Effect of Vitamin E on Platelet Aggregation in Diabetes Mellitus

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Vitamin E is known to be an inhibitor of platelet prostaglandin production and aggregation. The rate of platelet aggregation induced by adenosine diphosphate was significantly increased in diabetics with proliferative retinopathy, and the enhanced production of thromboxane B₂, a stable metabolite of thromboxane A₂, was demonstrated in those patients. On the other hand, vitamin E was significantly reduced in platelets obtained from diabetics as compared to age matched control subjects. In addition, it was shown that vitamin E content in platelets examined in diabetic and control subjects inversely correlated with both the rate of platelet aggregation and thromboxane B₂ production during aggregation. It is suggested that the reduced vitamin E levels in diabetic platelets can contribute to the mechanisms of the enhanced platelet thromboxane A₂ production and aggregation which lead to the development of vascular complications.

vitamin E; platelet aggregation; thromboxane B₂; diabetic retinopathy

It is widely recognized that platelets play an important role in the development of atherosclerosis (Ross and Glomset 1976). Diabetics are prone to develop microvascular complications (Bensoussan et al. 1975; Colwell et al. 1983). A number of abnormalities of platelet function including enhanced aggregation have been demonstrated in diabetes mellitus (Kwaan et al. 1972; Colwell et al. 1979; Umeda et al. 1982; Sugimoto et al. 1982).

In recent years, vitamin E has become known as an inhibitor of platelet aggregation. It has been reported that in vitro vitamin E addition to human platelet rich plasma causes reduced platelet aggregation (Fong 1976; Steiner and Anastasi 1976). The inhibitory effect on platelet aggregation was also demonstrated in animals supplemented with vitamin E (Karpen et al. 1981). However, there has been no report concerning the pathophysiology of vitamin E metabolism in diabetic platelets related to aggregation and prostaglandin metabolism.

In the present study, we have evaluated the role of vitamin E in platelets on
prostaglandin metabolism and adenosine diphosphate induced platelet aggregation. Abnormal vitamin E metabolism in platelet was discussed as one of the mechanisms of the enhanced platelet aggregation which accelerate vascular complications in diabetes mellitus.

**MATERIALS AND METHODS**

**Subjects**

Non-insulin dependent diabetics treated in the Third Department of Internal Medicine, Kyushu University Hospital, Fukuoka, Japan, were studied. Diabetics were divided into two groups, with or without proliferative retinopathy. Diabetic retinopathy was assessed ophthalmoscopically and by color photography of the fundi, and defined as multiple retinal hemorrhages, neovascularization and the presence of fibrous tissue. The characteristics of diabetic and control subjects are shown in Table 1. All subjects took diets containing 5 mg of vitamin E daily. They refrained from taking antiplatelet agent including vitamin E at least two weeks before the study.

**Materials**

The following chemicals were purchased from commercial sources: Adenosine diphosphate (ADP) (Boehringer-Mennheim, Germany); DL-α-Tocopherol (Nakarai Chemicals, Kyoto); \[5,6,8,9,11,12,14,15\] 3H-thromboxane B₂ (New England Nuclear, USA). Authentic thromboxane B₂ and thromboxane B₂ antibody were generous gifts from Ono pharmaceuticals (Osaka).

**Platelet Aggregation**

After overnight fasting, blood was collected from an antecubital vein into a plastic syringe without stasis. Nine parts of whole blood were mixed with 1 part of 120 mmol/liter sodium citrate. Blood samples were centrifuged at 150 × g for 10 min to obtain platelet rich plasma (PRP). The rest of whole blood was centrifuged at 1,500 × g for 15 min to obtain platelet poor plasma (PPP). The platelet count of PRP was determined in a hemocytometer and was adjusted to 20 × 10⁴ platelets/μl using autologous PPP. Platelet aggregation was achieved using Born’s turbidimetric method (Born 1962) with an Auto RAM-21 aggregometer (Rikadenki Kogyo Co., Tokyo) and recorded with an electronic recorder R-12 (Rikadenki Kogyo Co., Tokyo). Briefly, 250 μl of PRP were stirred magnetically at 1,100 rpm (37°C). After one minute preincubation, ADP (final concentration 2 μmol/liter) was added and the change in light transmission was recorded at a speed of 2 cm/min assuming that the PPP represented 100% aggregation, and that the PRP represented 0% aggregation. Five minutes after addition of ADP, platelet aggregation was determined and thromboxane B₂ in PRP was extracted as follows:

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number</th>
<th>Age (years)</th>
<th>Duration (years)</th>
<th>Fasting blood glucose (mmol/liter)</th>
<th>Hemoglobin A₁ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>18 (15M, 3F)</td>
<td>42 ± 6</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Diabetics</td>
<td></td>
<td></td>
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<tr>
<td>Without proliferative retinopathy</td>
<td>25 (15M, 10F)</td>
<td>52 ± 5</td>
<td>6 ± 2</td>
<td>9.7 ± 1.4</td>
<td>9.6 ± 1.0</td>
</tr>
<tr>
<td>With proliferative retinopathy</td>
<td>24 (12M, 12F)</td>
<td>54 ± 5</td>
<td>8 ± 2</td>
<td>10.1 ± 1.7</td>
<td>10.1 ± 0.6</td>
</tr>
</tbody>
</table>

Mean ± s.e.

**Table 1. Characteristics of subjects**
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Thromboxane B2 Determination

Thromboxane B2 (TXB2) production by platelets during aggregation was determined according to modified Siess' method (Siess et al. 1981). Briefly, 0.2 ml of supernatant exactly 5 min after platelet aggregation was acidified with 0.1 N HCl, then 3H-TXB2 was added for recovery estimation. The samples were extracted with 2 ml of ethylacetate three times. Collected organic solvents were evaporated at 40°C and dissolved in 99.5% ethanol and kept at -20°C. Stock solution was evaporated at 40°C and redissolved in 0.1 mol/liter phosphate buffer with 1 mol/liter NaCl, 1 g/liter gelatin, and 7.69 mmol/liter sodium azide (pH 7.2). The overall recovery was 77.0 ±2.7% (mean ±s.E.). To 0.1 ml of sample, 3H-TXB2 and TXB2 antibody were added and incubated for 24 hr at 4°C. The bound and free ligands were separated using dextran-coated charcoal, and the supernatant was counted in a LSC-700 liquid scintillation counter (Aloka, Tokyo).

Vitamin E Determination

PRP was centrifuged at 1,500 x g for 5 min (4°C). After removal of supernatant, platelets were resuspended in 0.138 mol/liter NaCl with 0.01 mol/liter Tris HCl buffer (pH 7.4). This step was repeated twice. Washed platelet suspension was sonicated with a W-185 sonifier (Branson Sonic Power Co., USA) in 0°C (22 Watts x 10 sec x 2 times), and then stocked at -70°C. Vitamin E in platelets and plasma was determined by Thompson’s method (Thompson et al. 1973). Plasma or washed platelet suspension (0.2 ml) was diluted with 1 ml of water and mixed with 1 ml of ethanol. Five ml of n-hexane was added and the samples were mixed thoroughly for 1 min. After centrifugation, the supernatant was measured fluorometrically (Ex 295 nm, Em 320 nm) using an RF-500 spectrofluorometer (Shimadzu, Kyoto). The same procedure was performed with an α-Tocopherol solution (20 mg/100 ml) for standard and water for blank. Vitamin E concentration was calculated as a total of α-Tocopherol. Platelet vitamin E content was expressed as μg/1010 platelets.

Statistical Methods

Statistical analyses were carried out using Student’s t test. All p values less than 0.05 were accepted as statistically significant.

Results

As shown in Table 1, diabetics were divided into two groups, with or without proliferative retinopathy. There was no difference in age, duration and treatment between these two groups.

Fig. 1 shows that the rate of ADP induced platelet aggregation in diabetics with proliferative retinopathy was 65 ±4% (mean ±s.E.), which was significantly greater than those in diabetics without proliferative retinopathy (41 ±4%) or the controls (36 ±4%). It is evident that the rate of platelet aggregation increased in diabetic patients with proliferative retinopathy.

Thromboxane A2, a main metabolite in platelet prostaglandin metabolism, is a potent activator of platelet aggregation. Since thromboxane B2 has been evaluated as a stable metabolite of thromboxane A2, the level of TXB2 following platelet aggregation was determined as a characterization of TXA2 production. Platelets obtained from diabetics with proliferative retinopathy generated a significantly greater amount of TXB2 during aggregation (442 ±61 pmol/1010 platelets, mean ±s.E.) than those obtained from diabetics without proliferative
retinopathy (277 ± 29 pmol/10¹⁰ platelets) or the controls (287 ± 23 pmol/10¹⁰ platelets), as shown in Fig. 2. Enhanced platelet aggregation with increased TXB₂ production was demonstrated in diabetics with proliferative retinopathy.

Since TXA₂ is a potent mediator of platelet aggregation, the correlation between TXA₂ production and platelet aggregation rate was examined. There was seen a significantly positive correlation between these two factors (Fig. 3). Increased TXA₂ production causes the increased platelet aggregation.

Our preliminary data demonstrated that the plasma vitamin E level in 65 diabetics (age 55 ± 1 years, mean ± s.e.) excluding renal insufficiency was 3.76 ±
Fig. 3. Correlation between ADP induced platelet aggregation and thromboxane B₂ production during aggregation in controls (○) and diabetics (●). Correlation coefficient: +0.561. Regression line: \( y = 181.65 + 6.11x \). Statistical significance: \( p < 0.01 \).

Fig. 4. Platelet vitamin E content in controls and diabetics (without proliferative retinopathy, with proliferative retinopathy). Results are expressed as mean ± s.e. The number of subjects in each group is given in parenthesis. *\( p < 0.02 \), †\( p < 0.01 \).
Fig. 5. Correlation between platelet vitamin E content and ADP induced platelet aggregation rate in controls (○) and diabetics (●).
Correlation coefficient: $-0.516$. Regression line: $y = 16.5 - 0.20x$.
Statistical significance: $p < 0.02$.

Fig. 6. Correlation between platelet vitamin E content and thromboxane $B_2$ production during platelet aggregation in controls (○) and diabetics (●).
Correlation coefficient: $-0.521$. Regression line: $y = 16.3 - 0.02x$.
Statistical significance: $p < 0.02$. 
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0.30 \( \mu g/ml \) (mean \( \pm \) S.E.), which was significantly lower than that of age matched 23 controls (7.50 \( \pm \) 0.40 \( \mu g/ml \)). In the present study, vitamin E level in platelets, which may reflect plasma vitamin E, was examined. Platelets obtained from diabetics with proliferative retinopathy contained significantly smaller amounts of vitamin E (2.6 \( \pm \) 1.3 \( \mu g/10^{10} \) platelets, mean \( \pm \) S.E.) than those from diabetics without proliferative retinopathy (10.3 \( \pm \) 2.3 \( \mu g/10^{10} \) platelets) and controls (15.0 \( \pm \) 3.4 \( \mu g/10^{10} \) platelets) (Fig. 4).

Furthermore, Fig. 5 shows that the relationship between ADP induced platelet aggregation and vitamin E content in platelets examined in both diabetics and controls. Significantly, there was an inverse correlation between these two factors in platelets.

The relationship was also studied between vitamin E content in platelets and TXB_2 production during aggregation. Vitamin E content in platelets was inversely correlated with TXB_2 production from platelets (Fig. 6).

**DISCUSSION**

In a previous report (Halushka et al. 1981), it has been demonstrated that platelet aggregation and thromboxane B_2 production are increased in diabetes mellitus, which may lead to diabetic vascular complications. In agreement, the present study shows that, in diabetics with proliferative retinopathy, ADP induced platelet aggregation was significantly increased, and this was positively correlated with the rise of TXB_2 production, a stable metabolite of TXA_2 in platelets. Platelets obtained from diabetics with proliferative retinopathy have a reduced amount of vitamin E. A significant inverse correlation was revealed between platelet vitamin E content and TXB_2 production during aggregation. These results suggest that the reduction of vitamin E in platelets may lead to an enhancement of prostaglandin metabolism and consequently platelet aggregation in diabetics. Karpen et al. observed that low platelet vitamin E causes an increase in thromboxane production in diabetic rats (Karpen et al. 1982), and that animals fed a vitamin E-rich diet also show reduced platelet aggregation (Karpen et al. 1981). In vitro addition of vitamin E to human PRP inhibits platelet aggregation (Fong 1976; Steiner and Anastasi 1976).

In platelets, vitamin E has been shown to inhibit phospholipase and calcium ion mobilization (Butler et al. 1979). So, the release of arachidonic acid (AA) is enhanced from the diabetic platelet membrane containing reduced vitamin E content on platelet activation, and AA is used as a substrate of platelet prostaglandin metabolism, which enhances aggregation. On the other hand, aorta obtained from animals supplemented with vitamin E generated a greater amount of 6-ketoprostaglandin F_1\alpha, a stable metabolite of prostacyclin (PGI_2), than that from vitamin E deficient rats (Karpen et al. 1981). It is thought that the mechanism of increased PGI_2 production from vascular intimal cells by vitamin E is due to the activation of cyclooxygenase and/or the reduction of lipoperoxide.
formation which inhibits PGI₂ synthetase (Panganamala and Cornwell 1982). Aorta obtained from vitamin E deficient rats showed a reduction of PGI₂-like substance (Sugimoto et al. 1980). Imbalance of TXA₂ and PGI₂, resulting in diabetic vascular complications, is present in the state of vitamin E deficiency. It is suggested that vitamin E deficiency in diabetics may be one of the pathogeneses of the abnormalities of diabetic prostaglandin metabolism and platelet function.

It is concluded that platelet vitamin E content in diabetics with proliferative retinopathy was reduced, and platelet vitamin E content was demonstrated to be closely related to both the activity of platelet prostaglandin metabolism and ADP induced platelet aggregation rate.

References


