Correlation Between Albuminuria and Spontaneous Platelet Microaggregate Formation in Type 2 Diabetic Patients

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OBJECTIVE — Albuminuria in type 2 diabetic patients is a risk factor for cardiovascular disease. We investigated the correlation between albuminuria and spontaneous microaggregation of platelets (SMAP) formed under shear stress.

RESEARCH DESIGN AND METHODS — The study subjects were 401 type 2 diabetic individuals (252 with normoalbuminuria and 149 with albuminuria) who were examined for SMAP under conditions of shear stress only (no agonist stimulation) and the reversibility of platelet microaggregation after stimulation with 1 μmol/L ADP, measured by a laser light-cattering method. Active glycoprotein IIb/IIIa (GPIIb/IIIa) and P-selectin expression levels on platelets as an index of platelet activation were measured by whole-blood flow cytometry.

RESULTS — SMAP formation was noted in 53% of diabetic patients. All patients with SMAP showed an irreversible pattern of platelet microaggregates by a low dose of ADP. SMAP was observed in 75% of diabetic subjects with albuminuria and in 39% of those with normoalbuminuria. Multivariate logistic regression analysis identified urinary albumin excretion rate and brachial-ankle pulse-wave velocity as independent factors associated with SMAP. The degree of SMAP correlated with active GPIIb/IIIa (γ = 0.59, P < 0.001) and P-selectin (γ = 0.55, P < 0.001) expression levels. These early-activated platelet profiles were significantly inhibited in albuminuric patients with aspirin intake, although the effect was incomplete.

CONCLUSIONS — Our study demonstrated an independent association between albuminuria and early changes in activated platelet profiles of type 2 diabetic patients. Further follow-up and intervention studies are needed to establish whether the inhibition of SMAP affects the course of cardiovascular disease in type 2 diabetic patients.

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There is growing evidence that increased urinary albumin excretion rate (AER) in type 2 diabetic patients is both a predictor of progression to chronic renal failure and an independent risk factor for cardiovascular disease (1). The U.K. Prospective Diabetes Study (UKPDS) group demonstrated that type 2 diabetic patients with microalbuminuria or overt proteinuria have a two- to three-fold greater risk of cardiovascular death, compared with those patients with normoalbuminuria (2). Furthermore, our group and others recently reported that a reduction of AER in type 2 diabetic patients was associated with a decreased occurrence of cardiovascular complications (3,4). Thus, albuminuria is considered an important therapeutic target for cardiovascular protection. However, the pathophysiological factors underlying this cardio renal interaction remain unknown. Identifying such factors and developing tools to identify patients at higher risk of both conditions might allow the design of new therapeutic strategies to improve outcomes in type 2 diabetic patients.

Platelet activation and aggregation are fundamental processes in the development of atherosclerosis and thrombosis, both of which contribute to cardiovascular risk (5,6). Platelets aggregate when activated, to form microaggregates of only a few cells as the initial response to various stimuli but large, tighter platelet aggregates appear with prolonged stimulation (7). In diabetic patients, platelets tend to hyperaggregate (8), although previous platelet aggregation measurements by optical density (9) or impedance methods (10) generally reflected the formation of large platelet aggregates in response to exogenous stimulation with various agonists. These conventional methods do not measure platelet microaggregate formation, which occurs at the initial process of platelet activation. Recently, a particle-counting method based on laser light scattering was developed to effectively detect platelet microaggregate formation (11). This new method also measures spontaneous microaggregation of platelets (SMAP), which occurs with a stirring force only and has no requirement for exogenous agonist stimulation. Using this new method, Matsuno et al. (12) reported SMAP as the predominant platelet aggregation event in type 2 diabetic patients. However, this study did not address the association between SMAP and albuminuria.

We therefore investigated the association between changes in early-activated platelet profiles and increased albuminuria in type 2 diabetic patients. The incidence of SMAP was investigated in relation to albuminuria status. To further evaluate the active state of SMAP, we simultaneously measured the cell surface expression levels of two markers of platelet activation: active fibrinogen receptor glycoprotein IIb/IIIa (GPIIb/IIIa), an...
important player in platelet adhesion, and P-selectin (CD62p), a transmembrane protein present in the α- (or dense) granules.

**RESEARCH DESIGN AND METHODS** — The diabetic subjects were recruited from type 2 diabetic patients who regularly visited the outpatient clinic of the Department of Medicine, Shiga University of Medical Science, in 2006–2007. Patients were clinically diagnosed with type 2 diabetes in accordance with the criteria of the World Health Organization. Patients were excluded based on the following criteria: complicating cancer, liver disease, infectious disease, collagen disease, nondiabetic kidney disease confirmed by renal biopsy, intake of nonsteroidal anti-inflammatory drugs with the exception of aspirin. Eligible patients were informed of the study protocol in oral and written forms. A total of 401 patients were finally enrolled in this study. Thirty healthy volunteers were also enrolled as the healthy control group to confirm the results of our previous study (17 male and 13 female, control group to confirm the results of our study). Thirty healthy volunteers were recruited from type 2 diabetic patients who regularly visited the outpatient clinic of the Department of Medicine, Shiga University of Medical Science, in 2006–2007. Patients were clinically diagnosed with type 2 diabetes in accordance with the criteria of the World Health Organization. Patients were excluded based on the following criteria: complicating cancer, liver disease, infectious disease, collagen disease, nondiabetic kidney disease confirmed by renal biopsy, intake of nonsteroidal anti-inflammatory drugs with the exception of aspirin. Eligible patients were provided written informed consent.

**Detection of platelet microaggregation**

Platelet-rich plasma was obtained from blood collected into sodium citrate (14 μmol/l) by immediate centrifugation at 155g for 12 min at room temperature. The platelet-rich plasma was removed and centrifuged at 1,750g for 10 min at room temperature in the presence of prostaglandin I2 (20 μmol/l). The resultant platelets were washed with HEPES-Tyrode buffer and resuspended to 4 × 10⁸ cells/ml. In this platelet suspension, platelets did not form the microaggregates without stirring force or any exogenous agonists. Platelet microaggregation was determined by measuring the light-scattering intensity (13) on a PA-200 aggregometer (Kowa, Tokyo, Japan). SMAP was observed under low shear stress conditions at a stirring speed of 1,000 rpm (26 dyn/cm²) without stimulation with an exogenous agonist. This degree of low shear stress is considered to be the same degree of shear stress occurring in the arterial stream, suggesting that this system provides a condition that mimicked the state of platelets in the artery. The reversibility of platelet microaggregation in response to a low dose of ADP (1 μmol/l) was also investigated. The data were recorded as a two-dimensional graph showing the change in total light intensity over time, expressed as a cumulative summation at 10-s intervals of scattered light intensity (Ii) and the number of particles corresponding to that intensity (Ni) in terms of particle size (intensity) (ΣIiNi) (volts × counts per second). Particles with an intensity of 25–400 mV represented small aggregates (9–25 μm). The degree of SMAP was described by measurement of the area under the curve (AUC) of each detection line for 5 min. AUC data were expressed as ×10⁵ particles. Each measurement was performed twice. All individuals showed identical results as to whether SMAP was formed. The mean values of two AUC measurements as the degree of SMAP in each individual were used for the analysis to minimize the variability.

**Statistical analysis**

Categorical variables were compared between the two groups using χ² tests, an unpaired Student t test was used for normally distributed variables, and the Mann-Whitney U test was used for non-normally distributed variables. For the nonparametric comparison among four groups, a Kruskal-Wallis test was first performed to find a measure of the aggregate degree to which the group means differed and then the Mann-Whitney U test with Bonferroni correction was used to compare the differences between groups. A Spearman correlation coefficient was used to analyze the association between two variables. A multivariate logistic regression model was applied to evaluate the independence of factors that were significantly different between the groups, using logarithmic transformed values of nonnormally distributed variables. All data were analyzed using the SPSS software package (version 11; SPSS, Chicago, IL). P <0.05 was considered statistically significant.

**RESULTS**

**Platelet microaggregation in diabetic patients**

SMAP was detected in 211 (52.6%) of 401 patients with type 2 diabetes (Fig. 1A) but not in any of the 30 healthy volunteers tested here (Fig. 1B) or in our previous study (12). The diabetic patients with SMAP showed an abnormal and irreversible...
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Figure 1—Typical patterns of SMAP and reversibility of platelet microaggregation by low-dose ADP, measured by a laser light-scattering system. The spontaneous formation of microaggregated platelets under low shear stress alone without any exogenous agonists was observed in 52.6% of diabetic patients (A), whereas it was not observed in 47.4% of diabetic patients and in any healthy volunteers (B). Diabetic patients with SMAP showed irreversible platelet microaggregation in response to ADP (1 μmol/L) (C), whereas the others showed the typical reversible pattern of platelet microaggregation within 5 min (D).

Table 1—Clinical characteristics of the study subjects according to the formation of SMAP

|                               | Diabetic patients without SMAP | Diabetic patients with SMAP |
|-------------------------------|-------------------------------|-----------------------------|
| n                             | 190                           | 211                         |
| Sex (male/female)             | 110/80                        | 119/92                      |
| Age (years)                   | 64 ± 11                       | 65 ± 9                      |
| Duration of diabetes (years)  | 17 ± 11                       | 16 ± 9                      |
| BMI (kg/m²)                   | 24.6 ± 4.3                    | 24.7 ± 3.8                  |
| Waist-to-hip ratio            | 0.93 ± 0.07                   | 0.95 ± 0.08*                |
| A1C (%)                       | 7.2 ± 0.8                     | 7.2 ± 1.0                   |
| Diabetes treatment (%) (diet/oral agents/insulin) | 11/55/34 | 7/49/44 |
| Systolic blood pressure (mmHg) | 133 ± 17                      | 134 ± 14                    |
| Diastolic blood pressure (mmHg) | 73 ± 10                      | 72 ± 10                     |
| Hypertension (%)              | 66                            | 74                          |
| Taking aspirin (%)            | 41                            | 59*                         |
| Total cholesterol (mg/dl)     | 194 ± 31                      | 197 ± 29                    |
| Triglycerides (mg/dl)         | 86 (61–116)                   | 92 (62–134)                 |
| HDL cholesterol (mg/dl)       | 54 (46–65)                    | 52 (45–62)                  |
| Taking statins (%)            | 52                            | 48                          |
| Urinary albumin excretion rate (mg/min) | 9 (6–16) | 23 (8–87)* |
| eGFR (ml/min per 1.73 m²)     | 74 ± 23                       | 70 ± 20*                    |
| baPWV (cm/s)                  | 1,606 (1,416–1,800)           | 1,726 (1,521–1,987)†        |
| Taking aspirin (%)            | 40                            | 41                          |
| Current smoking (%)           | 22                            | 21                          |

Data are means ± SD for normally distributed continuous variables or medians (25th–75th interquartiles) for skewed continuous variables. *P < 0.05; †P < 0.01 vs. diabetic subjects without SMAP.
moalbuminuria showed no such difference according to aspirin intake (0.00 [0.00–0.14] vs. 0.00 [0.00–0.21]) (Fig. 2). In addition, the strong correlation between the degree of SMAP and the levels of urinary AER as continuous variables was investigated in the patients without aspirin intake (γ = 0.53, P < 0.001), whereas the weak association between them was found in those with aspirin intake (γ = 0.27, P < 0.001).

**Correlation between platelet microaggregation and GPIIb/IIIa and P-selectin expression**

To further evaluate the active state of SMAP, we quantitated the expression levels of active GPIIb/IIIa and P-selectin on the platelets. The expression levels of active GPIIb/IIIa were higher in diabetic patients with SMAP than in those without SMAP (29.9% [interquartile range 19.3–42.2] vs. 15.5% [11.7–21.9]). Similarly, the expression levels of P-selectin were higher in diabetic patients with detectable SMAP than those without it (8.1% [5.1–16.0] vs. 4.4% [3.2–5.6]). The expression levels of both markers correlated significantly with the degree of SMAP (Spearman γ = 0.59 for active GPIIb/IIIa and γ = 0.55 for P-selectin).

**CONCLUSIONS** — The present study provided new evidence that both increased AER and baPWV are independent factors associated with the abnormal formation of SMAP in type 2 diabetic patients. Furthermore, the patients with SMAP showed an irreversible pattern of platelet microaggregation by ADP, and the degree of SMAP correlated with the enhanced expression of active GPIIb/IIIa and P-selectin, indicating that SMAP is pathophysiologically active. These early-activated platelet profiles were signifi-
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Dramatically inhibited in albuminuric patients with aspirin intake, although the effect was incomplete.

Assessment of inappropriate platelet activation is one way to risk-stratify patients who are at high risk of atherosclerosis and atherothrombosis. The microaggregates produced in the early phase of platelet activation are now considered to potentially aggravate thrombus formation (7). Under normal conditions, these platelet microaggregates dissolve within a few minutes, as shown in this study (Fig. 1D). In the present study, 53% of type 2 diabetic patients showed SMAP and abnormal irreversibility of the microaggregation after low-dose ADP. This high proportion of diabetic patients with abnormal platelet hyperaggregability is in agreement with the result from a cohort studied previously (12). The SMAP formation was observed under a low-shear stress that mimicked the state of the arterial bloodstream without the stimulation of exogenous agonists. In addition, the degree of SMAP was associated with enhanced expression of active GPIIb/IIIa and P-selectin. Thus, the formation of SMAP is considered to show the abnormal activated state of platelets in diabetic patients, which is enhanced according to the increase of albuminuria.

In the present study, SMAP occurrence was associated with both albuminuria and baPWV. In agreement with these results, other investigators reported that high levels of platelet microaggregation were associated with adverse outcomes in patients with cardiovascular disease (16) and the ankle-brachial index in patients with peripheral arterial disease (17). In addition, diabetic patients with SMAP showed overexpression of platelet surface markers, active GPIIb/IIIa, and P-selectin. Such upregulation of these molecules has also been associated with the development of atherogenesis (18). Taken together, the early platelet activation in type 2 diabetic patients may be a risk factor for cardiovascular disease.

A recent guideline of the American Diabetes Association recommended prophylactic use of antithrombotic agents, with low-dose aspirin, for diabetic subjects, especially those with albuminuria (19). However, Di Minno and Violi (20) indicated that aspirin alone is insufficient to prevent thrombosis in diabetic patients with angiopathy. In the present study, SMAP formation was inhibited in albuminuric patients taking aspirin compared with those not taking it. However, SMAP formation was still associated with albuminuria, even in patients taking aspirin, suggesting that the inhibitory effect of aspirin may be insufficient in diabetic patients with albuminuria. Further follow-up and intervention studies are required to investigate whether the incomplete inhibition of early-activated platelet profiles is a cardiovascular risk.

Several plausible mechanisms underlying the cardio-renal interaction have been proposed. Deckert et al. (21) proposed in the Steno hypothesis that excess leakage of albumin into urine reflects widespread vascular (endothelial) damage. This endothelial dysfunction is considered a common feature of cardio-renal interactions (22); it leads to platelet activation, adhesion, and subsequent platelet aggregate formation. Platelets are also a rich source of chemokines and cytokines, released within seconds of platelet activation (23). Thus, the formation of SMAP in diabetic patients with albuminuria might either cause or reflect systemic vascular endothelial dysfunction. An alternative explanation is that any pathophysiological alteration in a diabetic kidney could directly affect platelet activation. Glomerular hypertension, which induces mechanical shear stress (24), and excess renal production of type IV collagen in the kidney (25), a powerful activator of platelets, might induce activation of platelets that circulate into the kidney.

The present study had some limitations. It was not possible to ascertain whether the early-activated platelet profile was a cause or consequence of the increased urinary albumin excretion because of the nature of the cross-sectional study. The present study also could not address whether the incidence of future cardiovascular disease is higher in patients with SMAP and whether the inhibitory effect of aspirin on the degree of SMAP would be sufficient to prevent the development of cardiovascular disease.

In summary, the majority of type 2 diabetic patients with albuminuria showed an altered profile of early platelet activation including SMAP events. Given the growing concern over cardiovascular consequences in type 2 diabetic patients, further follow-up and intervention studies are needed to establish whether the inhibition of SMAP is a therapeutic target to prevent cardiovascular complications in type 2 diabetic patients.

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