AUGMENTED MALONDIALDEHYDE PRODUCTION BY PLATELETS FROM PATIENTS WITH CEREBROVASCULAR DISORDERS

KEI SATOH, M.D., SHIGERU TAKAMATSU, M.D., SHIGERU SAKUTA, M.D.
SEITOKU MIZUNO, M.D., HIROBUMI METOKI, M.D.*
AND MUTSU TAKAMATSU, M.D.**

A basic study was conducted on the method for the determination of thio-barbituric acid-reactive substances (TBARS) produced by platelets. Some modifications were introduced which enabled a precise semimicrodetermination.

The average values of platelet TBARS production in hypertensives and in patients with cerebral infarction were higher than that in healthy controls. Among patients with cerebral infarction, those with angiographically demonstrated obstruction of internal carotid or middle cerebral arteries showed an increased production as compared with non-obstructive cases. These results may suggest the important role of platelet arachidonate metabolism in obstructive cerebrovascular disorders.

PLATELET arachidonate peroxidation, which leads to the production of prostaglandin (PG) endoperoxides and thromboxane A2 (TXA2), is an essential metabolic process underlying platelet aggregation.1,2 On the other hand, the favorable effect of anti-platelet agents for the prophylaxis against stroke and for the treatment of transient ischemic attack (TIA) has been demonstrated by the recent trials.3,4 This may suggest the crucial role of platelets, especially arachidonate metabolites of platelets, in the pathogenesis of cerebrovascular disorders.

The production of thio-barbituric acid-reactive substances (TBARS) by platelets is known as an indicator of the production of PG endoperoxides and TX A2.5 Measurements of platelet TBARS production have been performed for the purpose of determining platelet life span6 besides the measurements appear to be useful for the observation of platelet function. We, therefore, have intended to apply them to the clinical practice as one of the platelet function tests that differs basically from the conventional ones, to observe the metabolic function of platelets. In this study, a clinically useful method for the determination of platelet TBARS production has been investigated, then the observations of platelets in hypertensives and cerebrovascular patients have been performed.

Key Words:
Cerebrovascular disorders
Platelet
Malondialdehyde
Plasma lipids
Hypertension

(Received April 2, 1981; accepted July 21, 1981)
Department of Pathologic Physiology, Institute of Cerebrovascular Diseases, Hirosaki University School of Medicine, Hirosaki, Japan
*The Reimeiyo Rehabilitation Hospital
**The Training Course of School Nursing, Faculty of Education, Hirosaki University
Address for correspondence: Kei Satoh, M.D., Department of Pathologic Physiology, Institute of Cerebrovascular Diseases, Hirosaki University School of Medicine, 5 Zaifucho, Hirosaki 036, Japan

MATERIALS AND METHODS

Subjects
Subjects studied consisted of 50 patients with cerebral infarction, 39 with cerebral hemorrhage, 34 patients with hypertension and 25 healthy subjects. Cerebrovascular patients were those in chronic stage who had passed more than 3 months after the acute events. The average age in patients with infarction was 61 ± 8.5 (mean ± S.D.). Those in hemorrhagic patients and in hypertensives were 58 ± 9.1 and 60 ± 10.1, respectively. The control group consisted of 18 healthy subjects age-matched with these patients (58 ± 11.0 years). The healthy subjects received no medication during the preceding 4 weeks. The cerebrovascular patients received no such agents as non-steroidal anti-inflammatory drugs, hemostatics, anticoagulants or fibrinolytics, but otherwise the type of treatment employed was not taken into consideration. Of 34 hypertensives, 15 cases were studied prior to the initiation of treatments, and medications in the remainder were diverse with various combinations of anti-hypertensive agents. Blood samples were obtained in the fasting state early in the morning.

Determination of Platelet TBARS Production
Venous blood was collected in a siliconized glass tube containing 1/13 volume of 77 mM EDTA-2Na. Platelet rich plasma (PRP) was separated by differential centrifugation. Aliquot (0.6 ml) of PRP was transferred into a polycarbonate tube and centrifuged at 600G for 15 min. Pelleted platelets were resuspended in 1.0 ml of Tris-saline-EDTA buffer (1 mM EDTA-2Na; 5 mM D-glucose, 0.134 M NaCl; 15 mM Tris-HCl, pH 7.4). Platelets pelleted by the second centrifugation were resuspended in 0.6 ml of Tris-saline buffer (5 mM D-glucose, 0.134 M NaCl; 15 mM Tris-HCl, pH 7.4). To this suspension, 0.05 ml of 100 U/ml thrombin in saline was added. After the incubation at 37°C for 10 min, 0.5 ml of this mixture was pipetted into a glass tube containing 2.5 ml of 0.12 N H₂SO₄ and 1.0 ml of 0.6 g/dl TBA in water. The tube was then heated in a boiling water bath for 30 min and cooled by tap water. After the extraction with 2.0 ml of n-butanol, clear upper organic phase was separated by centrifugation at 3000 rpm for 10 min. The optical density of the organic phase was determined at 530 nm. The solution of malondialdehyde (MDA) in 0.12 N H₂SO₄ was used as a standard. A platelet count was obtained on PRP and the determined value was expressed in terms of nanomole MDA per 10⁹ platelets (nmol/10⁹ platelets).

Contaminations of platelet suspension with other blood cells were less than one per 16,000 platelets for red cells and one per 40,000 platelets for white cells, and such contaminations gave no error to the estimated platelet TBARS production.

The intra-assay precision of this method was 3.1% (n = 15) and the inter-assay precision was 4.7% in 5 different assays (n = 15). Standard MDA solution, added to the thrombin-treated platelet suspension, to give a calculated TBARS production of 12.75 nmol/10⁹ platelets, showed a percent recovery range of 96.6–104.2%.

As a basic experiment, the effect of acetylsalicylic acid (Aspirin) on platelet TBARS production was studied. To 0.6 ml of PRP, 10 μl of Aspirin in methanol was added, and the incubation was performed at 37°C for 10 min. As shown in Fig. 1, Aspirin inhibited the production almost completely at the concentration of 0.1 mM. The other details of the method were described elsewhere.

Other Procedures
Plasma lipid peroxide level was determined by our method. Determinations of plasma total cholesterol (TCh) and triglyceride (TG) were conducted using the reagent kits purchased from Wako Pure Chemicals Co., Ltd., Tokyo. Low density lipoprotein (LDL), very low density lipoprotein (VLDL) and high density lipoprotein-cholesterol (HDL-Ch) were measured by the heparin-Ca²⁺ precipitation method.

Results

Blood chemical and hematological findings in controls and cerebrovascular patients are given in Table I. Significant differences between these 2 groups were found in HDL-Ch, LDL, VLDL, and lipid peroxides. The average value of platelet TBARS production in healthy subjects is shown in Table II. The value in the fifth decade was slightly high but not statistically significant difference was observed as compared with other age groups. The average value in 17 males (5.41 ± 1.96 nmol/10^9 platelets) was slightly, but not significantly, higher than in 8 females (5.00 ± 2.08 nmol/10^9 platelets).

The values in patients with hypertension are given in Table III. The average value in the patients was somewhat higher than that in age-matched healthy controls but the difference was insignificant. Among hypertensives, those studied prior to the initiation of anti-hypertensive treatment showed an increased production as compared with cases under the treatment. A significant difference was observed between the untreated cases and controls (p < 0.05).

Table IV shows the average values in cerebrovascular patients. The value in patients with cerebral infarction was significantly higher than those in hemorrhagic patients and in controls (p < 0.05, p < 0.05). No age or sex difference was found among the values in cerebrovascular patients. The average values in male and female patients were 6.52 ± 2.09 nmol/10^9 platelets (n = 32) and 6.20 ± 2.13 nmol/10^9 platelets (n = 32), respectively.

Cerebral angiographical examinations were performed on 27 cases with cerebral infarction. Figure 2 illustrates that among these patients, an increased production of platelet TBARS was observed in cases with angiographically demonstrated obstruction of internal carotid or middle cerebral arteries as compared to those without such findings. The difference between obstructive cases and controls was statistically signifi-
TABLE IV  PLATELET TBARS PRODUCTION IN CEREBROVASCULAR PATIENTS AND
HEALTHY CONTROLS

<table>
<thead>
<tr>
<th>Age</th>
<th>Patients with infarction</th>
<th>Patients with hemorrhage</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>Mean ± S.D.</td>
<td>n</td>
</tr>
<tr>
<td>40 – 49</td>
<td>4</td>
<td>7.60 ± 2.64</td>
<td>9</td>
</tr>
<tr>
<td>50 – 59</td>
<td>21</td>
<td>6.73 ± 2.10</td>
<td>15</td>
</tr>
<tr>
<td>60</td>
<td>25</td>
<td>6.81 ± 2.08</td>
<td>15</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>6.84 ± 2.10*</td>
<td>39</td>
</tr>
</tbody>
</table>

* p < 0.05: significantly higher as compared with controls and hemorrhagic patients

TABLE V  RELATIONSHIPS BETWEEN PLATELET TBARS PRODUCTION AND PLASMA
LIPIDS IN CONTROLS AND CEREBROVASCULAR PATIENTS

<table>
<thead>
<tr>
<th>Controls</th>
<th>N</th>
<th>r</th>
<th>Patients</th>
<th>N</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>25</td>
<td>+0.386</td>
<td>89</td>
<td>-0.100</td>
<td></td>
</tr>
<tr>
<td>Triglyceride</td>
<td>25</td>
<td>+0.453*</td>
<td>89</td>
<td>+0.041</td>
<td></td>
</tr>
<tr>
<td>LDL</td>
<td>25</td>
<td>+0.321</td>
<td>89</td>
<td>-0.049</td>
<td></td>
</tr>
<tr>
<td>VLDL</td>
<td>25</td>
<td>+0.267</td>
<td>89</td>
<td>-0.027</td>
<td></td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>25</td>
<td>-0.488*</td>
<td>89</td>
<td>-0.084</td>
<td></td>
</tr>
<tr>
<td>HDL-Ch/TCh ratio</td>
<td>25</td>
<td>-0.622**</td>
<td>89</td>
<td>-0.053</td>
<td></td>
</tr>
<tr>
<td>Lipid peroxides</td>
<td>25</td>
<td>+0.092</td>
<td>89</td>
<td>+0.227</td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05,  ** p < 0.01

![Graph showing platelet TBARS production](image)

Fig. 2. Platelet TBARS production in patients with cerebral infarction. The average value in cases with angiographically demonstrated obstruction of internal carotid or middle cerebral arteries is higher than that in those without such findings. The value in obstructive cases is significantly higher as compared with controls.

DISCUSSION

Once arachidonic acid in platelet phospholipids is released by the action of phospholipase, it is converted into PG endoperoxides by cyclooxygenase and then one of the endoperoxides is converted into TX A2 by TX synthetase. These products are known as the potent inducers of platelet aggregation and vascular constriction. TBA test has been regarded as a method to determine MDA produced mainly during the breakdown of PG endoperoxides. In our study, platelets without any pretreatment with thrombin produced almost no TBARS and Aspirin, an

inhibitor of cyclooxygenase, greatly depressed the production. Therefore, TBA test should be regarded, at least, as a method to determine the platelet arachidonate peroxidation, especially the cyclooxygenase products.

The process of the measurement consists of two parts, one is the activation of platelet arachidonate peroxidation and the other is the quantification of produced TBARS. As for the first point, one must settle which should be used for specimen, PRP or platelet suspension, and which agent should be employed for platelet activation. Determination on PRP might result in the overestimation caused by additional extraction of TBARS in plasma. It has been noted that exogenously added arachidonate causes platelet production of TBARS. Since release of endogenous arachidonic acid is a trigger process in platelet metabolism, added arachidonate may interfere with the precise evaluation of platelet activity to metabolize arachidonic acid. Thus platelet activation by exogenous arachidonate may be inappropriate except for the purpose of monitoring the activity of cyclooxygenase as in the case of determination of platelet life span. We, therefore, have designed a method employing washed platelet suspension as a specimen and thrombin as an agent for the activation of platelets.

As to the procedure for the quantification, there are many problem concerning the specificity of TBA reaction. TBA reacts with various compounds and the interference from sialic acids has been a critical problem in the determination of serum lipid peroxides. We applied the same principle used in the method for serum to the present one, i.e., TBA reaction should be performed in a weak acid medium. Another problem in this item is the difficulty in colorimetry caused by the turbidity of the reaction mixture. Okuma et al. dealt with this problem using a detergent. We employed the extraction with butanol which was also advantageous for the sensitivity of the method.

PG endoperoxides and TX A2 possess vasoconstrictive action, as well. Increased platelet arachidonate peroxidation observed in hypertensive patients may indicate a possible contribution of these products to hypertension and to hypertensive vascular damage. Drugs such as reserpine, clonidine, furosamide, etc., have been shown experimentally to depress platelet function. Our results demonstrating the augmented peroxidation in untreated hypertensives may reflect such an action of anti-hypertensive agents. Another possible explanation for this is that hypertension has brought about the activation of platelets secondarily which has been ameliorated by the control of blood pressure. In any case, platelet hyperfunction may predispose to the vascular damage in conjunction with high blood pressure.

A platelet hyperfunction in patients with cerebrovascular disorders has been observed in many reports. The large scale trial conducted by the Canadian Cooperative Study Group has demonstrated the effectiveness of Aspirin against the recurrence of stroke in patients with TIA or “Partial non-progressing stroke”. These facts may imply that platelet is one of the major factors causing the outbreak of cerebrovascular disorders. Our results suggest the possible involvement of cyclooxygenase products as a basic mechanism underlying such an important role of platelets. This may be further clarified by the fact that platelet TBARS production has exhibited a close relation with a pathophysiological state specific to cerebrovascular disorders: an increased production has been observed in patients with the obstruction of major cerebral arteries. Vascular constriction and platelet aggregation induced by cyclooxygenase products may be of physiological importance for the hemostatic mechanism. On the other hand, overproduction of these substances may initiate and sustain the thrombotic events and thereby contribute to the development of cerebrovascular disorders.

Carvalho and associates have reported the increased sensitivity of platelets to aggregating agents in hyperlipoproteinemias. This may be in accordance with our findings demonstrating the influence of some plasma lipids on platelet arachidonate metabolism. Influence of plasma lipids on platelet may be mediated via an alteration in the lipid composition of platelet membrane, as clearly demonstrated in the experimental study of Insel and associates. The membrane lipid composition may, in turn, affect the activity of some membrane-bound enzymes in the arachidonate metabolising system of platelets.

In this connection, plasma lipids may also affect the substrates for the syntheses of PGs and TX. The importance of precursor fatty acids has been suggested by the recent findings that dietary enrichment with eicosapentaenoic acid might protect against thrombosis. The change

*Japanese Circulation Journal Vol. 45, December 1981*
in the fatty acid composition of plasma lipids generally observed in cerebrovascular patients is that the relative amount of linoleic acid is smaller than normal\textsuperscript{26,27}. Whether the change in fatty acid composition of plasma lipids are related to the productions of PG and TX remains to be elucidated.

Recently, plasma content of TX B\textsubscript{2}, the stable metabolite of TX A\textsubscript{2}, has been quantified by radioimmunoassay methods\textsuperscript{28-30} and Sakanishi et al\textsuperscript{31} have disclosed that plasma TX B\textsubscript{2} level of healthy subjects is low in females as compared to males. Our results showing somewhat lower TBARS production in women than that in men, may accord with the view\textsuperscript{32,33} that sex hormones may affect thrombogenesis through their actions on platelets. Sakanishi et al\textsuperscript{31} have also reported higher than normal TX B\textsubscript{2} level in patients with atherosclerotic diseases, including stroke. An experimental evidence\textsuperscript{34} for the coincidental generations of TX B\textsubscript{2} and TBARS from platelets has presented, as well. However, measurement of platelets TBARS production may be clinically useful, for instance, in monitoring the activity of cyclooxygenase as a guide to the effective antiplatelet therapy.

REFERENCES


4. FIELDS WS, LEMAK NA, FRANKOWSKI RF, HARDY RJ: Controlled trial of aspirin in cerebral ischemia. \textit{Stroke} 8: 301, 1977


15. ROSSI EC, LEVIN NW: Inhibition of ADP-induced platelet aggregation by furosemide. \textit{J Lab Clin Med} 81: 140, 1973


Platelet MDA Production in Cerebrovascular Patients

790. 1961 (in Japanese)
27. LIN S-H, HORNING EC: Concurrent determination of \( \alpha \)-tocopherol and free fatty acids in human plasma by glass open tubular capillary column gas chromatography. *J Chromatogr* **112**: 465, 1975
29. FITZPATRICK FA, GORMAN RR, MCGUIRE JC, KELLY RC, WYNALDA MA, SUN FF: A radioimmunoassay for thromboxane B\(_2\). *Anal Biochem* **82**: 1, 1977