Platelet Adenine Nucleotides in Patients with Primary Glomerular Disease

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Department of Internal Medicine, Nakano General Hospital, Tokyo 164, *the Second Department of Internal Medicine, Tokyo Medical and Dental University, Tokyo 113 and †Division of Cardiovascular Research, Tokyo Metropolitan Institute of Medical Science, Tokyo 113

TOMURA, S., CHIDA, Y., IDA, T., TANOUÉ, K., MOTOMIYA, T. and YAMAZAKI, H. Platelet Adenine Nucleotides in Patients with Primary Glomerular Disease. Tohoku J. exp. Med., 1988, 156 (3), 221-227 — Total platelet adenosine diphosphate (ADP) and adenosine triphosphate (ATP) contents and amounts of ADP and ATP released from platelets by 3 μg/ml of collagen were studied in 20 patients with primary glomerular disease (PGD) to examine the metabolism of these platelet substances in the disease. ADP and ATP were measured by Holmsen’s firefly luciferase method. The patients had significantly lower total platelet ATP compared with controls and total platelet ATP was significantly lower in nephrotic patients than in patients whose serum albumin levels were normal. Releasable platelet ADP and ATP were both significantly decreased in patients. Releasable ADP : total ADP ratio and releasable ATP : total ATP ratio were both significantly lower in patients’ platelets than in normal platelets. There was no significant difference in platelet counts between patients and controls. Platelet aggregation induced by ADP and adrenaline were significantly higher in patients compared with controls. We conclude that total ATP content and the amount of ADP and ATP released by collagen are decreased in PGD patients’ platelets.

Recent evidence suggesting that platelet activation occurs in patients with glomerulonephritis (GN) is based on the following observations: increased platelet aggregation (Remuzzi et al. 1979; Tomura et al. 1982), accelerated platelet turnover (George et al. 1974), the release of platelet-specific proteins such as β-thromboglobulin (β-TG) and platelet factor 4 (PF-4) into the circulation (Parbtani et al. 1980a; Tomura et al. 1982), and the presence of platelet aggregates or platelet membrane antigens within the glomeruli (Duffy et al. 1970; Miller et al. 1980; Duffus et al. 1982).
The possible factors responsible for platelet activation in patients with GN are hypoalbuminemia (Yoshida and Aoki 1978; Remuzzi et al. 1979), hyperlipidemia (Colman 1978) and the interaction of platelets with immune complexes (Kasai et al. 1981) and damaged renal microvasculature (Duffy et al. 1970; Clark et al. 1975). Hypoalbuminemia is accompanied by platelet hyperaggregability via increased production of thromboxane A₂ (Yoshida and Aoki 1978; Stuart et al. 1980).

When platelets are stimulated, they release β-TG and PF-4 from the α granules and adenine nucleotides and serotonin from the dense bodies. It has been reported that plasma levels of β-TG and PF-4 are increased in patients with GN and nephrotic syndrome and the platelet contents of serotonin are reduced in patients with certain forms of GN (Parbtani et al. 1980a, b). However, no data have been published as to adenine nucleotide metabolism of platelets in GN. In this study, we measured total platelet contents of adenosine diphosphate (ADP) and adenosine triphosphate (ATP) and the amounts of ADP and ATP released from platelets by collagen in patients with primary glomerular disease (PGD) to examine the metabolism of these platelet substances in the disease.

**Patients and Methods**

Twenty patients with primary glomerular disease (PGD) (14 males and 6 females) including nephrotic syndrome and 10 normal volunteers were studied. The diagnosis of PGD was based on clinical evaluations and the histologic examination of renal biopsy materials by light and immunofluorescent microscopy. Histologically, there were three cases of minor glomerular abnormalities, two of focal glomerulonephritis (GN), six of mesangial proliferative GN, three of membranous GN, three of membranoproliferative GN and three of minimal change nephrotic syndrome. Nephrotic syndrome was present in four patients (two minimal change nephrotic syndrome, one membranous GN and one membranoproliferative GN). Nephrotic syndrome was defined as proteinuria greater than 3.5 g/24 hr with hypoalbuminemia less than 3.0 g/100 ml on consecutive determinations. Serum creatinine levels ranged from 0.7 to 1.7 mg/100 ml, serum urea nitrogen levels from 11 to 36 mg/100 ml and creatinine clearance from 44 to 180 l/24 hr.

Patients with end-stage renal failure were excluded, since advancing uremia may impair platelet function. None of the subjects had ingested any antiplatelet drugs such as aspirin, ticlopidine or indomethacin for a period of 10 days before the evaluation.

Nine parts of venous blood were collected utilizing a polypropylene disposable syringe containing one part of 3.8% sodium citrate solution. Platelet-rich plasma (PRP) was prepared from citrated blood by centrifugation at 170 G for 10 min at room temperature. The platelet count in PRP was adjusted to 30-35 × 10⁴/μl.

Platelet aggregation induced by 3 μM adenosine 5’diphoshate (ADP), 1 μg/ml of adrenaline and 3 μg/ml of collagen was measured by a platelet aggregation meter (Model DP-247 E, Sienco Co., Morrison, Co, USA). The optical density deflection at 5 min after the addition of reagents (5 min platelet aggregation) was taken as the value of platelet aggregation.

For analysis of total ADP and ATP contents in platelets, the following two procedures were done: (1) One part of PRP was mixed well at 0°C with one part of ethylenediaminetetraacetate (EDTA)-ethanol which consisted of nine vol of 99.5% ethanol and one vol of 0.1 M EDTA. The mixture was frozen at −80°C, thawed, and centrifuged at
10,000 G for 20 min at 0°C. The supernatant was stored at -80°C until assay. (2) (David and Herion 1972) 450 μl of PRP was added to 50 μl of EDTA (134 mM) and lysed with 50 μl of Triton X-100 for 5 min at 37°C. Another 450 μl of PRP was processed in the same manner, and the two samples were mixed together. The lysed PRP was centrifuged at 12,500 g for 15 min at 0°C and an extract of the supernatant containing ADP and ATP was prepared by mixing well with an equivalent volume of aqueous ethanol. The mixture was kept for 10 min at 0°C and centrifuged at 10,000 g for 20 min at 0°C. The supernatant was stored at -80°C until assay.

To determine the amount of ADP and ATP released during aggregation of platelets with collagen, 450 μl of PRP was stirred in a platelet aggregometer (Model DP-247 E) with 50 μl of collagen (Collagenreagent Horm, Hormon-Chemie, Munich, FRG). Collagen was used at a final concentration of 3 μg/ml. The intensity of aggregation was followed on a pen recorder for 5 min, after which 50 μl of EDTA (134 mM) was added. After the same procedure was done on another 450 μl of PRP, the two samples were mixed together and centrifuged at 12,500 g for 15 min at 0°C. Thereafter, the supernatant containing the released ADP and ATP was prepared in the same manner as previously described (2).

The concentrations of ADP and ATP in the supernatant were measured by the firefly luciferase method of Holmsen and co-workers (Holmsen et al. 1972).

Statistical analysis was performed using the t-test. Deviations were expressed as ±s.d.

RESULTS

There was a significant correlation between total platelet contents of ADP and ATP determined by methods (1) and (2) (ADP: r = 0.525, p < 0.01; ATP: r = 0.777, p < 0.01).

Total platelet content of ATP in patients as determined by methods (1) and (2) were 7.10 ± 0.20 and 7.07 ± 0.21 μmole/10¹¹ platelets, respectively, both significantly lower than the corresponding values in normal controls (7.87 ± 0.36 and 7.97 ± 0.41, respectively). Total platelet ATP content was significantly lower in nephrotic patients than in patients whose serum albumin levels were more than 4.0 g/100 ml. Patients had lower total platelet ADP contents compared with normal controls and total platelet ADP contents were lower in nephrotics than in non-nephrotics, although the differences were not significant. There was no significant difference in platelet counts between patients and controls and between nephrotics and non-nephrotics.

Releasable amounts of platelet ADP and ATP were significantly decreased in patients (1.00 ± 0.46, 1.02 ± 0.44 μmole/10¹¹ platelets, respectively) compared with normal controls (1.52 ± 0.42, 1.46 ± 0.36). The ratio of releasable ADP: total ADP and of releasable ATP: total ATP were both significantly lower in patients' platelets (0.36 ± 0.10, 0.14 ± 0.06, respectively) than in normal platelets (0.45 ± 0.10, 0.19 ± 0.03) (The results are summarized in Tables 1 and 2).

The intensities of platelet aggregation induced by 3 μM ADP and 1 μg/ml adrenaline were 64.2 ± 23.1 and 73.8 ± 12.3%, respectively, in patients. These values were both significantly higher (p < 0.01, p < 0.01, respectively) than those of controls (44.3 ± 27.1, 49.0 ± 29.3, respectively) (Table 3).
### TABLE 1. Comparison of platelet ADP and ATP in controls and PGD patients

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Patients</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count ( (\times 10^{11}/\mu l) )</td>
<td>20.9 ± 3.8</td>
<td>22.8 ± 6.7</td>
<td>n.s.</td>
</tr>
<tr>
<td>Total platelet ADP content</td>
<td>3.59 ± 0.72(1)</td>
<td>3.34 ± 0.79(1)</td>
<td>n.s.</td>
</tr>
<tr>
<td></td>
<td>3.72 ± 0.64(2)</td>
<td>3.50 ± 0.64(2)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Total platelet ATP content</td>
<td>7.87 ± 0.36(1)</td>
<td>7.10 ± 0.20(1)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td></td>
<td>7.97 ± 0.41(2)</td>
<td>7.07 ± 0.21(2)</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Total ATP : ADP ratio</td>
<td>2.22 ± 0.49(1)</td>
<td>2.21 ± 0.44(1)</td>
<td>n.s.</td>
</tr>
<tr>
<td>Releasable amount of ADP</td>
<td>1.52 ± 0.42</td>
<td>1.00 ± 0.46</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>Releasable amount of ATP</td>
<td>1.46 ± 0.36</td>
<td>1.02 ± 0.44</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Releasable ADP : Total ADP ratio</td>
<td>0.45 ± 0.10</td>
<td>0.36 ± 0.10</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>Releasable ATP : Total ATP ratio</td>
<td>0.19 ± 0.03</td>
<td>0.14 ± 0.06</td>
<td>&lt; 0.05</td>
</tr>
</tbody>
</table>

Values are means ± s.d. The amount of ADP and ATP is expressed as µmole/10^{11} platelets. (1) and (2) denote the values measured by methods (1) and (2), respectively.

### TABLE 2. Difference in total platelet ADP and ATP content in PGD patients' platelets, depending on serum albumin concentration or urine protein excretion

<table>
<thead>
<tr>
<th>Serum albumin (g/100 ml)</th>
<th>Urine protein (g/24 hr)</th>
<th>Platelet count ( (\times 10^{11}/\mu l) )</th>
<th>Total platelet ADP content</th>
<th>Total platelet ATP content</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alb ≤ 3.0</td>
<td>9.6 ± 1.9</td>
<td>27.5 ± 5.7</td>
<td>2.95 ± 1.01</td>
<td>6.79 ± 0.67*</td>
<td>4</td>
</tr>
<tr>
<td>3.0 &lt; Alb &lt; 4.0</td>
<td>3.8 ± 2.9</td>
<td>23.0 ± 6.2</td>
<td>3.30 ± 0.80</td>
<td>6.93 ± 0.82</td>
<td>10</td>
</tr>
<tr>
<td>4.0 ≤ Alb</td>
<td></td>
<td></td>
<td>3.67 ± 0.60</td>
<td>7.64 ± 0.85</td>
<td>6</td>
</tr>
</tbody>
</table>

ADP and ATP concentrations (µmole/10^{11} platelets) were measured by method (1).

Values are means ± s.d.

Single asterisk indicates \( p < 0.05 \) compared with patients whose serum albumin concentrations are equal to or more than 4.10 g/100 ml.

### TABLE 3. Platelet aggregation in controls and PGD patients

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Patients</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 min platelet aggregation (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 µM ADP</td>
<td>44.3 ± 27.1</td>
<td>64.2 ± 23.1</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>1 µg/ml Adrenaline</td>
<td>49.0 ± 29.3</td>
<td>73.8 ± 12.3</td>
<td>&lt; 0.01</td>
</tr>
<tr>
<td>3 µg/ml Collagen</td>
<td>57.8 ± 25.7</td>
<td>69.6 ± 12.4</td>
<td>n.s.</td>
</tr>
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</table>

Values are means ± s.d.
DISCUSSION

Previous studies have demonstrated that there are two pools of adenine nucleotides within platelets (Holmsen et al. 1969; Holmsen and Day 1971; Holmsen 1985). ADP and ATP stored in the dense bodies constitute a non-metabolic (storage) pool of nucleotides and account for about two thirds of total platelet adenine nucleotides. In response to various stimuli, the storage pool of ADP and ATP, serotonin and calcium are released from platelets. Another pool, the metabolic pool, participates in metabolism. The metabolic pool is present in the cytoplasm, membranes and mitochondria of platelets, and most of this pool of ADP and ATP is retained within platelets during the release reaction.

The present study showed that platelets from patients with PGD presented a significant decrease in the amount of ADP and ATP released after stimulation with collagen compared with normal platelets. Furthermore, releasable ADP : total ADP ratio and releasable ATP : total ATP ratio were both significantly lower in patients' platelets than in normal platelets. The decrease in releasable adenine nucleotides in patients' platelets seems to be due to the failure of releasing adenine nucleotides by collagen or due to diminished platelet stores of adenine nucleotides associated with the ongoing release.

The releasable ADP + ATP represented 26% (controls) and 19% (patients) of the total ADP + ATP content. These ratios are low in comparison to the amount of adenine nucleotides in the storage pool. It is, therefore, unlikely that 3 μg/ml of collagen used in this study can induce strong release of platelet adenine nucleotides. Antiinflammatory agents such as aspirin inhibits platelet release. Since none of the subjects had ingested any antiplatelet drugs for a period of 10 days before the evaluation, the effects of the drugs can be denied.

Patients' platelets showed significantly lower total ATP contents and a tendency of decrease in total ADP content. The decrease in total platelet ADP and ATP content was more marked in patients with nephrotic syndrome whose platelets seem to be activated. One explanation for the decrease in total platelet content of adenine nucleotides in patients is that, during platelet activation in vivo, the hemostatically reactive granule-rich platelets are selectively removed or patients' platelets are partially degranulated by release reactions.

Another explanation for the decrease in total ATP content in patients' platelets is that ATP has been used to provide the energy for the shape change, aggregation and release. The metabolic pool of ATP is converted to inosine monophosphate and hypoxanthine during the reactions and this consumption provides the energy for them (Holmsen and Day 1971; Holmsen 1985).

Although platelet adenine nucleotides were decreased in patients, patients' platelets were still hyperaggregable. It is likely the the extent of the decrease in platelet adenine nucleotides is too small to affect in vitro platelet aggregation in the patients and that the platelets are hyperaggregable since they are stimulated
by the following factors: enhanced production of thromboxane A2 in hypoalbuminemia, alteration in the lipid composition of the platelet membrane in hyperlipidemia, inhibition of the synthesis of prostacyclin in the vascular endothelium by increased β-TG release and platelet activation by endothelial damage or immune complexes.

Acknowledgments

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References


