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In Vitro Thromboxane Synthesis of Depleted Blood Platelets Following Renal Transplantation*

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Summary

Renal transplant rejection is associated with platelet activation in vivo which may lead to partially α- and δ-granule-depleted platelets that continue to circulate. These "exhausted" platelets are hemostatically defective. To quantitate the extent of platelet granule depletion following kidney transplantation, we determined intraplatelet levels of β-thromboglobulin (βTG), platelet factor 4 (PF4), and serotonin (5-hydroxytryptamine, 5-HT) ex vivo in Triton X-100-treated platelet lysates. To explore biochemical alterations of partially depleted platelets, we studied platelet thromboxane A_2 (TXA_2) synthesis in citrated platelet-rich plasma (PRP) upon stimulation with thrombin or collagen in 45 recipients of renal allografts and 10 healthy volunteers. The patients were divided into subjects with acute and chronic allograft rejection (N = 15), those with compensated renal failure after kidney transplantation but without evidence of allograft rejection (N = 15), and those with functioning renal transplant (N = 15). The mean intraplatelet content of βTG (38.6 ± 4.2 μg/10^9 platelets), PF4 (11.8 ± 1.8 μg/10^9 platelets), and 5-HT (274 ± 31 ng/10^9 platelets) in patients with acute or chronic renal allograft rejection was significantly lower than in other recipients of kidney transplants or healthy volunteers (βTG: 59.9 ± 4.7 μg/10^9 platelets; PF4: 20.4 ± 2.3 μg/10^9 platelets; 5-HT: 461 ± 48 ng/10^9 platelets; $p < 0.005$ in all cases). Platelet TXB_2 formation upon stimulation with thrombin (10 U/ml) or collagen (6.25 μg/ml) for 5 min was significantly reduced in patients with acute or chronic renal allograft rejection (2.25 ± 0.29 and 0.64 ± 0.08 nmol/10^9 platelets for thrombin- and collagen-stimulated platelets, respectively) compared to that of healthy volunteers (4.72 ± 0.60 and 1.35 ± 0.12 nmol/10^9 platelets, respectively; $p < 0.05$ in all cases). In contrast, platelet TXB_2 formation of patients with functioning kidney transplant or those with compensated renal failure but without evidence of transplant rejection did not differ significantly from that of normals. These results confirm that platelets with reduced levels of α- and δ-granular constituents are detectable in the circulation following kidney transplantation when acute or chronic renal allograft rejections occur. These platelets are incapable of forming normal amounts of thromboxane upon stimulation with thrombin and collagen in vitro. This dysfunction of thromboxane synthesis, due to alterations in the platelet arachidonate pathway, may reflect the previous activation of platelets in vivo associated with acute or chronic renal allograft rejection.

Introduction

Acute and chronic allograft rejection following renal transplantation is associated with activation of blood platelets (1–4) and, to some extent, with intra- and/or extravascular generation of thrombin (3, 4). Platelet activation with increased secretion of granular constituents may lead to circulating depleted platelets (5, 6). In some instances the occurrence of "exhausted" platelets (5) is associated with bleeding since these platelets are hemostatically defective (7, 8) due to partial or complete loss of granule contents ("acquired storage pool deficiency") and/or metabolic alterations. There is increasing evidence that acquired platelet storage pool deficiency can be associated with impaired platelet thromboxane synthesis (1, 9) and concomitant platelet dysfunction similar to that demonstrated in patients with congenital storage pool deficiency (10).

The present study was designed to determine platelet granular constituents ex vivo and platelet thromboxane formation in vitro upon stimulation with thrombin or collagen in patients after renal transplantation. We report that intraplatelet levels of α- and δ-granule constituents are significantly decreased when renal allograft rejection occurs, and that these depleted platelets are incapable of forming normal amounts of thromboxane upon stimulation with platelet agonists in vitro.

Patients and Methods

Patients

We studied 45 recipients of a renal cadaveric allograft, comprising 24 males and 21 females. Their mean age was 40 ± (SEM) 0.9 (range: 20 to 49) years. Fifteen patients were studied at the time of transplant rejection, including 10 subjects with acute, and 5 with chronic allograft rejection. The diagnosis of acute rejection was based on clinical criteria (fever, swelling and tenderness over the allograft, and reduction in urinary output), laboratory findings (increase in serum creatinine and blood urea nitrogen, decrease in urinary sodium excretion), and confirmed by histological examination of a transplant biopsy. In accord with Capitanio et al. (1), a slowly progressive, irreversible rise in serum creatinine (exceeding more than 25% of the patient's initial baseline level) was considered indicative of chronic allograft rejection. Fifteen patients presented with compensated renal failure but without clinical or histological evidence of acute or chronic allograft rejection, and 15 subjects (control patients) were studied at a time when the kidney transplant function was intact (serum creatinine: 1.2 ± 0.05 mg/dl) and stable. Patients with diabetes mellitus, systemic lupus erythematosus, congestive heart failure, artificial heart valves, clinically suspected or microbiologically proven infection, patients requiring hemodialysis, or those who received nonsteroidal antiinflammatory agents, were excluded.

Eight of the 15 patients with acute or chronic allograft rejection received hydralazine (25 mg 3 times daily), 2 were given prazosin (2 mg 2 times daily), and in 5 of the 10 patients, this medication was combined either with nifedipine (20 mg 3 times daily), or propranolol (40 mg 3 times daily). Among the transplant recipients with compensated renal failure but without evidence of rejection, 14 of 15 patients received at least two of the following agents: hydralazine, prazosin, propranolol, metoprolol, nifedipine, nisoldipine, diltiazem, and verapamil. In the majority, the doses administered were higher than those in patients with allograft rejection. Eleven of 15 patients with intact transplant function were given at least one of the agents noted above.

In all patients, primary immunosuppression therapy consisted of cyclosporine A (15 mg kg^{-1} day^{-1} in two daily doses) and a 5-day burst of methylprednisolone (beginning with 200 mg intraoperatively; dose reduc-

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Table 1 Intraplatelet α- and δ-granule contents and serum creatinine levels in recipients of kidney allografts and healthy volunteers

Patients	N	Serum creatinine (mg/dl)	Intraplatelet concentrations βTG μg/10^9 plts	PF4 μg/10^9 plts	5-HT ng/10^9 plts
Acute and chronic allograft rejection	15	3.1 ± 0.75	38.6 ± 4.2	11.8 ± 1.8	274 ± 31
Allograft with compensated renal failure	15	4.9 ± 0.85	53.1 ± 4.0	16.9 ± 1.6	391 ± 33
Functioning transplant (FT)	15	1.2 ± 0.05	52.7 ± 3.7	17.5 ± 1.5	384 ± 30
Healthy volunteers (HV)	10	0.9 ± 0.06	59.9 ± 4.7	20.4 ± 2.3	461 ± 48
Significance (vs. HV, FT)					
Acute and chronic rejection		p <0.001	p <0.005	p <0.005	p <0.005
Compensated renal failure		p <0.001	n.s.	n.s.	n.s.

All values are given as mean ± SEM.

Table 2 Platelet thomboxane formation in vitro upon stimulation with thrombin or collagen in recipients of kidney allografts and healthy volunteers

Patients	TXB_2 (nmol/10^9 platelets) Thrombin (10 U/ml)	Collagen (6.25 μg/ml)
Acute and chron. allograft rejection	2.25 ± 0.29 (0.81 – 3.85)	0.64 ± 0.08 (0.14 – 0.81)
Allograft with compensated renal failure	4.39 ± 0.40 (3.28 – 5.11)	1.25 ± 0.10 (0.83 – 1.72)
Functioning transplant (FT)	4.09 ± 0.15 (1.69 – 5.02)	1.17 ± 0.15 (0.54 – 1.50)
Healthy volunteers (HV)	4.72 ± 0.60 (2.02 – 6.15)	1.35 ± 0.12 (0.92 – 2.07)
Significance (vs. HV, FT)		
Acute and chron. rejection	p <0.05	p <0.05
Compensated renal failure	n.s.	n.s.

The amount of TXB_2 formed upon incubation of citrated PRP samples with thrombin or collagen for 5 min at 37 °C was determined in the supernatants after addition of 8 μM indomethacin and 10^{-5} M PGE_1 followed by subsequent centrifugation, as described in the Method section. The data were corrected for the concentrations of TXB_2 found in the supernatant of PRP samples treated with Tyrode instead of thrombin or collagen. All values are given as mean ± SEM (range).

tion by 40 mg/day) followed by low-dose prednisone (20 mg/day for maintenance). The cyclosporine A dose was adapted individually, using a radioimmunoassay (Sandoz, Basel, Switzerland), to achieve whole blood trough levels of approximately 500 to 800 ng/ml during the first 2 weeks posttransplantation and about 400 ng/ml (range: 250 to 500 ng/ml) thereafter (11). Patients with acute rejection episodes were treated with intravenous methylprednisolone (1 g) given daily for 3 days (12, 13). Those with chronic transplant rejection also received methylprednisolone in a dose of 0.6 to 0.8 mg kg^{-1} day^{-1}. In 9 of the 45 patients, a conversion of the standard immunosuppressive therapy to azathioprine was performed because of cyclosporine-related toxicity. Serum creatinine levels were determined according to the enzymatic method described by Boerner et al. (14).

For comparison purposes, renal function, platelet granule constituents ex vivo and platelet thromboxane formation in vitro were evaluated in 10 healthy volunteers (5 males, 5 females; mean age: 36 ± 2.7; range: 25 to 51 years).

In all patients, blood was collected with a 21-gauge butterfly infusion set (Venisystems, Abbott Ireland Ltd., Sligo, Ireland) in the fasting state between 8 and 9 a.m. prior to administration of immunosuppresssive, or antihypertensive drugs. All individuals gave their informed consent to participate in this study.

Methods

Evaluation of intraplatelet levels of βTG, PF4, and 5-HT. Blood (2.7 ml) obtained by clean venipuncture was drawn into precooled sample tubes (preloaded with 0.1 ml EDTA 90 mM, 0.1 ml theophylline 10 mM, and 0.1 ml forskolin 0.1 mM, pH 7.4; "ETF" cocktail) and kept on ice for 30 min as described previously (15). After preparing platelet-rich plasma (PRP; 110 × g for 15 min at 4 °C) and measuring the platelet concentration with a Thrombocounter (Coulter Electronics, Hialeah, FL), platelets were lysed with 1% v/v Triton X-100. ETF-PRP samples were frozen at –20 °C until assayed for βTG and PF4, using commercially available RIA kits (Amersham-Buchler, Braunschweig, FRG; Abbott Diagnostics, Wiesbaden, FRG). The ETP-PRP-Triton samples were diluted in phosphate-buffered saline containing 20% horse serum. The contribution of plasma βTG to intraplatelet βTG content was less than 1%. The same was true for PF4. Intraplatelet levels of βTG and PF4 are expressed as μg/10^9 platelets. Intraplatelet concentrations of 5-HT (ng/10^9 platelets) were determined in Triton-treated platelet lysates, using the fluorimetric method described by Drummond and Gordon (16). Triton X-100 at a final concentration of 1% was shown not to interfere with any of these tests (4).

Determination of platelet thromboxane synthesis in vitro. One ml aliquots of PRP (platelet concentration adjusted to 250 × 10^6/ml) obtained by centrifugation of citrated blood (3.2% trisodium citrate, 1 : 10) at room temperature were incubated with bovine thrombin (10 U/ml; specific activity 200 U/mg; Behringwerke AG, Marburg/Lahn, FRG), collagen (6.25 μg/ml; Hormon-Chemie, München, FRG), or Tyrode buffer, pH 7.35, for 5 min at 37 °C, using an aggregation module (Braun, Melsungen, FRG) with continuous stirring. Platelet TXB_2 formation was stopped by addition of 8 μM indomethacin (Serva GmbH, Heidelberg, FRG) and 10^{-5} M prostaglandin E_1 (PGE_1, Serva GmbH, Heidelberg, FRG) and subsequent centrifugation (9,000 × g for 1 min at room temperature).

TXB_2 in the supernatant was determined radioimmunologically according to the method of Smith (17), using rabbit anti-TXB_2 antiserum and TXB_2 standards both from Upjohn Co., Kalamazoo, MI, and [^{3}H]-TXB_2 (139 Ci/mmol) from Dupont NEN, Boston, MA. The amount of TXB_2 formed upon stimulation with the two agonists was corrected for the value obtained after 5 min incubation of PRP with Tyrode buffer, which was less than 0.02 nmol/10^9 platelets.

Statistical analysis. Statistical comparisons were made using Student's t test (two-tailed) when data were normally distributed (Wilk-Shapiro test). Otherwise, the Wilcoxon sign rank test was chosen. All data are given as mean ± SEM. Differences having a p value of <0.05 were considered significant.

Results

Intraplatelet Levels of βTG, PF4, and 5-HT Ex Vivo

Patients with acute and chronic renal allograft rejection showed a significantly lower intraplatelet concentration of α- and δ-granule constituents than recipients of renal allografts with compensated renal failure, those with functioning tranplants, or healthy volunteers (Table 1). There was no significant difference in the mean serum creatinine levels between patients with acute or chronic allograft rejection and recipients of kidney transplants with compensated renal failure (Table 1).

Platelet Thromboxane Synthesis In Vitro

Platelet TXB_2 production in patients with acute or chronic renal allograft rejection was significantly reduced upon stimulation with thrombin (10 U/ml) or collagen (6.25 μg/ml) at 37 °C for 5 min when compared with platelets from healthy volunteers (Table 2). In response to thrombin, there was no statistical significant difference between patients with acute renal allograft rejection (2.05 ± 0.26 nmol $TXB_2/10^9$ platelets) and those with chronic rejection (2.64 ± 0.63 nmol $TXB_2/10^9$ platelets). The same was true when collagen was used as stimulating agent. Prolongation of the incubation time with thrombin or collagen up to 30 min did not significantly increase platelet TXB_2 formation in patients with transplant rejection (data not shown). Platelets from patients with functioning renal transplant and those from kidney allograft recipients with compensated renal failure but without evidence of transplant rejection formed normal amounts of TXB_2 in response to thrombin or collagen (Table 2).

Discussion

Circulating platelets with reduced levels of α- and δ-granular constituents have been identified in various clinical conditions, including extracorporeal circuits (cardiopulmonary bypass, hemodialysis) (6, 7, 18, 19), artificial heart valves (20), autoimmune thrombocytopenia (5, 8, 21, 22), thrombotic thrombocytopenic purpura (8, 9), hemolytic uremic syndrome (8, 23), systemic lupus erythematosus (8), and disorders associated with disseminated intravascular coagulation (4, 8, 24, 25). We have previously shown that partially depleted platelets are detectable in recipients of renal allografts when acute or chronic rejections occur (2, 3). Similar observations have been reported by Capitanio et al. (1). Our earlier findings (2, 3) and those of Capitanio et al. (1) suggest that renal allograft rejection is associated with platelet activation in vivo, leading to secretion of platelet granular constituents and the circulation of partially depleted platelets. Some of the biochemical and morphological platelet abnormalities (4) observed in recipients of renal transplants at the time of acute or chronic allograft rejection may resemble those found in patients with congenital platelet storage pool deficiency (10, 26). In some clinical conditions associated with acquired storage pool defects, a dysfunction of the platelet cyclo-oxygenase pathway has been reported (9, 27).

The results of the present study demonstrate that acute and chronic allograft rejection after kidney transplantation is associated with impaired platelet arachidonate metabolism. In contrast, platelets obtained from recipients of kidney allografts with functioning transplant or those with compensated renal failure without evidence of allograft rejection were capable of forming normal amounts of TXB_2 in response to stimulation with platelet agonists in vitro (Table 2). The latter observation is in contrast to findings made by Capitanio et al. (1) who reported a reduction in platelet thromboxane production in all patient groups with kidney transplants, including those with functioning allograft. The reason for this discrepancy is unclear, especially since, in both studies, comparable procedures for evaluation of platelet thromboxane synthesis capacity (4, 28) and the same diagnostic criteria for classifying renal transplant recipients were used.

The data of the present study are consistent with the hypothesis that, in patients with acute or chronic renal allograft rejection, impaired platelet thromboxane synthesis in vitro is a consequence of platelet activation in vivo, leading to secretion of platelet granular constituents and the circulation of partially and/or completely depleted platelets. Indeed, activation of platelets in vitro can result in refractoriness, including a decreased capacity for thromboxane synthesis, upon further stimulation with platelet agonists (4, 29–32).

Drug intake may be another potential mechanism for the impaired platelet thromboxane synthesis caused by inhibition of the enzymes involved in platelet eicosanoid metabolism. None of the kidney allograft recipients included in this study were given nonsteroidal antiinflammatory agents for at least 8 to 10 days prior to blood collection. However, the majority of patients received cyclosporine A and methylprednisolone, 9 patients were given azathioprine in lieu of cyclosporine A, and most patients were on antihypertensive regimen, including β-blockers, calcium channel blockers and vasodilators. Among these drugs, in particular calcium channel blockers have been shown to inhibit platelet thromboxane production. The inhibitory potential of these agents was present upon platelet stimulation by low-dose collagen (e.g., 0.6 μg/ml) but not at collagen concentrations of greater than or equal to 2.5 μg/ml (33). Using high concentrations of thrombin and collagen, no correlation was found in this study between antihypertensive medication and impaired platelet thromboxane formation. For example, kidney transplant recipients with compensated renal failure had normal TXB_2 levels despite receiving higher doses of antihypertensive agents than patients with allograft rejection.

Methylprednisolone may also inhibit platelet arachidonate metabolism due to inactivation of phospholipase A_2 (34), thereby leading to decreased formation of thromboxane upon platelet stimulation in vitro. However, it is unlikely that the impaired platelet thromboxane synthesis observed in this study was caused by methylprednisolone for the following reasons. First, patients with functioning kidney transplants and recipients of renal allografts with compensated renal failure were given the same therapeutic regimen as those with acute or chronic rejection, although lower doses of corticosteroids were administered to patients without evidence of transplant rejection. Second, even the higher doses of methylprednisolone given to patients with acute (1 g daily for 3 days) and chronic rejection (0.4 to 0.6 mg/kg daily) are probably too small to inhibit phospholipase A_2 in vivo (34, 35).

Cyclosporine A may also affect platelet function and metabolism, however, little is known about effects of cyclosporine A on hemostasis. Vanrenterghem et al. (36) reported that ADP-induced platelet aggregation was significantly enhanced in renal transplant recipients who were treated with cyclosporine A compared to those who received azathioprine. However, no difference in plasma TXB_2 levels upon stimulation of anticoagulated whole blood with collagen was found between the cyclosporine A and azathioprine group (36). Thus, it is unlikely that the impaired platelet TXB_2 synthesis reported here is caused by the administration of cyclosporine A, especially since the therapeutic regimen of this study did not differ between different patient groups.

Finally, it has to be considered that the renal failure itself that was present in patients with acute or chronic allograft rejection may be responsible for the impaired platelet thromboxane synthesis observed upon platelet stimulation by thrombin or collagen in vitro. Thus, Remuzzi et al. (37) reported that a reduced platelet TXB_2 production due to a functional cyclo-oxygenase defect occurs in chronic renal failure. However, it is unlikely that a uremia-associated dysfunction of platelet arachidonate metabolism was responsible for the reduced platelet thromboxane formation in patients with acute or chronic allograft rejection since recipients of kidney transplants with compensated renal failure but without evidence of rejection displayed a normal platelet thromboxane synthesis capacity in vitro.

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