Circulating platelet-derived microparticles with procoagulant activity may be a potential cause of thrombosis in uremic patients

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Background. Clinical experience indicates that bleeding and thrombotic tendencies co-exist in uremic patients. Numerous studies have shown that platelet functional defects contribute to the bleeding tendency in uremic patients. In contrast, there are no solid studies clarifying the pathogenesis of the prothrombotic state in uremic patients. Platelet-derived microparticles (PMPs), which are small vesicles with procoagulant activity released from activated platelets, are thought to be involved in clinical thrombogenesis. This study addressed the question of why uremic patients are thrombophilic even though they have a bleeding tendency, focusing on the clinical significance of PMPs.

Methods. The subjects were pre-dialyzed patients, patients under hemodialysis (HD) or continuous ambulatory peritoneal dialysis (CAPD) therapy, and age-matched healthy controls. Analyses of PMPs were performed using a flow cytometer. Annexin V was used to probe procoagulant activity of PMPs. The impacts of the HD procedure, arteriovenous (AV) fistula, and recombinant human erythropoietin (rHuEPO) treatment on the release of PMPs were additionally assessed.

Results. Major results are: (1) PMP counts were significantly greater in each uremic group than in controls. The PMP counts were not different among three types of uremic groups; (2) PMP counts were significantly higher in uremic patients with thrombotic events than in those without thrombotic events; and (3) the HD procedure and existence of AV fistula did not affect PMP counts, but rHuEPO treatment possibly enhanced the PMP release in these patients.

Conclusions. Elevated PMP counts may trigger thrombosis in uremic patients. The primary cause of PMP elevation in uremia was not clarified in this study.

Key words: thrombosis, flow cytometric analysis, procoagulant activity, annexin V, bleeding tendency, platelet function, acute arterial occlusion.

Received for publication October 10, 2001 and in revised form June 10, 2002
Accepted for publication June 12, 2002
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Uremic patients seem to have two opposite aspects in hemostatic status, which are bleeding and thrombotic tendencies. A number of experimental studies have revealed platelet functional defects in uremia, such as diminished adherence to vascular subendothelium [1, 2], decreased expressions of platelet membrane glycoprotein GIb [3] and dysfunction of glycoprotein IIb/IIIa [4]. The bleeding tendency in such patients, therefore, may be due to the functional impairments of platelets and their vulnerability in physiological hemostatic functions. Platelets release small particles called platelet-derived microparticles (PMPs) after they are activated by some chemical stimuli or high shear stress [13–19]. Elevated counts of circulating PMPs have been reported in association with thrombotic disorders, such as cerebrovascular accidents [20], unstable angina [21], and acute myocardial infarction [22]. In addition, PMPs that adhered to vascular endothelium and leukocytes...
activate such cells and transport their chemical mediators to those cells, potentially leading to the development of thrombosis and atherosclerosis [23–26]. We hypothesized that an enhancement of PMP generation—if it exists—may participate in thrombogenesis and compensation for the bleeding tendency in uremia.

This study used flow cytometry [13, 14, 18] to evaluate the PMPs in healthy subjects and three types of uremic patients: those who were pre-dialyzed, on hemodialysis (HD), and on continuous ambulatory peritoneal dialysis (CAPD) therapy. The procoagulant activity of PMPs were especially evaluated by annexin V, a placental protein with a high affinity and specificity for aminophospholipids, which has been shown to be an appropriate probe for detecting such specific activity of PMPs [27]. In addition, the concomitant influences of the HD procedure, existence of internal arteriovenous (AV) fistula, and chronic recombinant human erythropoietin (rHuEPO) therapy on PMP formation were studied.

**METHODS**

**Reagents and antibodies**

Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (mAb) to GPIb, NNKY5-5 [28], was a generous gift from Dr. Shosaku Nomura, Kansai Medical School (Osaka, Japan). Phycoerythrin (PE)-conjugated annexin V was purchased from Pharmingen (San Diego, CA, USA). Isotype-matched mAbs of irrelevant specificity (FITC-labeled mouse IgG) were purchased from Becton Dickenson (San Diego, CA, USA). Calibrated latex beads (SPHERO™ Fluorescent Particles) were purchased from Pharmingen.

**Subjects**

Twenty-nine healthy subjects and 46 HD patients, 23 CAPD patients, and 20 pre-dialyzed uremic patients were studied. Diabetic patients were not included to estimate the simple influence of uremia on PMP generation [29]. Patients who had been receiving anti-platelet drugs were excluded. The subjects' profiles are shown in Table 1. Each patient population was matched for age with the healthy population (mean age, 52.3 ± 5.19 years). A majority of the dialysis patients had been treated with recombinant human erythropoietin (rHuEPO), which was intravenously injected three times a week in the HD patients, and subcutaneously administered once a week in the CAPD and pre-dialyzed patients. The HD patients were dialyzed using columns of different types of membrane materials: polysulfone, cellulose-triacetate, or polymethylmethacrylate. Heparin, as an anticoagulant agent, was continuously injected at a dose of 600 to 800 units per hour during HD treatment.

**Blood collection, preparation of washed platelets, and cell labeling**

Blood samples were obtained before the start of treatment at the first HD of the week in the HD patients, or when patients visited the hospital for their regular medical examination in the CAPD or pre-dialyzed patients. Two milliliters of venous blood were placed into a tube containing 0.2 mL of an EDTA-ACD solution [ethylene-diaminetetraacetic acid (EDTA)–2 Na, 1.0 g, trisodium citrate 2.2 g, citric acid 807 mg, dextrose 2.2 g in 100 mL of distilled water] and gently inverted twice. Platelet-rich plasma (PRP) was prepared by centrifugation at $180 \times g$ for 20 minutes at room temperature. An aliquot (15 μL) of the PRP that had been diluted up to 50 μL with 0.1% EDTA-saline was then incubated with 2 μL of FITC-GPIb and PE-annexin V for 30 minutes at room temperature in the dark. Nonspecific FITC-labeled mAb was used for the negative controls.

**Flow cytometry**

Samples were analyzed using a flow cytometer (FACS Calibur; Becton Dickinson). The light scatter and fluorescence channels were set at a logarithmic gain. The gating methods for PMPs are shown in Figure 1. Only cells and particles positive for GPIb were gated to distinguish platelets and PMPs from electronic noise as follows: The cells labeled with monoclonal antibody (mAb) to FITC-GPIb (R1) were gated on the side scatter (SSC) dot plot versus fluorescence-1 (FL-1) profile, which was determined by comparing with the dot plot profile of the samples treated with non-labeled mAb to GPIb (Fig. 1A). Regions corresponding to PMPs (R3) were then discriminated from the platelet islet contaminated (R2) by reviewing the cells in the gate R1 on SSC dot plot versus the forward scatter (FSC) dot plot profile (Fig. 1B). A known number of fluorescent latex beads (30,000) were added to each sample tube before analysis [30], and the time of analysis was defined by counting a pre-determined bead number (300 counts). The percentage of PMPs positive for PE-annexin V in the cells of the gate R3 was evaluated on histograms (Fig. 1C) constructed by the analysis software, CellQuest (Beckton Dickinson). The bar was determined as the specific positive region of PE-annexin V. The particles in the R3 were designated as crude PMPs. The absolute number of PMPs stained by annexin V was calculated by the following formula:

$$\text{Counts of specific PMPs} = (\text{crude PMP counts in the gate R3}) \times (\text{percentage of the stained cells in the gate R3}) \times 0.01.$$  

These specific PMPs with procoagulant activity were
Fig. 2. Comparison of PMP counts between three types of uremic patients (HD, CAPD, and pre-dialyzed uremic patients) and controls. Each patient population was matched for age with the controls. The significant differences were obtained between each uremic group and the controls. The PMP counts were not different among three types of uremic patients. Statistically significant difference is marked. **P < 0.01 vs. control.

simply designated as “PMPs” in the following description unless otherwise stated. The study was approved by the institutional review board of the hospital and was carried out according to the principles of the Declaration of Helsinki. Informed consent was obtained from all of the subjects included in this study.

Statistics

Data were expressed as mean ± SD unless otherwise stated. The differences between mean values were evaluated by the two-tailed Student t test for comparing two groups and by the analysis of variance (ANOVA) followed by Bonferroni-Dunn for comparing more than three groups. P < 0.05 was considered statistically significant.

RESULTS

PMP counts in uremic patients

The PMP counts were studied in 89 non-DM uremic patients and 29 age-matched controls. The PMP counts were significantly higher in each uremic group (pre-dialyzed, 142.3 ± 49.5; HD, 195.3 ± 103.1; CAPD, 161.0 ± 47.9) than in the controls (104.9 ± 46.3). The PMP counts were not different among three types of uremic groups (Fig. 2). The PMP counts did not correlate with platelet

from the platelet islet shown as R2. (C) The percentage of PMPs positive for PE-annexin V in the cells of R3 was evaluated with a histogram analysis. The bar was determined as the specific positive region of PE-annexin V by comparison with the histograms of the samples treated with non-labeled Ab to annexin V.
counts and serum levels of albumin, urea nitrogen, and creatinine in any uremic group.

**PMP counts in uremic patients with and without thrombotic events**

A clinical link between PMP counts and thrombotic events were examined in all uremic subjects. The thrombotic events were retrospectively investigated. Overall, the number of patients who had suffered thrombotic events for the last five years was 15 out of 89 patients (17.4%; 9 of 46 HD patients, 3 of 23 CAPD patients, and 3 of 20 pre-dialized patients). The PMP counts in the patients with thrombotic events (248.5 ± 91.1) were significantly greater than in the patients without such events (159.5 ± 74.5; Fig. 3).

**Effects of rHuEPO on the PMP count in dialysis patients**

The PMP counts were compared between the HD patients receiving chronic rHuEPO treatment (recipients; 220.0 ± 106.5, N = 30) and those receiving no such treatment (non-recipients; 148.9 ± 80.5, N = 16). The PMP counts of the EPO recipients were significantly higher than those of the EPO non-recipients (Fig. 4). Both groups were matched for age (59.2 ± 12.8 vs. 55.4 ± 11.4 years old). The mean dose of rHuEPO used was 2380 ± 921 units a week. Moreover, the significant difference in the PMP counts between the EPO recipients (178.7 ± 50.3, N = 13) and non-recipients (138.0 ± 34.3, N = 10) was found in the subgroup of CAPD (Fig. 4). This comparative study was not done in the subgroup of pre-dialized patients because of the small number of rHuEPO recipients, as shown in Table 1. No significant correlation was obtained between the PMP counts and rHuEPO dose administered in each dialysis subgroup. Regardless of rHuEPO use, the PMP counts were significantly higher in the EPO non-recipients of HD (N = 30; 148.9 ± 80.5), CAPD (N = 10, 138.0 ± 34.3), and pre-dialized (N = 18; 133.1 ± 42.3) patients than in the controls. In addition, PMP counts were significantly higher (P = 0.0260) even in the uremic patients without thrombotic events receiving no rHuEPO treatment (N = 38, 131.6 ± 48.7) than in the controls (N = 29, 104.9 ± 46.3).

**Influence of HD therapy on PMP generation**

**Changes in PMP counts during HD treatment.** The time courses in PMP counts were examined in 14 HD patients before and 10 minutes, 30 minutes, and 2, 3, and 4 hours after starting the HD treatment. Blood samples were collected from the outlet port of the HD circuit tube before the dialysis column. The counts of PMP did not significantly change during HD treatment. The data are shown in Figure 5A.

**Changes in PMP count before and after dialysis columns.** The changes of PMP counts before and after dialysis columns were specially examined to better understand the influence of blood contact with membrane materials on PMP counts at two hours after the start of HD. No significant changes in PMP counts were observed before and after the columns (before, 188.7 ± 22.3; after, 193.1 ± 41.5; Fig. 5B). The HD membrane materials used were polysulfone (N = 10) and cellulose triacetate (N = 4). There were no significant differences.
Table 1. Subject profiles

<table>
<thead>
<tr>
<th></th>
<th>Pre-dialyzed patients</th>
<th>HD patients</th>
<th>CAPD patients</th>
<th>Controls</th>
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<tbody>
<tr>
<td>Number</td>
<td>20</td>
<td>46</td>
<td>23</td>
<td>29</td>
</tr>
<tr>
<td>Age years</td>
<td>54.8 ± 10.1</td>
<td>57.9 ± 12.3</td>
<td>50.8 ± 8.31</td>
<td>52.3 ± 5.19</td>
</tr>
<tr>
<td>Dialysis duration years</td>
<td>—</td>
<td>12.3 ± 6.7</td>
<td>5.8 ± 3.2*</td>
<td>—</td>
</tr>
<tr>
<td>rHuEPO recipients/non-recipients</td>
<td>2/18</td>
<td>30/16</td>
<td>13/10</td>
<td>—</td>
</tr>
<tr>
<td>Hematocrit %</td>
<td>31.9 ± 3.8</td>
<td>32.1 ± 7.2</td>
<td>31.8 ± 6.6</td>
<td>44.1 ± 5.3</td>
</tr>
<tr>
<td>Platelet counts × 10^9/L</td>
<td>225.3 ± 60.1</td>
<td>196.2 ± 75.4</td>
<td>214.8 ± 61.0</td>
<td>232.6 ± 51.3</td>
</tr>
<tr>
<td>Serum albumin g/dL</td>
<td>3.5 ± 0.73</td>
<td>3.4 ± 1.8</td>
<td>3.1 ± 1.7</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>Serum urea nitrogen mg/dL</td>
<td>53.4 ± 8.89*</td>
<td>80.2 ± 11.1</td>
<td>56.6 ± 10.2*</td>
<td>17.3 ± 1.81</td>
</tr>
<tr>
<td>Serum creatinine mg/dL</td>
<td>6.2 ± 4.8*</td>
<td>10.3 ± 2.8</td>
<td>11.1 ± 3.8</td>
<td>1.1 ± 0.1</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. Abbreviations are: HD, hemodialysis; CAPD, continuous ambulatory peritoneal dialysis; rHuEPO, recombinant human erythropoietin.

*P < 0.05 versus HD patients

Table 2. Effects of internal arteriovenous (AV) fistula on platelet-derived microparticles (PMP) counts

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AV fistula side</th>
<th>Non-AV fistula side</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMP counts (N = 10)</td>
<td>213.8 ± 80.6</td>
<td>205.5 ± 88.56</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD.

DISCUSSION

The major findings of this study are (1) the PMP counts were significantly elevated in a resting state in uremic patients such as pre-dialyzed, HD, and CAPD patients, compared with the age-matched healthy subjects, the PMP counts were not significantly different among three types of uremic groups; (2) the HD procedure and existence of internal AV fistula did not affect the PMP counts, but rHuEPO treatment was possibly involved in the further increase of PMP release; and (3) the PMP counts in the uremic patients who had experienced thrombotic events were much higher than those in the patients without such events. This result indicates that an extreme increase of PMP counts may be responsible for a high incidence of thrombosis in uremic patients. However, the primary cause of PMP elevation in uremia was not clarified in this study.

Our data showed that the counts of PMP enriched with procoagulant activity are elevated in non-diabetic uremic patients, as compared with the controls, regardless of uremic stages or dialysis modality. Nomura et al previously showed that PMP counts were increased in uremic patients [31, 32], which was likely to support our current results. However, their data were deficient in several points. The profiles of their uremic subjects did not include the stage of uremia, modality of dialysis, ages, and complications (for example, diabetes mellitus). Moreover, their data were based upon so-called crude PMPs that might include a lot of debris and non-func-
tioning PMPs, as we described in our Methods section. In contrast, we clarified the patients’ characteristics and identified vesicles with procoagulant activity as PMPs. We believe that our current report is comprehensive and elaborates on the clinical significance of PMPs in uremic patients.

Our study shows the biological relevance of elevated PMP counts in uremia. PMPs provide competent surfaces for the assembly of coagulation factors and may interact with components of the vessel walls, contributing to thrombus and atheroma formation [13, 14, 23–26]. Although the clinical significance of circulating PMPs in the pathogenesis of thrombotic diseases is not sufficiently acknowledged yet, recent clinical evidence indicates that an increase of circulating PMP counts is involved in the incidence of ischemic heart diseases and cerebral strokes [20–22]. Our data show a positive link between PMP counts and thrombotic events in uremic patients, suggesting that an increase of PMP release in the circulatory system underlies the high incidence of thrombotic diseases in uremic patients [7, 8]. Alternatively, PMP could be a new marker or predictor of clinical thrombosis in uremia.

We expected that changes of PMP release might contribute to the pathogenesis of intra-dialyzer coagulation and vascular access thrombosis in HD patients. Several studies suggested that mechanical stress, particularly high shear stress by a cone-plate viscounter, stimulates the shedding of PMPs from platelets [15, 18]. Thus, we speculated that similar phenomena would occur in the HD procedure that may impose repetitive mechanical stress on platelets during extracorporeal circulation. However, the data proved opposite to this speculation. AV fistulas also were expected to stimulate platelets and to facilitate the data proved opposite to this speculation. AV fistulas on platelets during extracorporeal circulation. However, procedure that may impose repetitive mechanical stress elaborates on the clinical significance of PMPs in uremic levels of fluid shear stress that may occur in atherosclerotic arteries [15–19]. It would be natural to think that such physiological stress is easy to induce in uremic patients who commonly have hypertension and systemic arterio-atherosclerosis [8]. Alternatively, unknown uremic substances that are accumulated despite or by repetitive dialysis treatment may additionally impact the process of PMP formation from activated platelets.

In conclusion, the counts of circulating PMPs were elevated in three types of uremic patients compared with the healthy controls. The primary cause of PMP elevation was not clarified, but rHuEPO treatment could be involved as an enhancer of PMP release. In a uremic setting overlapped by unfavorable factors such as uremic toxins, pro-inflammatory cytokines, hypertension, and systemic atherosclerosis, an increase of PMPs may be a potential trigger of acute thrombotic accidents, even under circumstances where the hemostatic functions of platelets are impaired.

ACKNOWLEDGMENTS

We thank the research staff at Otsuka Pharmaceutical Co., Ltd. (Tokushima, Japan) for their excellent technical support.

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cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. J Biol Chem 263:18205–18212, 1988


