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Acta Medica Okayama

Volume 50, *Issue* 1 1996 *Article* 7

FEBRUARY 1996

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Abstract

Aggregation activity of platelets in umbilical blood is lower than that in adult blood, but the reason for this is not well understood. It has recently been clarified that calcium plays a role as a second messenger of platelet aggregation, and that glycoproteins of platelet surface membrane such as glycoprotein I b and IIb/IIIa are receptors for agonists inducing aggregation. We examined the concentrations of intracellular calcium and the membrane glycoproteins of platelets in umbilical and adult blood. The increase of intracellular calcium in umbilical platelets was lower than that in adult platelets when the aggregation was induced by ADP, collagen, thrombin and epinephrine. Only calcium ionophore A23187 induced aggregation of both umbilical and adult platelets. On the other hand, there were no qualitative differences between glycoproteins I b and IIb/IIIa of these two groups. Therefore, the low aggregation activity of umbilical platelets seems to be due to low responsiveness of the intracellular calcium system, not to the disorder of functional surface membrane glycoprotein.

KEYWORDS: umbilical blood, fura-2, calcium ion, platelet aggregation, glycoprotein

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*PMID: 8701781 [PubMed - indexed for MEDLINE]
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ACTA MED OKAYAMA 1996; 50(1): 47-52

Effect of Intracellular Free Calcium Mobilization on Aggregation of Umbilical Cord Blood Platelets

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Aggregation activity of platelets in umbilical blood is lower than that in adult blood, but the reason for this is not well understood. It has recently been clarified that calcium plays a role as a second messenger of platelet aggregation, and that glycoproteins of platelet surface membrane such as glycoprotein I b and IIb/IIIa are receptors for agonists inducing aggregation. We examined the concentrations of intracellular calcium and the membrane glycoproteins of platelets in umbilical and adult blood. The increase of intracellular calcium in umbilical platelets was lower than that in adult platelets when the aggregation was induced by ADP, collagen, thrombin and epinephrine. Only calcium ionophore A23187 induced aggregation of both umbilical and adult platelets. On the other hand, there were no qualitative differences between glycoproteins I b and IIb/IIIa of these two groups. Therefore, the low aggregation activity of umbilical platelets seems to be due to low responsiveness of the intracellular calcium system, not to the disorder of functional surface membrane glycoprotein.

Key words: umbilical blood, fura-2, calcium ion, platelet aggregation, glycoprotein

It is well recognized that the aggregation activity of umbilical blood platelets is lower than that of adult blood platelets (1). Certain functional differences, such as the hemocoaggulative or fibrinolytic capacities, are known to exist between adult and umbilical platelets (2). However, the exact mechanism underlying these observed differences has not been completely elucidated. It has been recognized that intracellular free calcium ion is important as a second messenger for the activation of adult platelets (3), and that intracellular free calcium ion is an important modulator of platelet aggregation. In the present study, we have investigated the correlation between platelet aggregation and the intracellular free calcium ion concentration in umbilical blood platelets and compared the results with those of adult controls.

Glycoproteins (GP), present on the outer surface of the platelet membrane, function in the aggregating and releasing reactions of these cells. So far, the major glycoproteins, GPIIb/IIIa and GP I b/IX, have been studied extensively. GPIIb and IIIa derive from a Ca^{2+}-dependent complex which serves both as the platelet fibrinogen receptor and as the principal site for high affinity Ca^{2+} binding on the platelet surface (4). GP I b, a receptor for von Willebrand factor, is thought to be most responsible for the platelet adhesion to the subendothelial matrix in a flowing system (5). We investigated the difference between GPs in umbilical and adult platelets by electrophoresis and immunoblotting.

Materials and Methods

Measurement of Platelet Aggregation and Intracellular Free Calcium Concentration ($[Ca^{2+}]_i$)

Preparation of platelets. In this study, appropriate-for-date fetuses at full-term delivery without maternal complications and full-term normal pregnant women were used and informed consent was obtained from all volunteers. Blood samples from adult antecubital vein and newborn umbilical vein blood before delivery of the placenta were withdrawn with plastic syringes containing 0.1 volumes of acid-citrate-dexrose solution (ACD;

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Terumo Co., Tokyo, Japan) as the anticoagulant. Platelet-rich plasma (PRP) was prepared by centrifugation of the blood at 120 × *g* for 15 min at room temperature after the addition of 0.01 volumes of apyrase (1 U/ml) (grade 5; Sigma Chemical Co., St. Louis, Mo, USA), followed by the separation of plasma and the platelet pellet. The platelet pellet was resuspended in Hepes-Tyrode's buffer (129 mM NaCl, 2.8 mM KCl, 0.8 mM KH_2PO_4, 8.9 mM $NaHCO_3$, 0.8 mM $MgCl_2$, 10 mM Hepes, 5.5 mM glucose, pH 7.4) at a concentration of 2×10^8 cells/ml. This suspension is referred to as the platelet suspension.

Measurement of platelet aggregation induced by agonists. Platelet aggregation was measured by recording light transmission using aggregometer (NBS HEMA TRACER MODEL 601; Niko Bioscience, Tokyo, Japan). The degree of aggregation (%) was standardized taking the light transmission of Hepes-Tyrode's buffer as 100 % and that of platelet suspension as 0 %. $CaCl_2$ was added at a final concentration of 1 mM before the addition of agonists. Aggregation was induced by adding either ADP (Boehringer-Mannheim, Mannheim, Germany) (1 μM), collagen (Hormon-Chemie, Munich, Germany) (1 μg/ml), thrombin (Sigma Chemical Co.) (0.5 U/ml), epinephrine (Sigma Chemical Co.) (10 μM), ristocetin (Sigma Chemical Co.) (1 mg/ml), or Ca^{2+} ionophore A23187 (Sigma Chemical Co.) (1 μM).

Fura-2 loading and measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured using fura-2. Platelets were loaded with fura-2 by incubating PRP for 20 min at 37 °C in the presence of fura-2 acetoxymethyl ester (Dojin Chemicals, Kumamoto, Japan) at a final concentration of 5 μM. The platelets were then centrifuged at 1500 × *g* for 10 min at room temperature after the addition of 0.01 volumes of 1 U/ml apyrase. The separated platelet pellet was resuspended in Ca^{2+}-free Hepes-Tyrode's buffer, pH 7.4. Fura-2-loaded platelets and washed platelets were then adjusted to a concentration of 2×10^8 cells/ml. Fura-2 fluorescence was measured at 37 °C, using a spectrofluorophotometer (MODEL FP-777; NIHONBUNKO, Tokyo, Japan). $CaCl_2$ was added at a final concentration of 1 mM before the addition of agonists. Fura-2-loaded platelets were mixed with each agonist at 30 sec after the addition of $CaCl_2$. The emission wavelength was 500 nm, and the excitation wavelengths were 340 nm and 380 nm. Maximal fluorescence was determined at the end of each incubation by the addition of 10 % Triton X-100. Minimal fluorescence was determined by the addition of 3 mM EGTA. $[Ca^{2+}]_i$ was calculated as described for fura-2 using Ca^{2+}-dye dissociation constants of 224 nM. All studies were performed within 4 h after blood sample collection.

Electrophoresis and Immunoblotting of Platelet Glycoprotein

Preparation of platelets. Umbilical blood samples from normal full-term infants and venous blood samples from healthy adults were analyzed for comparison. Whole blood (10 ml) was drawn with a syringe containing 1/7 volumes of ACD solution as the anticoagulant. PRP was obtained by separation of the supernatent after centrifugation of whole blood at 120 × *g* for 10 min at room temperature. PRP was centrifuged at 1500 × *g* for 10 min after the addition of 20 ng/ml of prostaglandin E_1 (PGE_1) (Sigma Chemical Co.), followed by the separation of plasma and platelet pellets. The platelet pellets were resuspended in phosphate-buffered saline (PBS; 80 mM Na_2HPO_4, 1.5 M NaCl, 20 mM KH_2PO_4, 30 mM KCl, pH 7.4) containing 10 mM EDTA. After the platelet count, this suspension was centrifuged at 1500 × *g* for 10 min, to separate the platelet pellets. The platelet pellets were lysed by resuspension in 100 μl of PBS/TW solution (1/15 M PBS with 0.05 % Tween 20) containing 5 mM EDTA to 1×10^8 platelets and complemented with 0.1 volumes of 10 % Triton-X. This platelet lysate was centrifuged at 10,000 × *g* for 15 min and the resulting supernatant was separated. The supernatant samples were used immediately or were stored at −20 °C.

Electrophoresis. Samples were mixed with a half-volume of a buffer solution containing 36 % sucrose solution, 8.4 % sodium dodecyl sulfate (SDS), 0.26 M Tris-HCl (pH 6.8) and 0.01 % bromophenol blue (BPB) and heated at 100 °C for 10 min. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (6) using 10 % SDS-polyacrylamide gels at a constant current of 40 mA for 90 min.

Immunoblotting. Immunoblotting was performed by the method of Burnette (7) using 0.45-μm pore polyvinylidene fluoride (PVDF) membranes (Millipore Corp.). Following electrophoresis, gels were equilibrated in Tris-glycine buffer (25 mM Tris, 192 mM glycine, 10 % SDS, pH 8.3) containing 20 % methanol for 10 min and then in a transfer buffer containing 25 mM Tris, 192 mM glycine and 9 % methanol for 10 min. The PVDF membrane was then placed in contact with the gel, and air bubbles between the gel and the PVDF membrane were

carefully removed. The membrane/gel was then placed into the electroblotting device (ISS-semidry electroblotter; Daiichi Chemicals, Tokyo, Japan) and electrophoretic transfer was performed at 24 V (7 V/cm) for 4 h. After the transfer, the membrane was washed by three 10-min immersions in PBS/TW. Then, the PVDF membrane was incubated overnight at 4 °C with 10 mM Tris, 1 M NaCl, and 3 % skim milk, pH 7 (blocking buffer). After blocking, the membrane was washed three times as above. The PVDF membrane was immersed in a primary monoclonal antibody (anti-GP I b, Takara Biomedicals, Kyoto, Japan; anti-GPIIb/IIIa, CMN Biomedicals, Ohtsu, Japan) solution (10 μg/ml in 0.5 % skim milk/PBS). After 1 h at 37 °C, the membrane was washed three times with PBS/TW as above. The PVDF membrane was then probed with the indicated primary antibodies, and the bound antibodies were detected using a VECTASTAIN ABC KIT (Vector Laboratories, Burlingame, CA, USA) using peroxidase-labeled anti-mouse IgG (10 μg/ml; Hyclone Laboratories, Tokyo, Japan) as the secondary antibody. For the development of color bands, the PVDF membrane was incubated with a mixture of avidin and biotinylated peroxidase (HYPERFILM-ECL: Western blotting, Amersham Japan).

Results

Platelet aggregation. Platelet aggregation in response to agonists (ADP, collagen, thrombin, epinephrine, ristocetin and A23187) were studied with paired adult and umbilical platelets as shown in Table 1. Platelet aggregation induced by ADP, collagen, thrombin, epinephrine and ristocetin in the umbilical blood platelets were significantly lower ($P < 0.05$ or $P < 0.01$) than those in adult blood. However, there was no significant difference in platelet aggregation induced by A23187 between umbilical and adult blood platelets.

Changes in $[Ca^{2+}]_i$ caused by agonists. Figure 1 shows typical changes in $[Ca^{2+}]_i$ after stimulation with various agonists in fura-2-loaded paired adult and umbilical blood platelets. Each one of them showed a response to agonists at a final concentration under the same conditions. Elevation of $[Ca^{2+}]_i$ caused by ADP, collagen and thrombin in umbilical blood platelets decreased significantly compared to that in the blood of adults. In response to stimulation by epinephrine, $[Ca^{2+}]_i$ was scarcely elevated in the umbilical blood platelets, while the adult blood platelet $[Ca^{2+}]_i$ increased substantially. In response to stimulation by ristocetin, no change was observed in $[Ca^{2+}]_i$ in either adult or umbilical platelets.

Increase of $[Ca^{2+}]_i$ caused by agonists. Table 2 shows the resting $[Ca^{2+}]_i$ and the increase of $[Ca^{2+}]_i$ (Δ $[Ca^{2+}]_i$) caused by stimulation with various agonists in fura-2-loaded paired adult and umbilical blood platelets. As shown in Table 2, the resting $[Ca^{2+}]_i$ in the umbilical blood platelets was significantly lower than that in adult blood platelets. The Δ $[Ca^{2+}]_i$ in the umbilical platelets in response to stimulation by collagen and thrombin were also significantly lower than that in adult platelets. In response to stimulation by ADP and epinephrine, Δ $[Ca^{2+}]_i$ in the umbilical platelets tended to decrease compared to that in adult platelets. The Δ

Table 1. Platelet aggregation rate induced by agonists[a]

Subjects	ADP (n = 25) (1 μM)	Collagen (n = 32) (1 μg/ml)	Thrombin (n = 13) (0.5 U/ml)	Epinephrine (n = 15) (10 μM)	Ristocetin (n = 19) (1 mg/ml)	A23187 (n = 6) (1 μM)
Adult blood platelets (%)	45.9 ± 22.5	70.5 ± 15.8	62.2 ± 23.8	60.2 ± 16.2	61.8 ± 11.4	66.0 ± 8.5
	*	**	**	**	**	
Umbilical blood platelets (%)	31.5 ± 20.1	47.8 ± 26.6	34.6 ± 28.1	34.9 ± 27.3	48.4 ± 19.5	68.2 ± 5.1

[a]: Platelet aggregations induced by agonists in paired adult and umbilical platelets were measured. Aggregation was determined as % light transmission at 3 min after the addition of agonists taking Hepes-Tyrode's buffer as 100% and platelet suspention as 0%. Each value represents the mean ± SD. Statistically, significant difference was derived by Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$.

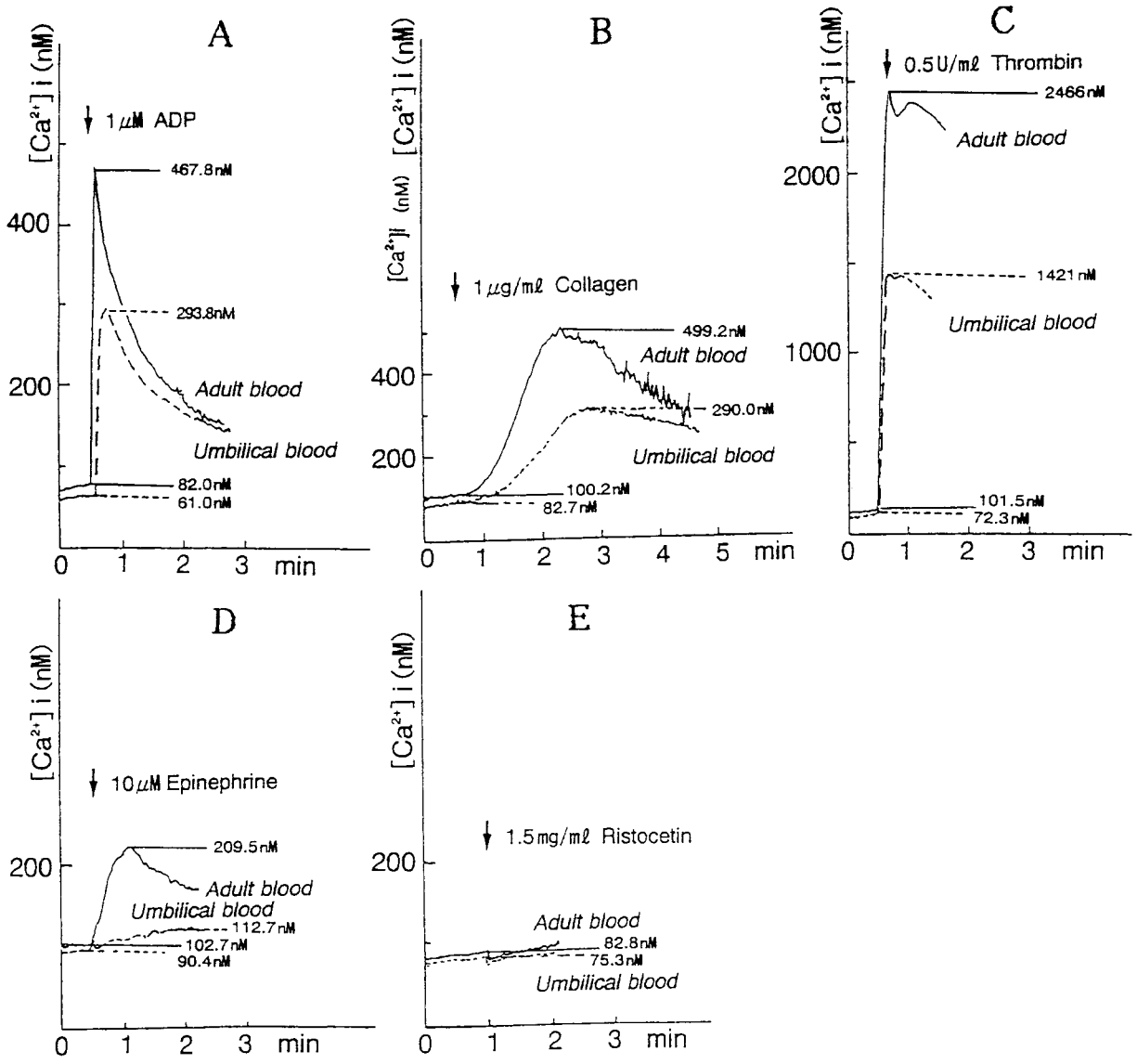

Fig. 1 Changes in $[Ca^{2+}]_i$ caused by agonists. Typical changes in platelet intracellular calcium concentration after each agonist's stimulation in fura-2-loaded paired adult and umbilical blood platelets are demonstrated. Solid lines represent the adult blood platelet. Dashed lines represent the umbilical blood platelet. Fura-2-loaded platelets were mixed with each agonist at a final concentration in the presence of 1 mM external Ca^{2+}. Each agonist was added at 30 sec following resting levels. Agonists used were: **A**, ADP; **B**, collagen; **C**, thrombin; **D**, epinephrine and **E**, ristocetin.

$[Ca^{2+}]_i$ in both adult and umbilical platelets in response to stimulation by epinephrine was at a lower level than those caused by other agonists (collagen, thrombin, ADP). The $[Ca^{2+}]_i$ in adult and umbilical platelets did not change after stimulation by ristocetin and remained at resting levels.

Platelet glycoprotein. Immunoprecipitation of glycoproteins from adult and umbilical platelets is shown in Fig. 2. Bands corresponding to GP I b and GPIIb and IIIa obtained by immunoprecipitation did not show any

Table 2. Increase of $[Ca^{2+}]_i$ (Δ $[Ca^{2+}]_i$) caused by agonists[a]

Subjects	Resting $[Ca^{2+}]_i$ (n = 21)	Δ $[Ca^{2+}]_i$				
		ADP (n = 19) (1 μM)	Collagen (n = 15) (1 μg/ml)	Thrombin (n = 19) (0.5 U/ml)	Epinephrine (n = 8) (10 μM)	Ristocetin (n = 6) (1 mg/ml)
Adult blood platelets (nM)	86.9 ± 20.1	211.5 ± 129.8	323.3 ± 128.4	1641.4 ± 1296.3	42.8 ± 27.3	no change
	**		**	*		
Umbilical blood platelets (nM)	61.9 ± 13.6	161.5 ± 114.1	177.9 ± 91.6	911.8 ± 403.1	28.1 ± 5.9	no change

[a]: The resting $[Ca^{2+}]_i$ and the increase of $[Ca^{2+}]_i$ (Δ $[Ca^{2+}]_i$) caused by stimulation with various agonists in fura-2-loaded paired adult and umbilical blood platelets were measured. Each value represents the mean ± SD. Statistically significant differences were evaluated by Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$

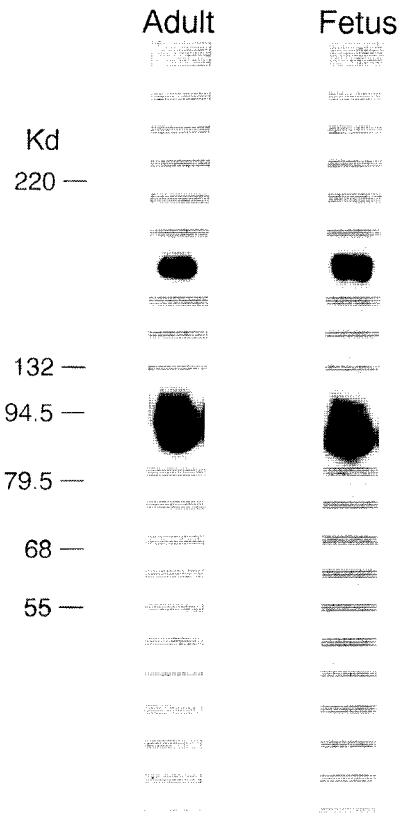

Fig. 2 Immunoprecipitation of glycoproteins from adult and umbilical platelets. Electrophoresis and immunoblotting were performed under nonreducing conditions. Fragments of GP I b (above) and GP II b and IIIa (below) in adult and umbilical platelets are immunoprecipitated.

difference between adult and umbilical platelets.

Discussion

Transient physiological alterations have been described in many components of the newborn's blood, *i.e.*, decreased plasma coagulation factors, red cell enzyme changes, and low immunoglobulins (8). This hypofunction may be defense mechanisms against intravascular coagulation (1). The suppression of platelet functions including aggregation and the release reactions have long been recognized to be present in neonatal platelets when compared to maternal platelets, and it has been recognized that a rise in $[Ca^{2+}]_i$ is important as a second messenger for platelet activation. The platelet aggregation activity and Δ $[Ca^{2+}]_i$ induced by ADP, collagen, thrombin and epinephrine in the umbilical platelets were lower than those in adult platelets. Ca^{2+} ionophore A23187 does not activate platelets through the surface receptor system and crosses directly platelet membranes into the platelet cytoplasm, that is, A23187 directly changes the membranous transmission, increases Ca^{2+} influx, and consequently $[Ca^{2+}]_i$ becomes elevated (9). There was no significant difference in platelet aggregation induced by A23187 between umbilical and adult platelets. These results suggest that, in response to stimulation by some agonists, Δ $[Ca^{2+}]_i$ in umbilical platelets decreases compared to that in adult platelets. As a result of suppression of platelet activation, the aggregation activity in umbilical platelets decreases compared to that in adult platelets. The decrease of Δ $[Ca^{2+}]_i$ in umbilical platelets may be related to the suppression of phos-

pholipase C activation and subsequent formation of inositol 1,4,5-trisphosphate or thromboxane A_2 synthesis or may be associated with the agonist or agonist-receptor coupling. In response to stimulation by epinephrine, $\Delta [Ca^{2+}]_i$ in umbilical platelets decreased compared to that in adult platelets, but $\Delta [Ca^{2-}]_i$ in both were evidently lower than those caused by ADP, collagen or thrombin stimulation. These findings suggest that activation by epinephrine, unlike ADP, collagen or thrombin stimulation, is associated with Ca^{2+}-independent pathways to platelet activation. Following stimulation with ristocetin, $[Ca^{2+}]_i$ did not change in either adult or umbilical platelets, and remained at resting levels. Ristocetin, in the presence of human factor VIII and a specific platelet membrane receptor, causes clumping of human platelets. On the platelet aggregation induced by ADP, collagen, thrombin or epinephrine, platelet adhesion is caused by platelet membranous changes or arachidonic acid metabolism as a result of the exteroceptive stimulus. In other words, "aggregation" is dependent on platelet energy metabolism. On the other hand, the platelet reaction caused by stimulation with ristocetin, unlike stimulation with ADP, collagen, thrombin or epinephrine, is independent of platelet energy metabolism and extracellular ADP, which is usually associated with platelet agglutination (10). Ristocetin-induced agglutination does not require the intra-platelet reactions, but is caused by electric potential changes on the platelet membrane, and it is speculated that agglutination is related to a Ca^{2+}-independent mechanism.

The relationship between suppression of platelet aggregation and suppression of $\Delta [Ca^{2+}]_i$ in umbilical blood compared to that in adult platelets can be explained as follows. The response of the umbilical platelet receptors is lower than that of adults, for example, the difference of platelet glycoproteins between umbilical blood and adult blood. The difference in stimulation-mediated mechanism on the platelet membrane or in cytoplasm, the difference of Ca^{2+} influx through Ca^{2+} channels, or Ca^{2+} release from the intracellular Ca^{2+} storage sites, are all possible explanations. As a result of suppression of $\Delta [Ca^{2+}]_i$ in umbilical platelets, the aggregation activity in umbilical platelets decreases compared to that in adult platelets. There is a report stating that the amount of platelet GP I b and GPIIb and IIIa in umbilical blood was not significantly different from that of adult platelets (11). In the present report, the possibility that there are no qualitative differences in GP I b and GPIIb and IIIa between umbilical and adult platelets was suggested. Furthermore, the resting $[Ca^{2+}]_i$ levels in the umbilical platelets were lower than those in adult platelets. This result suggests that umbilical platelets and adult platelets are already in different states before activation. We found no reports on $[Ca^{2+}]_i$ in the resting state in umbilical or adult platelets. In the present study, normal pregnancy without maternal complications was studied. Studies in the hyper-coagulative state such as toxemia or the hypo-coagulative state remain to be investigated. Much more research is needed to elucidate the difference between umbilical and adult platelets.

Acknowledgements. The author is deeply indebted to Prof. Toshihiko Ubuka, Department of Biochemistry, Okayama University Medical School, for his suggestions.

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Received October 18, 1995; accepted January 5, 1996.