Thromboxane A₂ Synthesizing Activity of Platelets in Coronary Artery Diseases

HIROH YAMAZAKI, M.D., SHIGERU KAWAGOE, M.D*, ATSUMI YAMAGUCHI, Ph.D.
JUNJI MUNAKATA, M.D*, SHINICHI KUROKI, M.D.*
AND KENJIRO TANOUE, M.D.

To observe platelet TXA₂ synthesizing activities in acute myocardial infarction (AMI, 38 cases) and effort angina (EA, 23 cases), radioimmunoassay was used to measure the amount of synthesized TXB₂ at 5 min after an addition of arachidonic acid or thrombin to the platelet suspension. The amount of TXB₂ in AMI patients did not show a significant difference from that of 13 healthy controls. However, there were significant changes during the time course of AMI. It increased in the super acute phase within the first 12 hrs from the onset of AMI. The activity decreased in 4 days, increased again in 10 days and then gradually recovered to the normal value. Platelet aggregation was elevated immediately after the onset of AMI and recovered during the time course. A significant negative correlation was observed between aggregation and TXA₂ production in AMI, but not in the healthy controls, suggesting that platelets with hyper TXA₂ synthesizing activity are consumed selectively in AMI. In EA, activity increased after treadmill exercise. Under ticlopidine-treatment, activity was depressed, and these patients were able to tolerate a longer exercise time than before ticlopidine. Since the pressure rate products did not change under treatment, changes in microcirculation such as the appearance of platelet aggregates may be important in the occurrence of anginal attacks.

THE role of platelet aggregation and prostaglandins (PG), especially thromboxane A₂ (TXA₂), in coronary artery diseases is of wide interest.¹ Although there have been a number of reports on TXA₂ changes in the diseases,²⁻⁵ the results of these studies did not show similar changes. One reason for this may be differences in the methods used for the determination of TXA₂ levels in vivo. Many recent studies have been published where the PG content of plasma

was measured with radioimmunoassay. However, the extreme rapidity of the biosynthesis of PG makes it doubtful whether it is possible to determine the *in situ* levels of these compounds in the plasma. The great variations in published plasma levels suggest that the finally measured PG levels may be influenced by many technical factors such as sampling techniques, extraction techniques and others.⁶ The recovery of the extracted PG cannot be determined with any higher degree of accuracy. Moreover, presences of other kinds of lipids, fatty acids and proteins in plasma can affect the measurements of PG.⁶ In order to eliminate these problems, we have examined TXA₂ synthesizing activity of platelets which were separated from plasmal components

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Department of Cardiovascular Research, The Tokyo Metropolitan Institute of Medical Science and *Tokyo Metropolitan Komagome Hospital, Tokyo, Japan

Mailing address: Hiroh Yamazaki, M.D., The Tokyo Metropolitan Institute of Medical Science, 18-22, Honkomagome, 3-chome, Bunkyo-ku, Tokyo 113, Japan

in coronary artery diseases such as myocardial infarction and angina pectoris.

MATERIALS AND METHODS

1. *Observations on Acute Myocardial Infarction (AMI)*

The subjects were 38 admitted patients with acute myocardial infarction (AMI). There were 23 males (44–78 yrs) and 15 females (57–82 yrs). Their stages of AMI were from 6 hrs to 34 days from the onset of AMI. The AMI diagnosis was confirmed according to the criteria recommended by WHO.⁷ Blood samples from patients with AMI were obtained for platelet study before the starting any kind of treatment. Patients were excluded from the study if they showed severe heart failure, if they were suspected of having decreased liver and/or kidney function, and when they had received any kind of antiplatelet drug such as aspirin and ticlopidine. The control group consisted of 13 healthy individuals (7 males and 6 females, 18–55 yrs), and the results from their platelets were compared to those from patients with AMI.

2. *Observations on Effort Angina (EA) during Exercise*

Twenty-three patients with effort angina (EA) were subjects of this study. There were 15 males (51–79 yrs) and 8 females (45–72 yrs). They have typical attacks of EA. Coronary angiograms were performed on 15 of the 23 patients, and 13 of them showed over 50% stenosis in more than two main branches of the coronary artery. The control group in this study consisted of 15 healthy individuals (9 males and 6 females, 27–67 yrs). All individuals underwent symptom-limited treadmill exercise testing according to Bruce's protocol.⁸ Before and immediately after the exercise, blood samples were collected for platelet study. During the two week period before exercise testing, the subjects were prohibited from taking any drugs, except nitroglycerin. After the exercise testing, ten of the 23 patients with EA were given ticlopidine (300 mg/day, PO) for a period of two weeks. These 10 patients were given a second treadmill test similar to the first one, and the results of the platelet study and the exercise test from the two tests were compared.

3. *Platelet Study*

For the platelet study, 18 ml of blood was

collected from the patient's cubital vein without stasis and placed in a plastic syringe containing 2 ml of 3.8% sodium citrate solution. Platelet-rich plasma (PRP) was prepared from the citrated whole blood by centrifugation at 160g for 15 min at room temperature. The platelet count and volume in the blood were measured by a Coulter Counter (Model ZBI) with a Coulter Channelyzer (C-1000) with a 70 μ m aperture tube (Coulter Electronics, Hialeah, FL, USA). After adjusting the pH from 6.0 to 6.1, the PRP was centrifuged at 800g for 15 min and kept at room temperature for 30 min. For the platelet study of AMI, platelets in the pellet were suspended in Ca^{2+} , Mg^{2+} free Tyrode solution (137 mM NaCl, 2.7 mM KCl, 5.6 mM dextrose, 0.4 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 11.9 mM NaHCO_3 , pH 7.3) to a concentration of 3×10^5 platelets/ μ l. For the platelet study of EA, platelets in the pellet were suspended in the following buffer solution modified from Timmons and Hawiger's method⁹: 137 mM NaCl, 2.7 mM KCl, 5.6 mM dextrose, 1.0 mg/ml of bovine serum albumin, 2 units/ml of potato apyrase and 3.8 mM HEPES, pH 7.5. The suspension was kept at 37°C for 15 min and then centrifuged at 800g for 15 min. The platelets were resuspended in the buffer containing 3 mM CaCl_2 and no apyrase at a concentration of 3×10^5 platelets/ μ l.

To determine the TXA_2 synthesizing activity of platelets, the platelet suspension (180 μ l) was incubated with 20 μ l of arachidonic acid (AA) solution or thrombin of the appropriate concentration in the cuvette of the platelet aggregometer (Sienco, DP 247E, Morrison, CO, USA) at 37°C with stirring. Observing the platelet aggregation curve, 20 μ l of the suspension was collected at 0.5, 1 and 5 min after the addition of the aggregating agent. The collected suspension was mixed immediately with 780 μ l of radioimmunoassay buffer (1 M NaCl, 0.1% gelatin and 0.1 mM indomethacin in 0.1 M phosphate buffer, pH 7.3), rapidly frozen in methanol-dry ice and stored at -80°C for 2 to 3 weeks until the radioimmunoassay was performed. Antiserum against TXB_2 was purchased from Ono Pharmaceutical Co., Ltd. (Osaka, Japan). The procedure to measure TXB_2 was described previously.¹ The intensity of platelet aggregation was measured at 5 min after the addition of agents.¹⁰

4. *Reagents*

Arachidonic acid (Sigma, St. Louis, MO, USA)

Fig.1. Time courses of TXB₂ production (○-○) and aggregation (●-●) of platelets collected from healthy volunteers induced by various concentrations of arachidonic acid (AA) and thrombin.

was dissolved in a small amount of ethanol and diluted with sodium carbonate solution! Bovine thrombin and potato apyrase were purchased from Parke Davis (Ann Arbor, MI, USA) and Sigma, respectively. The other reagents employed were of the first grade.

5. Statistics

All results were expressed in terms of their mean $\pm$ standard error. Statistical analyses were performed by Student's *t* test.

RESULTS

1. TXA₂ Synthesizing Activity of Normal Platelets

Figure 1 shows the time course of TXB₂ production (dotted lines) and aggregation of platelets (solid lines) collected from healthy volunteers induced by various concentrations of AA and thrombin. When AA was added into the platelet suspension, amounts of TXB₂ in the supernatant increased depending on the incuba-

tion time and the doses of added AA. There were no platelet aggregation and elevation of TXB₂ at 0.5, 1 and 5 min after an addition of 3.2 μ M AA. When more than 6.3 μ M AA was added, the elevation of TXB₂ level in the supernatant was observed accompanied by an appearance of aggregation. Both phenomena occurred in parallel after additions of 6.3 and 12.5 μ M AA. When 25 μ M AA was added, the maximum production of TXB₂ was observed 5 min after. At the same time, platelet aggregation decreased. Aggregation curves induced by 25 and 50 μ M AA decreased, while TXB₂ productions were prominently observed. When 100 μ M AA was added, the aggregation curve recovered, while the TXB₂ production was decreased.

On the contrary, when a small amount of thrombin was used as the aggregating agent, platelets aggregated without TXB₂ production. More than 0.06 u/ml of thrombin were needed to induce platelet aggregation accompanied with TXB₂ production. When larger doses of thrombin were added, TXB₂ production increased in a

Fig. 2. TXA₂ synthesizing activity of platelets induced by AA in the time course of AMI. The production of TXB₂ from the platelets changed during the time course.

TABLE 1 AMOUNT OF TXB₂ PRODUCTION AND INTENSITY OF AGGREGATION OF PLATELETS COLLECTED FROM HEALTHY CONTROL AND AMI

	Healthy (n = 13)	AMI			
		6-12 Hrs (n = 2)	13 Hrs-4 Days (n = 10)	5-10 Days (n = 10)	11-34 Days (n = 16)
<i>AA 25 μM</i>					
TXB ₂ (ng/10 ⁸ plat)	189.6 ± 16.3	254.5 ± 44.5	137.7 ± 10.5*	242.4 ± 16.5**	154.6 ± 17.2
Aggregation (%)	47.4 ± 6.4	59.0 ± 4.0	70.9 ± 3.8*	50.4 ± 6.3	56.7 ± 7.4
<i>Thrombin 0.1 U/ml</i>					
TXB ₂ (ng/10 ⁸ plat)	65.2 ± 6.0	40.5 ± 2.5	119.7 ± 41.9	58.8 ± 6.9	65.5 ± 13.6
Aggregation (%)	81.8 ± 1.5	83.5 ± 0.5	84.3 ± 2.1	89.3 ± 0.7	82.9 ± 2.2

AMI: Acute Myocardial Infarction

*: $p < 0.05$, **: $p < 0.01$ Compared to the healthy control.

dose-dependent manner. At the same time, the aggregation curve reached the maximum level, and further enhancement of aggregation could not be detected.

When each of the aggregating agents was added to the platelet suspension, TXB₂ level increased sharply during the first 30 sec and reached a plateau after 5 min. From these results, we determined the TXA₂ synthesizing activity of platelets as the amount of TXB₂ in the supernatant 5 min after the addition of 25 μM AA or 0.1 u/ml of thrombin.

2. TXA₂ Synthesizing Activity of Platelets in AMI

Figure 2 shows changes in TXA₂ synthesizing activity of platelets induced by 25 μM AA in the time course of AMI. The shaded area represents the mean and SEM in the healthy controls. At 6 and 10 hrs after the onset of AMI, the production of TXB₂ from the platelets showed elevated values (299 and 210 ng/10⁸ platelets). From 12 hrs to 4 days after the onset, the production decreased. It increased again from 5 to 10 days and gradually returned to the normal value after 11 to 34 days after the onset. Platelet count and

Fig.3. Relationship between TXB_2 production and intensity of aggregation of platelets collected from AMI (●) and normal controls (○). A significant negative correlation was observed in the cases of AMI.

TABLE II CHANGES IN PLATELET COUNT, VOLUME, TXB_2 PRODUCTION AND AGGREGATION INDUCED BY TREADMILL EXERCISE

	Healthy		EA		EA Under Ticlopidine	
	Before	After	Before	After	Before	After
Count	33.7 ± 1.0	$35.4 \pm 1.2^*$	38.1 ± 2.8	$40.7 \pm 3.3^*$	43.2 ± 6.1	45.8 ± 7.4
Volume	5.3 ± 0.1	5.3 ± 0.1	5.2 ± 0.1	5.1 ± 0.1	5.2 ± 0.1	5.2 ± 0.1
AA 50 μM						
TXB_2 (ng/ 10^8 plat)	65.6 ± 9.5	64.5 ± 9.4	72.5 ± 3.3	69.9 ± 6.9	$55.8 \pm 4.3^\#$	58.3 ± 5.6
Aggregation (%)	81.8 ± 1.1	78.5 ± 3.0	77.3 ± 1.8	79.1 ± 2.7	$69.2 \pm 1.6^\#$	$69.2 \pm 1.8^\#$
Thrombin 0.5 U/ml						
TXB_2 (ng/ 10^8 plat)	38.0 ± 5.9	$31.8 \pm 5.0^*$	33.3 ± 6.1	34.9 ± 6.7	$22.5 \pm 4.2^\#$	$20.8 \pm 2.7^\#$
Aggregation (%)	81.4 ± 1.0	81.4 ± 1.1	78.3 ± 1.9	79.0 ± 2.8	78.3 ± 1.2	79.4 ± 1.6

EA: Effort Angina,

*: $p < 0.05$ between before and after.

#: $p < 0.05$ compared to the value without ticlopidine.

volume did not show significant changes.

Table I shows the changes in TXA_2 synthesizing activity and aggregation of platelets during the time course of AMI. TXA_2 synthesizing activity of platelets in the healthy controls (13 cases) was 189.6 ± 16.3 ng/ 10^8 platelets induced by 25 μM AA, and was 178.7 ± 11.6 in AMI (38 cases); there was no statistically significant

difference between these groups. However, there were significant changes in the amounts of TXB_2 during the time course of AMI. The amounts showed a significant decrease from 12 hrs to 4 days after and a significant increase from 5 to 10 days after the onset. On the other hand, platelet aggregation showed different changes. Aggregation elevated at the onset of AMI and

Fig. 4. Duration of exercise time and pressure rate product in 10 patients with EA obtained by symptom-limited treadmill exercise testing according to Bruce's protocol. Each patient was tested two times. The first one ($\circ$) was undertaken before ticlopidine-treatment and the second one ($\bullet$) after two week period of ticlopidine administration (300 mg/day). The mean of exercise time prolonged significantly after the ticlopidine-treatment (thick arrow). Pressure rate product did not change.

decreased during the time course of AMI. A significant negative correlation ($r = -0.59$, $p < 0.01$) was observed between the production of TXB_2 and the intensity of aggregation in the cases of AMI (Fig. 3). Such a correlation was not found in the healthy controls. Using thrombin (0.1 u/ml) as an aggregating agent, significant changes were not observed in the TXB_2 production and aggregation of platelets.

3. TXA_2 Synthesizing Activity of Platelets from Patients with EA During Exercise

Platelet count, volume, TXA_2 synthesizing activity and aggregation of platelets collected from 23 patients with EA and 15 healthy controls were measured before and just after the symptom-limited treadmill exercise testing. We added 50 μM AA and 0.5 u/ml of thrombin as aggregating agents to platelets suspended in the HEPES-Tyrode solution containing 3 mM Ca^{2+} and 0.1% bovine serum albumin. Using this system, we obtained better reproducibility in TXB_2 production, even with lower levels, than those measured by the method used in the AMI studies. As shown in Table II, platelet count increased significant in both EA and control groups after exercise ($p < 0.05$). Platelet volume did not change. In EA and healthy control groups, TXA_2 synthesizing activity induced by AA showed a slight decrease after the exercise.

Thrombin-induced TXA_2 synthesizing activity decreased significantly ($p < 0.05$) in the control, while it somewhat increased in EA after the exercise. The increase, however, was not statistically significant. Platelet aggregation did not show significant changes after the exercise in both groups.

Ten of the 23 EA patients were given ticlopidine (300 mg/day for 2 weeks) after the first exercise test, and a second treadmill test was performed during the ticlopidine-treatment. AA- and thrombin-induced TXA_2 synthesizing activities decreased significantly before the second exercise ($p < 0.05$) as well as after the exercise. Platelet aggregation induced by AA also decreased under the ticlopidine-treatment. In the second exercise test, exercise time was prolonged in 7 out of 10 patients (Fig. 4). On the average, the exercise time increased significantly ($p < 0.05$) from 294 ± 32 sec without ticlopidine to 348 ± 38 sec with ticlopidine. However, their pressure rate products were not changed by treatment with ticlopidine, and sigma ST was also not affected.

DISCUSSION

Several reports on measurements of platelet TXA_2 synthesis in vitro noted an enhancement in patients with MI^{11,12} EA¹² and diabetes mellitus¹³. It was also reported that TXB_2 production from platelets decreased within the first 48 hrs after AMI and was accompanied by a decrease in aggregation induced by AA in some patients¹⁴. In these observations, large amounts of AA of more than 500 μM were used as the aggregating agent. It is known that AA has a detergent effect¹⁵. We have observed the time course of TXB_2 production in platelet suspensions induced by various concentrations of AA and found a biphasic response in TXB_2 production and aggregation. When 100 μM AA was added to platelets, the amount of TXB_2 in the supernatant was smaller than those induced by less than 50 μM AA. We also found leakage of LDH from the platelets. Using the electron microscope to investigate this finding, we noted that the cytoplasm of the platelets had almost disappeared, showing injuries to the platelet membrane induced by AA¹⁶. Taking these results into consideration, we measured TXA_2 synthesizing activity of platelets as the amount of TXB_2 in the supernatant 5 min after the addition of 25 μM AA. Moreover, we used thrombin as

an aggregating agent, because thrombin on the PG metabolism in platelets is different from that of AA. Observing the time course of TXB₂ production from platelets induced by thrombin, a small dose of thrombin could aggregate platelets without production of TXA₂. According to these results, we used 0.1 u/ml of thrombin to determine the TXA₂ synthesizing activity of platelets.

The production rate of TXB₂ was changed according to the concentrations of Ca²⁺ and albumin in the suspension. When we used suspending solution containing more albumin, the platelets produced a lesser amount of TXB₂. In this condition, we could obtain better reproducibility increased in the presence of Ca²⁺ (3 mM). Because of this, we measured platelet TXA₂ synthesizing activity in EA during exercise using a system different from that used in the observations on AMI.

In AMI, we have found an increase in TXA₂ synthesizing activity of platelets during the super acute phase within the first 12 hrs from the onset. This suggests that TXA₂ has a role in the genesis of MI. Then the activity decreased at 4 days and again increased at 10 days after the onset. After this, it gradually recovered to the normal value. On the other hand, platelet aggregation showed a different pattern of change. It showed an elevation immediately after the onset and recovered gradually to the normal value during the time course. A significant negative correlation was observed between aggregation and TXB₂ production in platelets. The correlation was not observed in the healthy controls. We have reported that hypersensitive platelets were selectively consumed after the attacks of thrombosis, while the aggregation of circulating platelets with hyper TXA₂ synthesizing activity which may play an important role in the generation of MI are consumed selectively during the early phase of AMI. After the consumption, platelets showed a hypersensitive character as a whole again. Production of PGI₂ from the vessel walls may be partially related to this phenomenon.

In EA, platelets showed somewhat different behaviors. We could not find any differences in count, volume, aggregation and TXA₂ synthesis of platelets between EA patients and those in the healthy controls at the resting stage. After the treadmill exercise, platelet count increased significantly in both groups. This may be due to the release of platelets from the splenic pool by

epinephrine which was increased during the exercise. At the same time, AA-induced TXA₂ synthesizing activity slightly decreased in both the groups. It is suggested that the activity of platelets in the splenic pool may be weak compared to that of the circulating platelets. Thrombin-induced TXA₂ synthesizing activity also decreased in the control, while it increased in EA. There may be some factors which enhance platelet TXA₂ synthesis in coronary sclerosis.

We have reported earlier that platelet aggregation was enhanced in angina pectoris after Master's two step test¹⁸ and isometric exercise^{19,20}. This phenomenon was prevented by the pretreatment with antiplatelet drugs.¹⁸⁻²⁰ In the present observation, we could not find differences in aggregation after the exercise. Because large doses of aggregating agents were used to observe TXA₂ synthesizing activity mainly, the aggregation curves showed almost the maximum response in this measurement. In such conditions, it is hard to detect changes in aggregation by comparing the intensities before and after exercise. Considering the difference in responses induced by AA and thrombin, platelets collected from EA may be easily affected by a stimulation on the platelet membrane, where a mechanism related to the release of AA from membrane phospholipids is present.²¹

Ticlopidine, one of the potent antiplatelet drugs,²² inhibited the TXA₂ synthesizing activity of platelets. Although the preventive mechanism of ticlopidine on TXA₂ synthesis is yet not clear, it was reported that ticlopidine might indirectly affect PG synthesis by depressing the liberation of AA from membrane phospholipids through activation of cAMP production in platelets.²³ EA patients, under ticlopidine-treatment, could tolerate exercise for a longer time than they could without ticlopidine, without any change in the pressure rate products. After exercise, their TXA₂ synthesizing activity did not change markedly, and these values were significantly lower in comparison with those of same patients after exercise without ticlopidine. These results suggest that the function of platelets, especially their TXA₂ synthesizing activity, may be related to an appearance of anginal attacks. Since the pressure rate products did not change under the ticlopidine-treatment, changes in the microcirculation such as the appearance of small platelet aggregates, which can be inhibited by ticlopidine, may be impor-

tant in the occurrence of anginal attacks.

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