INTERACTION OF PLATELETS WITH COLLAGEN SUBSTRATE: ROLE OF PLATELET PROSTAGLANDIN ENDOPEROXIDES AND THROMBOXANE A_2

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UDC 616-005.6-092:[616.155.25-008.939.959.6:616-008.939.629

KEY WORDS: thrombus formation; adhesion of platelets; prostaglandins; collagen substrate.

Prostaglandin (PG) endoperoxides and thromboxane A_2 (TA_2), formed in platelets during metabolic conversions of arachidonic acid (AA), are powerful inducers of changes of shape and aggregation of platelets in suspension [6, 7]. AA appears in platelets as a result of its removal from membrane phospholipids through the action of specific phospholipases [2, 4] or on the addition of exogenous AA to platelets. AA is converted successively in platelets into PG-endoperoxides and TA_2 in reactions catalyzed by cyclo-oxygenase and thromboxane synthetase, respectively [7, 12]. The formation of PG endoperoxides and TA_2 is an essential stage in platelet activation by exogenous AA [3, 6, 7]. These same metabolites, formed from endogenous AA, potentiate platelet aggregation, stimulated by ADP, collagen, thrombin, and other inducers, in suspension [3, 8]. The action of AA and its derivatives on changes in shape and aggregation of platelets in suspension has been investigated in detail by photometric aggregometry [3, 5-7].

Interaction between platelets and a collagen-coated surface is a convenient model with which to study the mechanisms of formation of juxtamural thrombi. The various stages of interaction of platelets with a collagen substrate can be analyzed quantitatively by scanning electron microscopy: 1) initial attachment of platelets to the substrate, 2) changes in shape of the adherent platelets induced by substrate and by soluble inducers (conversion of discoid platelets into spherical and their spreading), 3) the formation of stratified (thrombus-like) aggregates bound with the substrate [1, 11].

In the investigation described below the role of PG-endoperoxides and TA_2 in interaction of platelets and a collagen-coated surface was studied. For this purpose the effects of exogenous AA, of U46619, a stable analog of PG endoperoxides, which simulates the action of PG endoperoxides and TA_2 on platelets at the receptor level [5], and of aspirin, a cyclo-oxygenase inhibitor [13], on platelet–surface interaction were investigated.

EXPERIMENTAL METHOD

Blood was taken from the cubital vein of healthy blood donors, using dextrose acid citrate as the anticoagulant [11]. Platelet-enriched plasma was obtained by centrifugation of the blood at 180 g for 12.5 min at 20°C. Platelets were separated from plasma by gel-filtration on Sepharose 2B by the method in [14]. Platelets were eluted with Tyrode solution without Ca^{++} and Mg^{++}, but containing 0.35% bovine serum albumin (from "Sigma," USA). $CaCl_2$ and $MgCl_2$ were added to the platelets after gel-filtration to final concentrations of 2 and 1 mM, respectively. The platelet concentration was determined with a PL-100 TOA automatic platelet counter (from "Medical Electronics," Japan). The platelets were incubated with the collagen substrate and interaction between platelets and the substrate was subjected to morphometric analysis as described previously [1, 11]. A commercial preparation of acid-soluble collagen from calf skin (No. C3511, from "Sigma") was broken up into fibrils and adsorbed on the bottom of 16.4-mm wells of "Multiwell" cultural plates (from "Falcon," USA). Gel-filtered platelets in a volume of 200 µl ($1 \cdot 10^7$ to $4 \cdot 10^7$ cells) were added to the wells and incubated for 30 min at 37°C in a "Dubnoff Metabolic Shaking Incubator" (CCA, from "Precision Scientific," USA),

Institute of Experimental Cardiology, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR E. I. Chazov.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 98, No. 11, pp. 563-566, November, 1984. Original article submitted April 22, 1984.

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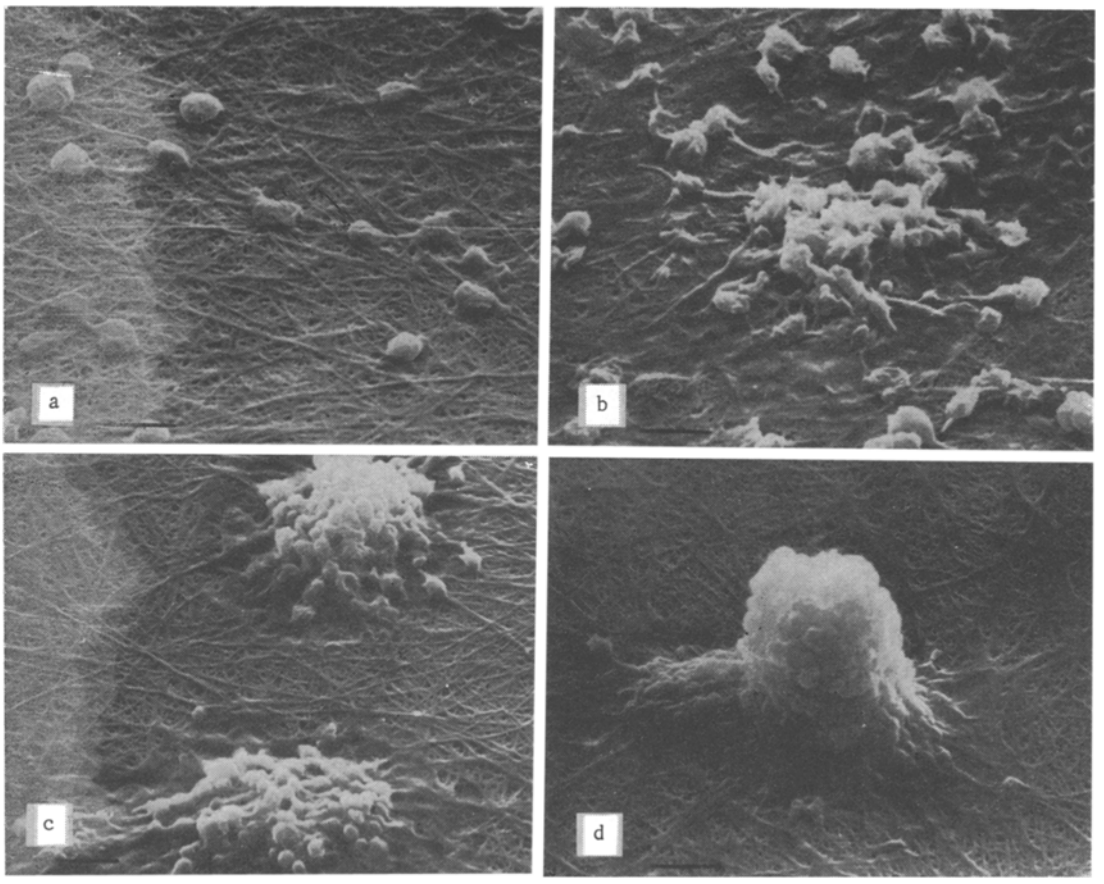

Fig. 1. Spread out platelets and formation of thrombus-like aggregates on surface of collagen substrate, stimulated by AA and U46619. a) Incubation in absence of AA and U46619. Adherent platelets are mainly single discoid and spherical forms of platelets with pseudopodia, not spread out. Scale: 5 μ; b) incubation in presence of 200 μM AA. Fields of confluent, spread out platelets cover the surface of fibrillary collagen. Areas of collagen substrate free from spreading platelets can also be seen. On surface of spread out platelets there are single platelets which have not spread, and also aggregates. Thrombus-like aggregate consisting of partially and completely confluent platelets located in center. Scale: 5 μ; c) incubation in presence of 1 μM U46619. Two platelet-like aggregates consisting of several layers of partly and completely confluent platelets can be seen. Sheets of spread out platelets at base. Scale: 10 μ; d) incubation in presence of 200 μM AA. Height of thrombus-like aggregate and degree of fusion of components of platelet aggregates increased with an increase in the rate of mixing. Sheet of spread out platelets at base. Platelets incubated with collagen substrate without mixing (a, b) and with mixing at a speed of 20 (c) and 40 cycles/min (d). Scale: a, b) 5 μ, c, d) 10 μ. Scanning electron microscopy.

with or without mixing of the platelets, by shaking the "Multiwell" backward and forward in the horizontal plane at a speed of 20-60 cycles/min. AA and U46619 (from "Upjohn," USA) were added immediately after the platelets to the wells, whereas aspirin (from "Sigma") was added 3-5 min before addition of the platelets. Interaction between platelets and substrate was analyzed on a "Phillips" PSEM 500X electron microscope (The Netherlands). In the absence of AA and U46619 the number of platelets adherent to 1 mm^2 of collagen substrate was counted and the percentages of discoid (native) platelets, spherical platelets containing pseudopodia, and spread out platelets were calculated. In the presence of AA and U46619 the area covered by spread out platelets was determined and expressed as a percentage of the total area of the substrate, and the number of thrombuslike aggregates per mm^2 of substrate was determined.

The quantity of TA_2 synthesized in the platelets was determined by measuring accumulation of its stable product TB_2. After adhesion of the platelets the incubation medium was removed,

TABLE 1. Effect of Aspirin on Adhesion of Platelets to Collagen Substrate and on Substrate-Induced Change in Shape of Adherent Platelets (M ± m)

Dose of Aspirin, mM	Number of platelets adherent to 1 mm² of collagen substrate	Shape of platelets		
		discoid	spherical	spread out
		% of number of adherent platelets		
0	5400±1200	16,7±10,5	75,2±10,3	8,1±5,3
1	4600±600	41,3±11,4*	57,4±11,4	1,3±0,8*

Legend. No soluble platelet inducers (AA and U46619) were present in incubation medium. Platelets incubated with collagen substrate without mixing. Significance of effects of aspirin calculated by Student's paired t test. *P < 0.05.

TABLE 2. Stimulation of Spreading out of Platelets and Formation of Thrombuslike Aggregates on Surface of Collagen Substrate by AA and U46619 (M ± m)

Inducer	Area of substrate covered with spread out platelets, %	Thrombus-like aggregates per mm² of substrate
Absent	6,1±4,0	0
AA, 200 μM	45,8±7,2*	197±29**
U46619, 1 μM	43,7±4,3	216±53**

Legend. Spread out platelets studied in absence of mixing of platelet suspension, but formation of thrombus-like aggregates studied after mixing at 40 cycles/min. Significance of stimulating effects of AA and U46619 determined by Student's t test for means. *P < 0.005, **P < 0.001.

nonadherent platelets were destroyed by freezing and thawing, and TB_2 was determined by radioimmunoassay [15].

EXPERIMENTAL RESULTS

A surface coated with commercial fibrillary collagen from calf skin was used as the substrate for platelet adhesion. Compared with highly purified human collagens of types I and III, this substrate has relatively low ability to induce changes of shape and aggregation of platelets. In its adhesive properties it occupies an intermediate position between collagens of basement membranes (types IV and V) [9], which are located closer to the lumen of the vessel [10]. Platelets adherent to a surface of collagen from calf skin are mainly single discoid and spherical forms (Fig. 1a, Table 1). Spread out platelets account for about 10% of the total number of adherent platelets and they cover about 6% of the area of collagen (Table 2).

Incubation of platelets with a collagen-coated surface in the presence of AA or U46619 led to marked stimulation of spreading of the platelets. Spread out platelets joined together to form extensive areas on the surface of the collagen substrate (Fig. 1b) which covered up to 40-50% of the area of the substrate (Table 2). Single platelets, not spread out, and also aggregates were present on the surface of the spread out platelets (Fig. 1b). AA and U46619 also stimulated the formation of large stratified (thrombus-like) aggregates, which were separate from each other, consisted of confluent platelets, and contained mainly sheets of spread out platelets (Fig. 1, b-d). In the absence of soluble inducers thrombus-like aggregates were never formed on this type of collagen substrate (Table 2).

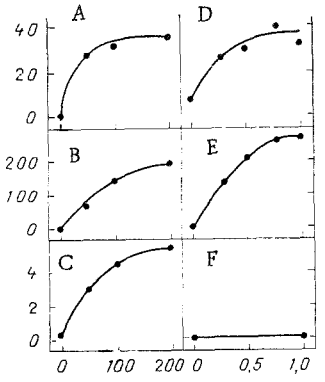

Fig. 2

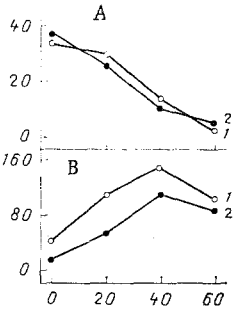

Fig. 3

Fig. 2. Dependence of effects of AA (A-C) and U46619 (D-F) on spreading of platelets (A, D), formation of surface thrombus-like aggregates (B, E), and TB_2 formation in platelets (C, F). Abscissa, concentration (in μM) of AA (A-C) and U46619 (D-F): ordinate, area of substrate (in %) covered by spread out platelets (A, D), number of thrombus-like aggregates per mm^2 of substrate (B, E), and TB_2, in nanomoles/10^6 platelets (C, F). Platelets incubated with collagen substrate without mixing (A, C, D, F) or with mixing at a speed of 40 cycles/min (B, E). C, F) Mean results of three determinations.

Fig. 3. Effect of mixing on spreading of platelets (A) and formation of thrombus-like aggregates (B) on surface of collagen substrate stimulated by 200 μM AA (1) and 1 μM U46619 (2). Abscissa, speed of shaking "Multiwell" plates in horizontal plane (number of cycles/min); ordinate, area of substrate (in %) covered with spread out platelets (A) and number of thrombus-like aggregates per mm^2 of substrate (B). Mean results of two experiments.

Depending on the concentration of exogenous AA the area covered by spread out platelets and the number of thrombus-like aggregates bound with the surface increased parallel to stimulation of synthesis of platelet TA_2 (Fig. 2A-C). U46619 activated interaction between platelets and the surface (Fig. 2D, E) by a different mechanism, without TA_2 formation (Fig. 2F), simulating the action of PG endoperoxides and of TA_2 at the receptor level [5].

The effectiveness of stimulation of spreading and formation of thrombus-like aggregates depended on the rate of mixing during incubation of platelets with the collagen substrate. Maximal spreading was observed in the absence of mixing. An increase in the rate of mixing to 60 cycles/min led to an approximately sevenfold decrease in the area covered by spread out platelets (Fig. 3A). The number of thrombus-like aggregates bound with the surface increased with an increase in mixing speed, to reach a maximum at 40 cycles/min (Fig. 3B). These effects were evidently due to gradual predominance of platelet–platelet interaction over platelet–substrate interaction with an increase in the speed of mixing. Thus in the presence of AA and U46619, by changing the conditions of incubation of platelets with substrate it was possible to stimulate selectively either spreading out of platelets (in the absence of mixing) or the formation of platelet-like aggregates (on mixing at a speed of 40 cycles/min).

The effects of aspirin, a cyclo-oxygenase inhibitor, on interaction between platelets and the collagen-coated surface were investigated in both the presence and the absence of exogenous AA. Aspirin in a concentration of 1 mM completely prevented spreading of platelets, the formation of thrombus-like aggregates bound with the surface, and TA_2 synthesis induced by AA (Table 3).

In the absence of soluble inducers aspirin did not change the number of platelets adherent to collagen (Table 1), i.e., it did not affect the process of initial adhesion of inactivated platelets from the suspension to the substrate. Meanwhile aspirin inhibited substrate-induced changes in shape of adherent platelets, by increasing the percentage of discoid (native) platelets and reducing the percentage of spread out platelets. The number of spherical platelets showed no significant change (Table 1). Inhibition of substrate-induced changes in shape of adherent platelets by aspirin is evidence that AA metabolites, namely PG endoperoxides and TA_2, take part in this process.

TABLE 3. Inhibition of Spreading of Platelets, Formation of Thrombus-Like Aggregates Bound with the Surface, and TA_2 Synthesis Induced by AA by Means of Aspirin

Dose of aspirin, mM	Area of substrate covered with spread out platelets, %	Thrombus-like aggregation per mm^2 substrate	TA_2 synthesis, nmoles TB_2/ 10^9 platelets
0	19,8	94	1,10
1	2,3	0	0,07

Legend. AA concentration 150 mM. Spreading of platelets and TB_2 formation measured in absence of mixing, formation of thrombus-like aggregates on mixing at a speed of 40 cycles/min. Mean results of two determinations shown.

The results indicate that the above-mentioned AA metabolites stimulate not only processes characteristic of platelet activation in suspension, namely conversion of discoid platelets into spheres with pseudopodia and interaction between platelets which have not spread out (aggregation), but also processes specific for interaction of platelets with a solid surface, such as spreading out of platelets and deposition of platelet aggregates on spread out platelets. The action of aspirin on platelet adhesion in the absence of soluble inducers suggests that initial adhesion of unactivated platelets to the substrate is not accompanied by stimulation of metabolism of endogenous AA, and that substrate-induced changes of shape depend on conversion of AA into PG endoperoxides and TA_2.

LITERATURE CITED

1. V. L. Leitin and D. D. Sviridov, in: The Cell Wall in Atherogenesis and Thrombogenesis, eds. E. I. Chazov and V. N. Smirnov [in Russian], Moscow (1982), p. 155.
2. R. L. Bell, D. A. Kennerly, N. Stanford, et al., Proc. Natl. Acad. Sci. USA, 76, 3238 (1979).
3. L. C. Best, T. K. Holland, B. B. Jones, et al., Thromb. Haemostas., 43, 38 (1980).
4. T. K. Billis, J. B. Smith, and M. J. Silver, J. Clin. Invest., 60, 1 (1977).
5. G. Di Minno, V. Bertelle, I. Bianchi, et al., Thromb. Haemostas., 45, 103 (1981).
6. M. Hamberg, J. Svensson, and B. Samuelsson, Proc. Natl. Acad. Sci. USA, 72, 2994 (1975).
7. M. Hamberg, J. Svensson, T. Wakabayashi, et al., Proc. Natl. Acad. Sci. USA, 71, 345 (1974).
8. R. L. Kinlough-Rathbone, M. A. Packham, H. J. Reimers, et al., J. Lab. Clin. Med., 90, 5037 (1977).
9. V. L. Leitin (V. L. Leytin), F. Misselvitz, S. P. Domogatsky, et al., J. Cell. Biol., 97, 464a (1983).
10. J. A. Madri, B. Dreyer, F. A. Pitlick, et al., Lab. Invest., 43, 303 (1980).
11. A. V. Mazurov, V. L. Leitin (V. L. Leytin), V. S. Repin, et al., Thromb. Res., 32, 189 (1983).
12. P. S. Needleman, S. Moncada, S. Bunting, et al., Nature, 261, 588 (1976).
13. G. J. Roth, N. Stanford, and P. W. Majerus, Proc. Natl. Acad. Sci. USA, 72, 3073 (1975).
14. O. Tangen, H. J. Berman, and P. Marfey, Thromb. Diathes. Haemmorh. (Stuttgart), 25, 268 (1971).
15. C. Taube, P. Hoffman, and W. Forster, Prost. Leukotr. Med., 9, 411 (1982).