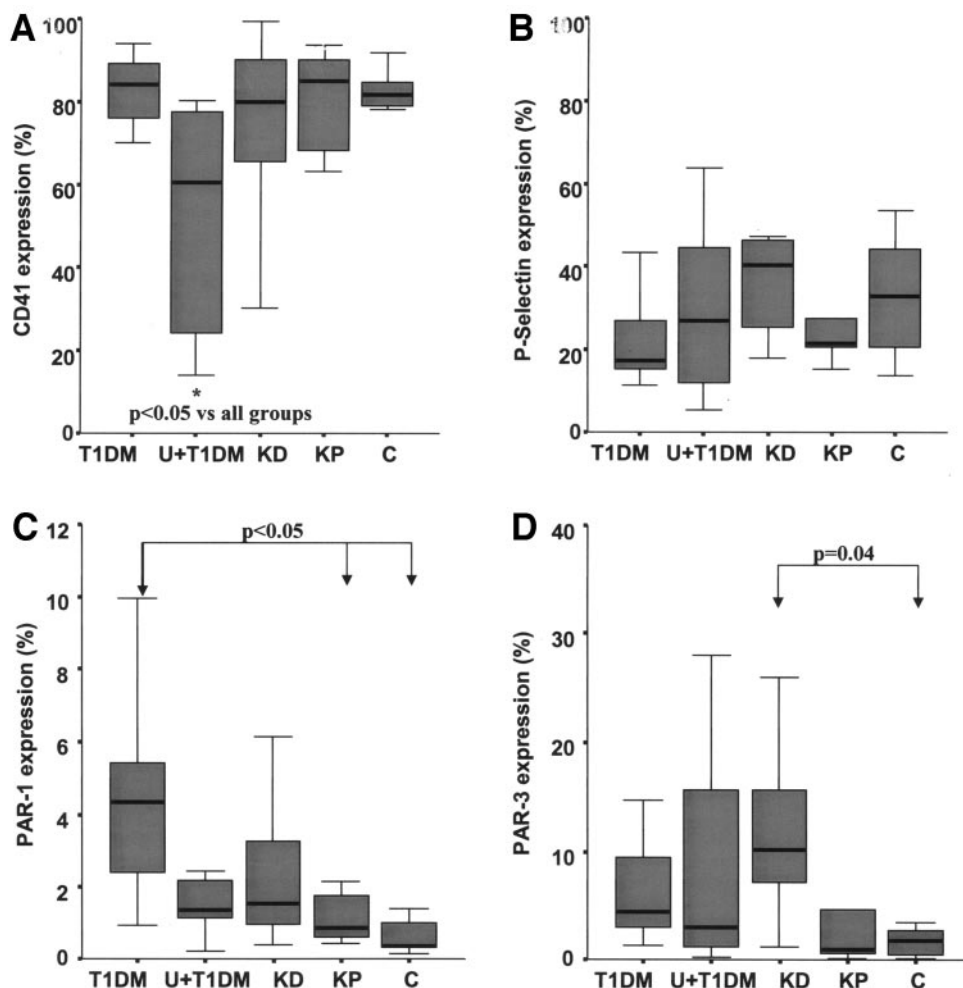
FIG. 4. CD41 (*A*), P-selectin (*B*) PAR-1 (*C*), and PAR-3 (*D*) expression at FACS analysis in control subjects (C), type 1 diabetic patients (T1DM), uremic type 1 diabetic patients (U+T1DM), patients who received a kidney-alone transplant (KD), and patients who received a kidney-pancreas transplant (KP). Platelets from the four groups of patients and the control subjects exhibited a different percentage of CD41 (*P* < 0.01), PAR-1 (*P* < 0.01), and PAR-3 (*P* < 0.05) but not P-selectin expression (*A*–*D*). Platelets from U+T1DM patients showed a significant reduction in CD41 expression compared with platelets from all other patient groups (*P* < 0.05; *A*). Notably, CD41, PAR-1, and PAR-3 platelet expression was similar in KP and in C.

1 diabetic patients have increased volume is important, given that platelet morphology and size may have a role in the evolution of cardiovascular events (17,19–22). The increased platelet size that we observed in these patients could at least partially explain the elevated rate of cardiovascular and cerebrovascular events typically found in uremic type 1 diabetic patients (both thrombotic and hemorrhagic) (31). Previous studies demonstrated a favorable role of pancreas transplantation toward progression of some features of macrovascular disease in uremic diabetic patients, leading to a better survival of type 1 diabetic patients who had a kidney-pancreas transplant than of uremic type 1 diabetic patients who had a kidney-alone transplant and uremic type 1 diabetic patients, with a reduction in cardiovascular life-threatening events (31,43). This lower incidence of cardiovascular events in the type 1 diabetic patients who had a kidney-pancreas transplant is consistent with the better hemostatic profile observed in our study. All uremic type 1 diabetic patients, uremic type 1 diabetic patients who had a kidney-alone transplant, and type 1 diabetic patients who had a kidney-pancreas transplant were on antiplatelet medications; this could exclude a bias among these three groups. It is possible that some of the hemostatic abnormalities observed in these three groups could be smoothed by medications when compared with type 1 diabetic patients and control subjects.

Prolonged platelet activation, as shown in our study by the higher levels of resting $[Ca^{2+}]_i$ found in uremic type 1 diabetic patients, may lead to or suggest a platelet malfunction. The increased bleeding tendency of uremic type 1 diabetic patients has been attributed to this platelet dysfunction. However, reports on various platelet functions in uremic patients have been conflicting (10,44–48). A previous report showed that uremic patients had longer bleeding time (44). It is difficult to establish how bleeding tendencies and prothrombic state can coexist in uremia. In uremic type 1 diabetic patients, activation of the clotting system induces a prothrombic state but creates a malfunction of platelets (44–46). Platelets become hyporesponsive to stimulation (44) as a result of a decreased GPIIb/IIIa availability for receptor occupancy by Fg/vWF fragments (45). Fg and fibrin degradation products were significantly increased, and the activity of PAI was slightly reduced, denoting an activation of fibrinolysis in uremic patients (10,47). The increased intravascular generation of thrombin and/or plasmin can mediate the defects in primary hemostasis, prolongation of the bleeding time, and, probably, bleeding in uremia (10,47). Furthermore, high levels of Fg, vWF, and D-dimer were associated with cardiac events (48), so high levels of vWF, D-dimer, and Fg induce a risk for both cardiovascular events and bleeding. This disturbed coagulation state seen in patients with uremia could contribute also to thrombotic events in kidney grafts after transplantation (46).

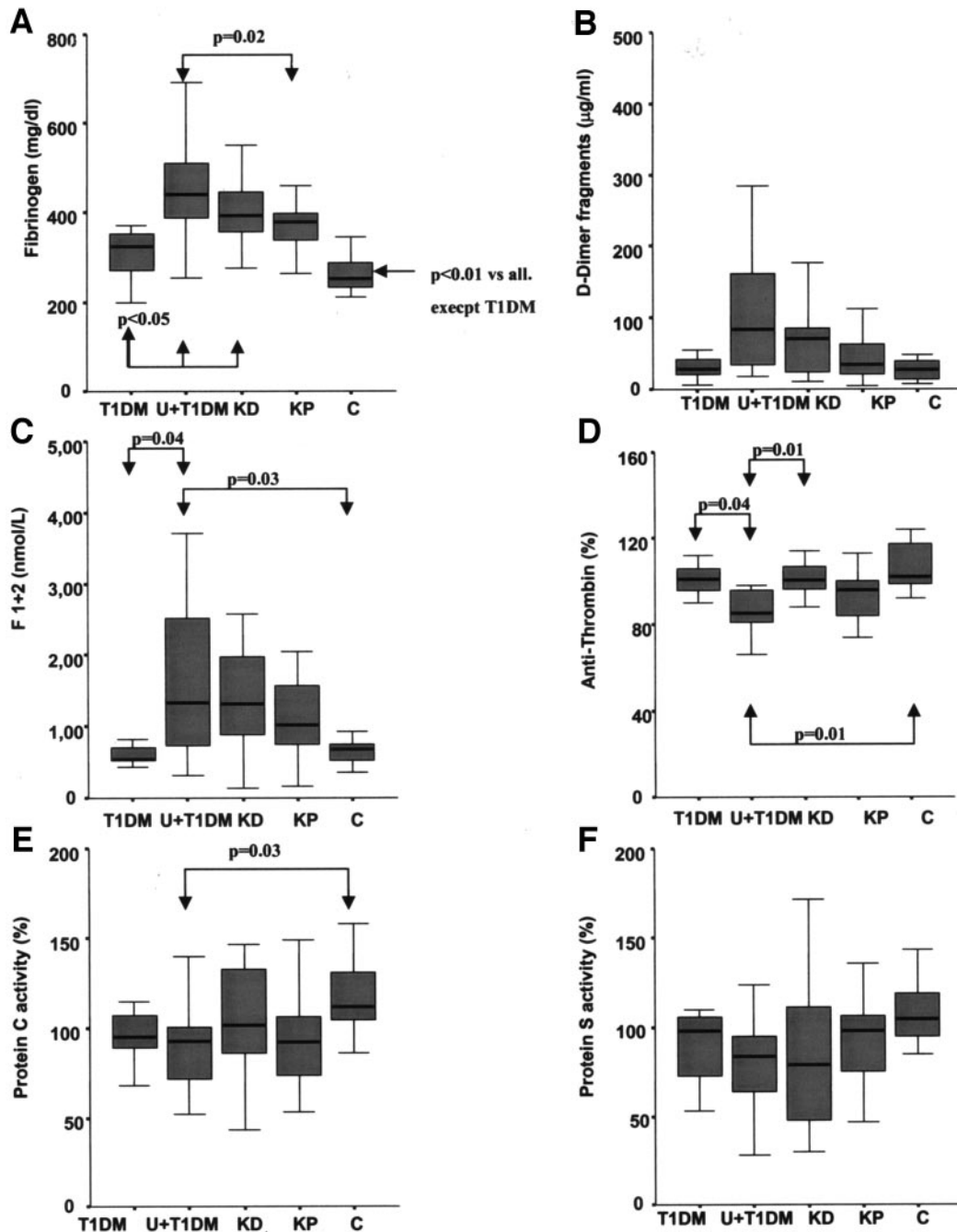Another hypothesis is that the global reduction of plate-

FIG. 5. Hypercoagulability markers (Fg [mg/dl; *A*], D-dimer [µg/ml; *B*], and F_{1+2} [nmol/l; *C*]) and natural anticoagulants (AT [%; *D*], PC [%; *E*], and PS [%; *F*]) in control subjects (C), type 1 diabetic patients (T1DM), uremic type 1 diabetic patients (U+T1DM), patients who received a kidney-alone transplant (KD), and patients who received a kidney-pancreas transplant (KP). Fg levels were significantly higher in the U+T1DM, KD, and KP groups than in the C group ($P < 0.01$; *A*). Patients in the KP group showed lower levels of Fg compared with patients in the U+T1DM group ($P < 0.05$; *A*). F_{1+2} levels were higher in U+T1DM patients compared with patients in T1DM and C groups ($P < 0.05$; *C*). AT activity was significantly reduced in U+T1DM patients compared with C, T1DM, and KD groups ($P < 0.05$; *D*). Finally, U+T1DM showed lower levels of PC activity compared with the C group ($P < 0.05$; *E*).

let nitric oxide found in uremic type 1 diabetic patients (49,50) could cause platelet activation by diminishing Ca-ATPase–dependent restoration of intracellular calcium stores or by activating phosphatidylinositol 3-kinase (51, 52). Alternatively, prothrombin fragments (F_{1+2}), which are increased in type 1 diabetic patients (53) and in uremic type 1 diabetic patients (54), could chronically stimulate PAR-1 and PAR-3, which are upregulated in these patients, resulting in malfunctioning platelets. The disruption of calcium homeostasis in platelets from uremic type 1 diabetic

patients suggests a possible role for the PAR-1/PAR-3 pathways. Generally, higher levels of both of these receptors were seen in diabetic patients in our study than in nondiabetic patients.

Finally, the reduction in protein C anticoagulant activity that was noted in uremic type 1 diabetic patients was not observed in the transplant patients. Apparently, transplantation is able to overcome the deleterious effect of diabetes and uremia on protein C, a protein of hepatic origin. In conclusion, alterations of hemostatic abnormalities are

TABLE 2
Synopsis of hemostatic biomarkers in kidney-pancreas transplant in type 1 diabetic, uremic type 1 diabetic, kidney transplant, and kidney-pancreas transplant patients

	Type 1 diabetic patients	Uremic type 1 diabetic patients	Patients who received a kidney-alone transplant	Patients who received a kidney-pancreas transplant
n	12	30	12	27
Platelet size, granule size, and granule number	↑	↑↑	↑	N
Calcium	N	↑↑	↑	↑
CD41	N	↓	N	N
P-selectin	N	N	N	N
PAR-1	↑	N	↑	N
PAR-3	↑	↑	↑	N
Hypercoagulability markers	N	↑↑	↑	N
AT	N	↓↓	↓	↓
PS and PC	↓	↓↓	↓	↓↓

Symbols represent variations vs. normal group (N, normal; ↑, increased; ↑↑, very increased; ↓, decreased; ↓↓, very decreased.

evident in uremic type 1 diabetic patients (Table 2). Uremic type 1 diabetic patients who receive kidney-alone and, to a greater extent, kidney-pancreas transplants have a lower prothrombic state and have a general improvement in the clotting system (Table 2). This could probably play a positive role in protecting against cardiovascular events and death in kidney-pancreas and kidney-alone transplant patients compared with uremic type 1 diabetic patients who do not receive a transplant.

ACKNOWLEDGMENTS

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