

## Agonist-Induced Platelet Aggregation Does Not Correlate with the Varying Severity of Microangiopathy in Patients with Type 2 Diabetes Mellitus

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**Abstract:** We investigated agonist-induced platelet aggregation in 23 age- and sex-matched control subjects and in 74 patients with type 2 diabetes mellitus. Platelet aggregation was measured by a particle counting method using light scattering (LS) and by a conventional light transmission method (LT) using epinephrine (Epi) and adenosine-5'-diphosphate (ADP) as agonists. Agonist-induced platelet aggregations measured by LS or LT did not show significant differences between control subjects and patients with type 2 diabetes mellitus as a whole. In addition, agonist-induced platelet aggregations by LS or LT did not show significant differences between control subjects and three diabetic patients subgroups with the varying severity of retinopathy, nephropathy or neuropathy. We recently reported that platelet aggregation in the absence of agonists (spontaneous aggregation) correlates well with the varying severity of microangiopathy. The results of the present study also support that spontaneous platelet aggregation measured by particle counting method using light scattering is more discriminative than agonist-induced platelet aggregation in relation to diabetic microangiopathy.

**Key words:** Agonist-induced platelet aggregation, Diabetes mellitus, Microangiopathy, Particle counting method, Light scattering

### INTRODUCTION

Platelet hyperfunction has been implicated as a risk factor for both microvascular and macrovascular diseases<sup>1-4)</sup>. Among various platelet functions, platelet aggregation has been most extensively investigated. Concerning the role of platelet hyperaggregability in the severity of microangiopathy, there are many controversial reports<sup>5-9)</sup>. Platelet aggregation in most of these studies was carried out by the conventional light transmission method in the presence of

agonists<sup>10,11)</sup>. However, the light transmission method is quite insensitive to observe small aggregates of platelets<sup>12,13)</sup>. So, a new sensitive method to observe platelet aggregation has been in demand for long time. Ozaki *et al.* invented a very sensitive method to evaluate platelet aggregation by particle counting method using light scattering, which allows us to observe aggregates as few as two platelets<sup>14,16)</sup>. By the application of this method, we recently reported that platelet aggregation without agonists (spontaneous aggregation) well correlates with the varying severity of microangiopathy in patients with type 2 diabetes mellitus<sup>17)</sup>. In the

present study, we evaluated agonist-induced platelet aggregations in relation to the increasing severity of microangiopathy in patients with type 2 diabetes mellitus.

## SUBJECTS AND METHODS

*Subjects, diagnosis and classifications of diabetic complications:* The present study was performed according to the principles of the Declaration of Helsinki, and informed consent was obtained from the subjects before the study. The study design was approved by the Ethical Committee of Yamanashi Medical University. We recruited patients with type 2 diabetes mellitus in the order of their visits to the out-patient clinic of Yamanashi Medical University Hospital. All patients were diagnosed to have diabetes mellitus based on the World Health Organization criteria<sup>18)</sup>. Diabetic retinopathy was diagnosed by expert ophthalmologists at Yamanashi Medical University Hospital and classified into no retinopathy, simple and proliferative retinopathy according to the report by Fukuda<sup>19)</sup>.

Diabetic nephropathy was diagnosed by measuring urinary albumin excretion/day<sup>20)</sup>. Those with albuminuria less than 28 mg/day, those more than 28 mg/day and less than 280 mg/day and those more than 280 mg/day were classified as normoalbuminuria, microalbuminuria and overt albuminuria, respectively. However, those who satisfy the conditions reported by Yum *et al.* were excluded from the present study<sup>21)</sup>.

Diabetic neuropathy was diagnosed based on subjective symptoms, diminished or loss of deep tendon reflexes and classified by measuring vibratory threshold (VT) at the right internal malleolus using an SMV-5 vibrometer (Teknologue, Tokyo, Japan) as we previously reported<sup>22,23)</sup>. Those with VT values less than

$29 \times 10^{-2}$  gravity (G), those more than  $30 \times 10^{-2}$  G and less than  $99 \times 10^{-2}$  G and those more than  $100 \times 10^{-2}$  G were classified as no neuropathy, mild and severe neuropathy, respectively. Patients with neuropathy due to toxic, cancers or metabolic causes other than diabetes mellitus or patients who were taking medicines such as vitamins B1, B6, B12, E, prostanoids, aspirin, cilostazol, antidepressants or tranquilizers were excluded from the present study.

Finally we recruited 74 patients (46 males and 28 females with the mean age of  $55.2 \pm 1.6$  years) with type 2 diabetes mellitus. Twenty-four of them were on the diet regimen only, 30 were taking oral hypoglycemic agents and 20 were injecting insulin. As a normal control group, age- and sex-matched 23 healthy volunteers (12 males and 11 females) with the mean age of  $55.2 \pm 2.8$  years were also recruited. All the subjects in this study are identical with those of the previous report<sup>17)</sup>.

*Measurement of agonist-induced platelet aggregation:* Blood was obtained in the presence of 1/10 volume of 3.8 % citric acid after overnight fasting and platelet rich plasma (PRP) was prepared by centrifugation at  $150 \times g$  for 10 min at room temperature. We used an instrument which simultaneously measures platelet aggregation by particle counting method using light scattering (LS) and light transmission method (LT)<sup>24)</sup>. We used  $0.3 \mu\text{M}$  of epinephrine (Epi) and  $1 \mu\text{M}$  of adenosine-5'-diphosphate (ADP) as agonists.

*Statistical analysis:* All numerical variables are expressed as the mean  $\pm$  SEM. The values of platelet aggregation of control subjects and diabetic patients as a whole were compared by Kruskal-Wallis test, and nonparametric Bonferroni test was used for the comparison between the groups.

## RESULTS

Fig. 1 shows platelet aggregations measured by LT and LS in the presence of Epi or ADP in age- and sex-matched control subjects and in diabetic patients as a whole. Agonist-induced platelet aggregation by either methods did not show a significant difference between diabetic patients and control subjects.

Then, agonist-induced platelet aggregations measured by the both methods were compared between control subjects and three subgroups of the varying severity of microangiopathy. The four groups in each comparison (Tables 1-3) were age- and sex- matched except in case of neuropathy, in which there was a significant difference in age. The LT and LS values of platelet

aggregation by Epi or ADP in relation to retinopathy did not show significant differences between these groups by Kruskal-Wallis test (Table 1). Agonist-induced platelet aggregations in relation to diabetic nephropathy (Table 2) or neuropathy (Table 3) showed similar results as in diabetic retinopathy. Although agonists increased the values of platelet aggregation in each group, there were no significant differences between each group and were not discriminative in relation to the varying severity of microangiopathy<sup>(17)</sup> and data not shown).

## DISCUSSION

Although many investigators observed platelet hyperaggregability in diabetic patients

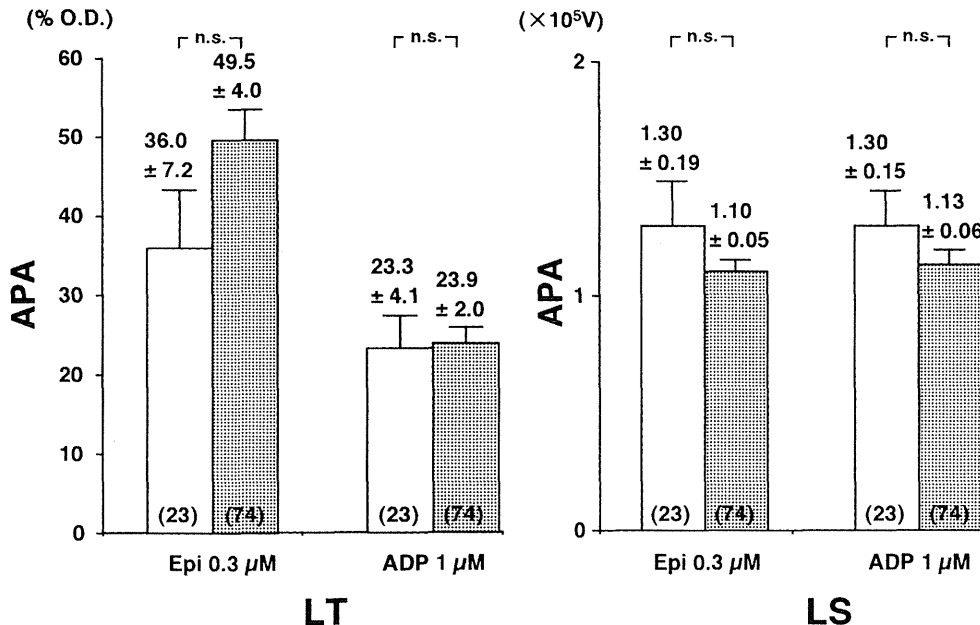


Fig. 1. Agonist-induced platelet aggregation (APA) in age- and sex-matched healthy control subjects and diabetic patients.

Open and dotted columns indicate healthy control subjects and diabetic patients, respectively. LT and LS indicate agonist-induced platelet aggregation measured by light transmission method and by particle counting method using light scattering, respectively. The concentrations of Epi and ADP are 0.3 μM and 1 μM, respectively. The numerical variables are expressed as the mean ± SEM. The numbers of subjects were indicated in parentheses.

Table 1. Agonist-Induced platelet aggregation in relation to retinopathy

|                       |            | DM          |                |                    |                           |
|-----------------------|------------|-------------|----------------|--------------------|---------------------------|
| Agonist               |            | Controls    | No Retinopathy | Simple Retinopathy | Proliferative Retinopathy |
| (n)                   |            | 23          | 27             | 29                 | 18                        |
| M/F                   |            | 12/11       | 22/11          | 12/6               | 12/11                     |
| Age (years)           |            | 55.0 ± 2.7  | 52.2 ± 2.2     | 59.3 ± 3.9         | 56.5 ± 2.4                |
| I.T                   | Epi 0.3 µM | 36.0 ± 7.2  | 41.3 ± 6.4     | 53.8 ± 7.7         | 57.8 ± 6.3                |
| (% O.D.)              | ADP 1 µM   | 23.3 ± 4.1  | 20.6 ± 2.6     | 21.3 ± 2.7         | 30.6 ± 4.4                |
| I.S                   | Epi 0.3 µM | 1.30 ± 0.19 | 1.03 ± 0.08    | 1.12 ± 0.08        | 1.17 ± 0.08               |
| (× 10 <sup>5</sup> V) | ADP 1 µM   | 1.30 ± 0.15 | 1.08 ± 0.08    | 1.25 ± 0.12        | 1.12 ± 0.11               |

The numerical variables are expressed as the mean ± SEM. The Kruskal-Wallis test was applied to compare values for the 4 groups as a whole, shown as the P values. n.s.: not significant.

Abbreviations: DM, diabetes mellitus; M/F, male to female ratio.

Table 2. Agonist-Induced platelet aggregation in relation to Nephropathy

|                       |            | DM          |                   |                   |                  |
|-----------------------|------------|-------------|-------------------|-------------------|------------------|
| Agonist               |            | Controls    | Normo-albuminuria | Micro-albuminuria | Over albuminuria |
| (n)                   |            | 23          | 27                | 29                | 18               |
| M/F                   |            | 12/11       | 15/12             | 21/8              | 10/8             |
| Age (years)           |            | 55.2 ± 2.8  | 56.3 ± 2.9        | 54.2 ± 2.5        | 55.2 ± 2.6       |
| I.T                   | Epi 0.3 µM | 36.0 ± 7.2  | 45.6 ± 6.8        | 52.3 ± 6.6        | 50.7 ± 7.4       |
| (% O.D.)              | ADP 1 µM   | 23.3 ± 4.1  | 24.2 ± 3.1        | 19.2 ± 1.7        | 30.9 ± 5.8       |
| I.S                   | Epi 0.3 µM | 1.30 ± 0.19 | 1.11 ± 0.08       | 1.09 ± 0.08       | 1.08 ± 0.09      |
| (× 10 <sup>5</sup> V) | ADP 1 µM   | 1.30 ± 0.15 | 1.23 ± 0.09       | 1.13 ± 0.09       | 1.00 ± 0.13      |

The numerical variables are expressed as the mean ± SEM. The Kruskal-Wallis test was applied to compare values for the 4 groups as a whole, shown as the P values. n.s.: not significant.

Abbreviations: DM, diabetes mellitus; M/F, male to female ratio.

Table 3. Agonist-Induced platelet aggregation in relation to Neuropathy

|                       |            | DM          |               |                 |                         |
|-----------------------|------------|-------------|---------------|-----------------|-------------------------|
| Agonist               |            | Controls    | No Neuropathy | Mild Neuropathy | Severe Neuropathy       |
| (n)                   |            | 23          | 25            | 20              | 29                      |
| M/F                   |            | 12/11       | 14/11         | 13/7            | 19/10                   |
| Age (years)           |            | 55.0 ± 2.7  | 46.7 ± 1.8    | 53.4 ± 3.3      | 63.9 ± 1.9* P < 0.00005 |
| I.T                   | Epi 0.3 µM | 36.0 ± 7.2  | 45.5 ± 7.5    | 46.1 ± 6.9      | 55.2 ± 6.3              |
| (% O.D.)              | ADP 1 µM   | 23.3 ± 4.1  | 22.4 ± 3.2    | 22.1 ± 2.9      | 26.4 ± 3.7              |
| I.S                   | Epi 0.3 µM | 1.30 ± 0.19 | 1.01 ± 0.07   | 1.11 ± 0.09     | 1.15 ± 0.08             |
| (× 10 <sup>5</sup> V) | ADP 1 µM   | 1.30 ± 0.15 | 1.18 ± 0.09   | 1.11 ± 0.09     | 1.11 ± 0.11             |

The numerical variables are expressed as the mean ± SEM. The Kruskal-Wallis test was applied to compare values for the 4 groups as a whole, shown as the P values. n.s.: not significant. The nonparametric

Bonferroni test was used to compare between each group. \*P < 0.01 versus no neuropathy.

Abbreviations: DM, diabetes mellitus; M/F, male to female ratio.

compared with normal control subjects<sup>25-28</sup>), there are many inconsistent reports on platelet hyperaggregability in relation to the severity of microangiopathy<sup>5,9</sup>). We speculate that these inconsistent reports may have resulted from the fact that these investigators employed insensitive light transmission method to observe platelet aggregation in the presence of agonists. It is also reported that platelets show different responses depending on agonists<sup>25,29</sup>). We recently demonstrated that spontaneous platelet aggregation measured by particle counting method using light scattering, which is developed by Ozaki *et al.*<sup>14</sup>), significantly correlates with the severity of diabetic microangiopathy, while the light transmission method did not show convincing results<sup>17</sup>). We think that our previous report<sup>17</sup>) is the first convincing data to support the postulation that platelet hyperfunction may contribute to complications in diabetic patients.

In this study, we did not observe any significant differences in agonist-induced platelet aggregations even by the particle counting method using light scattering.

Although other agonists might have brought different results, the results of the present study clearly demonstrate that spontaneous platelet aggregation measured by particle counting method is a more sensitive parameter than Epi- or ADP-induced platelet aggregation in relation to diabetic microangiopathy.

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