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Role of Glycoprotein IIb-IIIa (αIIbβ3-integrin) in Stimulation of Secretion from Platelet Granules

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Abstract—The involvement of glycoprotein (GP) IIb-IIIa (αIIbβ3-integrin) in the stimulation of secretion from platelet dense and α-granules was investigated. Fibrinogen binding with GP IIb-IIIa and platelet aggregation were inhibited by fragments of anti-GP IIb-IIIa monoclonal antibodies (monAB)—Fab fragment of antibody c7E3 (preparation ReoPro) and $F(ab')_2$ fragment of antibody FraMon (preparation FRAMON). Suppression of GP IIb-IIIa receptor activity by both preparations led to 100% inhibition of [^{14}C]serotonin secretion from dense granules upon platelet activation with ADP, to partial inhibition upon activation with thromboxane A_2 analog U46619 (by 60-70%) and thrombin at 0.1 U/ml (by 40-50%), but did not decrease serotonin secretion induced by thrombin at 1 U/ml. ReoPro and FRAMON completely inhibited ADP-induced release of soluble P-selectin from platelet α-granules, but did not influence P-selectin secretion stimulated by U46619 and by both thrombin concentrations. MonAB CRC54 against GP IIb-IIIa, which induced its interaction with fibrinogen and platelet aggregation, also stimulated serotonin and P-selectin secretion. Both types of release reactions were completely suppressed by ReoPro and FRAMON. Aspirin, the cyclooxygenase inhibitor, also prevented CRC54-induced secretion, proving the dependence of this process on thromboxane A_2 synthesis. Upon platelet activation by concanavalin A (Con A), caused by clusterization of membrane glycoproteins, GP IIb-IIIa blockade only slightly (by 15-20%) decreased serotonin secretion. High level of Con A-induced secretion was also detected in a patient with hereditary deficiency of GP IIb-IIIa. Thus, neither clusterization nor occupation of GP IIb-IIIa are essential for the stimulation of Con A-induced release reaction. The data indicate that GP IIb-IIIa binding with fibrinogen leads to the stimulation of secretion from platelet granules. When the level of secretion does not depend on GP IIb-IIIa interaction with the ligands or its presence on platelets full-scale release reaction is presumably stimulated by activating signals formed without GP IIb-IIIa involvement.

Key words: platelets, glycoprotein IIb-IIIa, secretion, serotonin, P-selectin

Platelet membrane glycoprotein (GP) IIb-IIIa complex (αIIbβ3-integrin) serves as a receptor for fibrinogen, von Willebrand factor, and several other RGD-containing adhesive proteins. GP IIb-IIIa is present in inactive form on the surface of resting platelets and gains the ability to bind protein ligands only after platelet activation by such agonists as ADP, thromboxane A_2, thrombin, etc. Agonists bind with their receptors on platelet membrane and initiate transduction and spreading of the activating signal within the platelet via second messenger system (intracellular calcium, cyclic nucleotides, different kinases, etc.). This signal stimulates changes of the cytoskeleton, synthesis of arachidonic acid metabolites, secretion from different types of granules, and other reactions. Platelet activation also induces changes in the cytoplasmic tails of GP IIb-IIIa, which cause conformational changes in the extracellular part of the protein and exposure of the ligand binding site of this receptor (inside-out signaling). (The molecular nature of the signal that affects cytoplasmic domains and stimulates GP IIb-IIIa receptor activity remains unclear.) Subsequent binding of polyvalent protein ligands, fibrinogen in particular, with GP IIb-IIIa leads to the formation of molecular bridges between activated platelets and to their aggregation. The interaction of ligands with GP IIb-IIIa also induces formation of signals that are transduced into the platelet and promotes further platelet activation (outside-in signaling). Representatives of several tyrosine kinase families are involved in the spreading of this signal and activation of these systems requires not only occupation but also clusterization of GP IIb-IIIa, which occurs upon its interaction with polyvalent proteins. It is supposed that signal initiated due to the GP IIb-IIIa occupation and clusterization plays essential roles in such reactions as platelet spreading, irreversible aggregation, and clot

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retraction (see reviews [1-3]). However the role of the signal transduced into the platelet by GP IIb-IIIa in the stimulation of secretion from intracellular granules remains uncertain. It is still unclear whether this signal is required for the induction of full-scale release reaction upon platelet activation by different agonists and whether it in itself is able to initiate secretion from different types of platelet granules.

In the present study the participation of GP IIb-IIIa in the formation of the signal stimulating platelet release reactions has been investigated. The effects of agents, which inhibit or stimulate GP IIb-IIIa, on the secretion from dense granules and α-granules have been studied. Dense granules contain low molecular weight compounds (serotonin, ADP, calcium, etc.) and α-granules contain protein components (fibrinogen, von Willebrand factor, platelet derived growth factor, and others) [4].

MATERIALS AND METHODS

Reagents and antibodies. ADP, human thrombin, concanavalin A (Con A), and aspirin were from Sigma (USA), thromboxane A_2 analog, U46619, from ICN (USA), bovine serum albumin from Serva (Germany), [^{14}C]serotonin (5-hydroxy-2-[^{14}C]tryptamine creatinine sulfate) from Amersham (USA), and streptavidin-peroxidase from IMTEK (Russia). Other reagents were from Sigma, ICN, Merck (Germany), or Reakhim (Russia).

Anti-GP IIb-IIIa monoclonal antibody (monAB) FraMon (earlier used name CRC64) inhibits interaction of GP IIb-IIIa with fibrinogen and other ligands and GP IIb-IIIa dependent platelet aggregation [5-7]. Preparation FRAMON is the $F(ab')_2$ fragment of monAB which could effectively inhibit platelet aggregation in a same way as antibody FraMon [5, 7-9]. Preparation ReoPro (Centocor/Eli Lilly, USA) is the Fab fragment of recombinant chimeric antibody c7E3 (chimeric 7E3), consisting of variable parts of anti GP IIb-IIIa murine monAB 7E3 and constant parts of human IgG. ReoPro in a same way as FRAMON inhibits GP IIb-IIIa receptor activity and platelet aggregation [10, 11]. MonAb CRC54 is directed against LIBS (Ligand-Induced Binding Site) epitope in GP IIIa [6, 12]. Upon interaction with GP IIb-IIIa this antibody changes the conformation of GP IIb-IIIa, stimulating binding of high molecular weight ligands with this receptor and subsequent platelet aggregation [6].

Platelets. Platelet rich plasma (PRP) was obtained from the blood of healthy donors using 3.8% sodium citrate as anticoagulant at the ratio blood/anticoagulant 9 : 1. The blood was centrifuged at 150*g* for 10 min at room temperature, PRP was collected, and the remaining blood was centrifuged at 1000*g* for 15 min at room temperature. Plasma was used for dilution of PRP to necessary platelet concentration ($2.5 \cdot 10^8$ per ml). When serotonin concentration was studied [^{14}C]serotonin was added to PRP at the concentration of 2 μM and incubated with PRP for 20 min at 37°C. Washed platelets from the blood of healthy volunteers were obtained and labeled with [^{14}C]serotonin as described earlier [13]. Washed platelets were also obtained from the blood of a previously characterized patient with Glanzmann's thrombasthenia (hereditary deficiency of GP IIb-IIIa) [14].

Platelet aggregation. Platelet aggregation and secretion from granules were studied in PRP or washed platelet suspension at platelet concentration of $2.5 \cdot 10^8$ and $3 \cdot 10^8$ per ml, respectively. [^{14}C]Serotonin-labeled platelets were used when serotonin secretion was investigated. Platelet suspension (300 μl) was added into the cuvette of the aggregometer (BIOLA Ltd, Russia) and incubated at 37°C and stirring at 800 rpm. Platelets were activated by different agonists at 30 sec after starting the incubation. In PRP platelets were activated by ADP (20 μM), U46619 (2 μM), and monAB CRC54 (450 μg/ml) and washed platelets were activated by thrombin (0.1 and 1.0 U/ml) and Con A (100 μg/ml). Thrombin and Con A were added to washed platelets because after addition to PRP thrombin caused formation of a fibrin clot and Con A interacted not only with platelet surface proteins but also with many blood plasma glycoproteins. In preliminary experiments it was shown that concentrations of CRC54 and Con A used in this study (450 and 100 μg/ml, respectively) were saturating and caused maximal platelet aggregation. After addition of ADP, U46619, and thrombin, platelets were incubated in the aggregometer cuvette for 4.5 min, and after addition of CRC54 and Con A, which induce slower aggregation, for 9 min. In respective control samples platelets were incubated without inducers also for 4.5 and 9 min. Aggregation was followed by changes of light transmission (% T) in the platelet suspension. When the effects of the inhibitors of platelet aggregation (ReoPro, FRAMON, aspirin) were studied platelets were preincubated with these preparations for ≥2 min at 37°C. The same incubation regimens were used for secretion measurements.

Serotonin secretion. After incubation of platelets (300 μl of PRP or washed platelets) with the agonists and registration of aggregation (see above) 60 μl of ice cold 100 mM EDTA solution (pH 7.4) was added into the aggregometer cuvette. Platelet suspension was taken from the cuvette, platelets were spun down at 10,000*g* for 5 min and the supernatant was collected for subsequent [^{14}C]serotonin determination. Total amount of [^{14}C]serotonin in platelet suspension (incorporated into platelets + free) was determined after platelet lysis (300 μl of PRP or washed platelets + 60 μl of 100 mM EDTA solution) with 1% SDS (final concentration) for 5 min at room temperature. For determination of free, not incorporated into platelets,

[^{14}C]serotonin its amount was measured in the supernatant obtained after addition of 60 μl of 100 mM EDTA to 300 μl of platelet suspension and sedimentation of platelets at 10,000*g* for 5 min. Before the measurement of radioactivity all samples containing platelet supernatant were supplemented with 1% SDS. The amount of [^{14}C]serotonin incorporated into platelets was calculated as total minus free [^{14}C]serotonin and this value was considered as 100%. The amount of released serotonin was estimated as [^{14}C]serotonin in the supernatant of activated platelets minus free [^{14}C]serotonin and was expressed in percent of platelet incorporated [^{14}C]serotonin. The amount of free [^{14}C]serotonin in PRP was not higher than 10% and in washed platelet suspension not higher than 3% of total [^{14}C]serotonin. When [^{14}C]serotonin secretion was measured in PRP in all samples protein was precipitated with trichloroacetic acid (TCA). TCA was added to the samples at final concentration of 5%, samples were incubated for 10 min at 4°C, non-dissolved material was precipitated (5 min at 10,000*g*) and [^{14}C]serotonin was measured in the supernatants. Measurements of the radioactivity were performed in an LKB counter (USA) using ZhS-8 scintillation cocktail (Reakhim, Russia).

Secretion of soluble P-selectin. After incubation with agonists and measurement of platelet aggregation (see above) platelets were spun down at 10,000*g* for 5 min. Supernatant was collected and centrifuged at 100,000*g* for 60 min at 4°C for the precipitation of possible contamination of membrane P-selectin containing membrane microparticles. Determination of soluble P-selectin in the 100,000*g* supernatants was performed using previously developed sandwich ELISA [15].

RESULTS

Inhibitors of GP IIb-IIIa receptor activity, FRAMON and ReoPro, at the concentration of 10 μg/ml completely blocked platelet aggregation induced by physiological platelet activators—ADP, thromboxane A_2 (substituted by its stable analog U46619), or thrombin (Fig. 1). Inhibition of platelet aggregation induced by ADP was also accompanied with the full inhibition of secretion from dense granules which was registered by [^{14}C]serotonin release. Upon platelet activation with U46619 release of serotonin was inhibited by FRAMON and ReoPro by 60-70% and upon activation with 0.1 U/ml of thrombin—by 40-50%. When platelets were activated with thrombin at 1.0 U/ml serotonin secretion was not decreased in the presence of GP IIb-IIIa blockers (Table 1). Secretion from α-granules was followed by the release from platelets of the soluble form of cell adhesion molecule P-selectin. When platelets were activated with ADP, GP IIb-IIIa antagonists completely suppressed P-selectin secretion. The amount of soluble P-selectin in the plasma obtained after sedimentation of platelets activated with ADP in the presence of FRAMON and ReoPro was significantly lower than in the absence of GP IIb-IIIa antagonists and approximately the same as in plasma collected after sedimentation of control non-activated platelets (Table 2). However, upon platelet activation with other inducers, U46619 and thrombin at both concentrations, the level of P-selectin secretion was not decreased in the presence of GP IIb-IIIa blockers (Table 2).

MonAB CRC54 interacting with GP IIb-IIIa changes its conformation and stimulates fibrinogen binding to GP IIb-IIIa and subsequent aggregation [6]. CRC54-induced aggregation, like aggregation induced by physiological inducers, was completely blocked by GP IIb-IIIa antagonists (Fig. 2). Studies of secretion have shown that CRC54 stimulated not only platelet aggregation but also release reactions—secretion of serotonin from dense granules and soluble P-selectin from α-granules (Tables 1 and 2, respectively). Both types of CRC54-induced secretion were less expressed than those induced

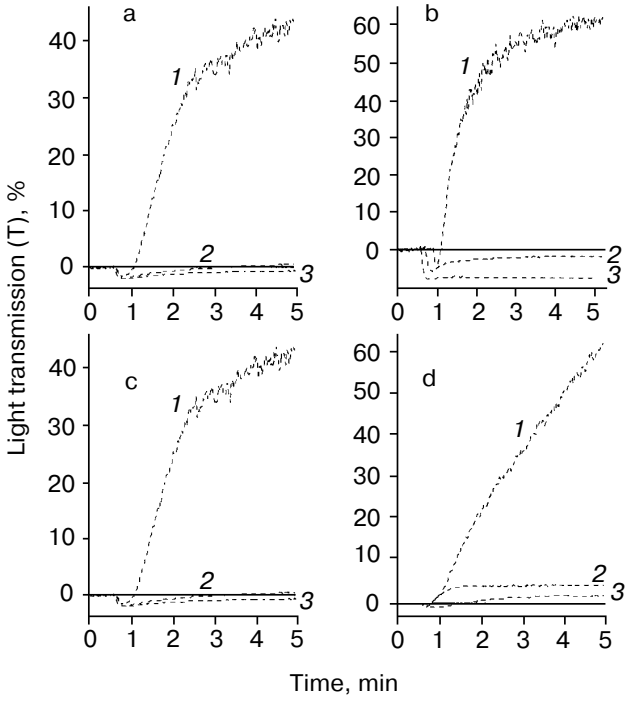

Fig. 1. Inhibition of platelet aggregation by GP IIb-IIIa antagonists—FRAMON and ReoPro. Platelets in PRP (a, b) and washed platelets (c, d) were preincubated without additions (curves *1*) or in the presence of 10 μg/ml of FRAMON (curves *2*) or 10 μg/ml of ReoPro (curves *3*) and then platelet aggregation was induced by 20 μM ADP (a), 2 μM U46619 (b), and 0.1 U/ml (c) or 1.0 U/ml thrombin (d).

Table 1. Effects of GP IIb-IIIa antagonists on serotonin secretion

Inducers of platelet activity	Secretion of [^{14}C]serotonin, % of platelet incorporated		
	without GP IIb-IIIa antagonists	FRAMON	ReoPro
ADP	57.2 ± 10.9	0.6 ± 0.6 $p = 0.022$	0 ± 0 $p = 0.021$
U46619	44.5 ± 4.1	13.9 ± 2.6 $p = 0.010$	6.3 ± 3.2 $p = 0.004$
CRC54	15.3 ± 5.1	0 ± 0 $p = 0.040$	1.0
Thrombin (0.1 U/ml)	52.0 ± 7.1	23.2 ± 12.6 $p = 0.019$	25.7
Thrombin (1.0 U/ml)	80.4 ± 2.7	75.2 ± 3.2 $p = 0.080$	73.5
Con A (100 μg/ml)	44.8 ± 9.4	37.6 ± 10.4 $p = 0.010$	n.d.

Note: Mean ± SEM for $n \geq 4$ (4-7) and mean values for $n < 4$ (2-3) are presented. p, significance of differences between the level of [^{14}C]serotonin secretion in the absence of GP IIb-IIIa antagonists and in the presence of FRAMON and ReoPro estimated for $n \geq 4$ (paired t-test); n.d., not determined. Secretion of [^{14}C]serotonin in control samples (incubation without inducers of platelet activity) did not exceed 2-3% of platelet incorporated [^{14}C]serotonin.

by activation of platelets with physiological agonists (ADP, U46619, and thrombin) and were blocked by FRAMON and ReoPro (Tables 1 and 2). CRC54-induced secretion from both types of granules was also completely suppressed in the presence of aspirin (Fig. 3), which inhibited synthesis of thromboxane A_2 in platelets [16]. Inhibition of so-called second wave of platelet aggregation was also observed after addition of aspirin (Fig. 2). This wave is developed as a result of the autocrine action of platelet-synthesized thromboxane A_2 and secreted from dense granules endogenous inducers of aggregation like ADP and serotonin [4]. Thus, these data indicated that effects of CRC54 on secretion processes depend on the formation of thromboxane A_2 in platelets.

It is known that platelet activation after addition of Con A is caused by the clusterization of membrane protein receptors, which interact with this lectin [17-19], including GP IIb-IIIa [19, 20]. Con A-stimulated aggregation of washed platelets was only partially inhibited by FRAMON and ReoPro (Fig. 4) and so was only partially dependent on the interaction of GP IIb-IIIa with fibrinogen, which corresponded to our previous results [13]. (Residual aggregation that did not depend on GP IIb-IIIa receptor activity represented passive agglutination of platelets caused by interaction of the polyvalent lectin with glycoproteins localized on the surface of adjacent platelets [13].) Con A-induced platelet activation and aggregation were accompanied by secretion of serotonin from dense granules. This secretion was decreased upon inhibition of GP IIb-IIIa receptor activity by FRAMON by only 15-20% (Table 1). To evaluate the contribution of not only ligand binding but also of the clusterization of GP IIb-IIIa in the stimulation of Con A-induced release reactions, we measured serotonin secretion from platelets of the patient with Glanzmann's thrombasthenia—the hereditary deficiency of GP IIb-IIIa. Despite the absence of GP IIb-IIIa addition of Con A caused agglutination of the patient's platelets. The level of this agglutination was about the same as the level of agglutination of platelets

Table 2. Effects of GP IIb-IIIa antagonists on soluble P-selectin secretion

Inducers of platelet activity	Soluble P-selectin, ng/ml		
	without GP IIb-IIIa antagonists	FRAMON	ReoPro
PRP			
ADP	293 ± 28	168 ± 23 $p = 0.009$	145 ± 42 $p = 0.010$
U46619	376 ± 33	318 ± 43 $p = 0.152$	344 ± 59 $p = 0.172$
CRC54	242 ± 29	151 ± 21 $p = 0.007$	150
Without inducers	180 ± 24	n.d.	n.d.
Washed platelets			
Thrombin (1.0 U/ml)	159 ± 15	159 ± 25 $p = 0.987$	148 ± 19 $p = 0.232$
Thrombin (0.1 U/ml)	149 ± 22	125 ± 27 $p = 0.305$	144 ± 41 $p = 0.412$
Without inducers	19 ± 7	n.d.	n.d.

Note: Soluble P-selectin was determined in plasma (PRP) or in the incubation medium (washed platelets). Mean ± SEM for $n \geq 4$ (4-7) and mean values for $n < 4$ (3) are presented. The amount of soluble P-selectin in plasma or incubation medium in the presence of inducers of platelet activity (without GP IIb-IIIa antagonists) in all cases was higher than without inducers ($p < 0.05$, paired t-test). p, significance of differences between concentrations of soluble P-selectin in the absence of GP IIb-IIIa antagonists and in the presence of FRAMON and ReoPro estimated for $n \geq 4$ (paired t-test); n.d., not determined.

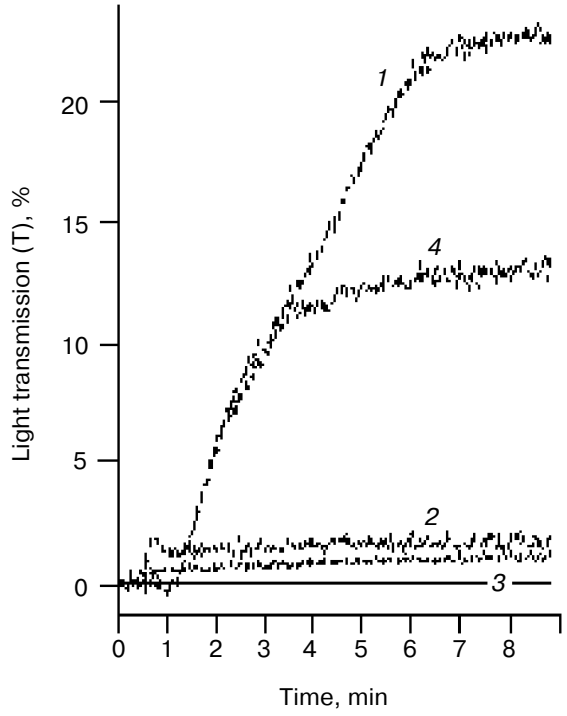

Fig. 2. Effects of FRAMON, ReoPro, and aspirin on platelet aggregation induced by monAB CRC54. Platelets (PRP) were preincubated without additions (curve *1*) or in the presence of 10 μg/ml FRAMON (curve *2*), 10 μg/ml ReoPro (curve *3*) or 1 mM aspirin (curve *4*) and then aggregation was stimulated with 450 μg/ml CRC54.

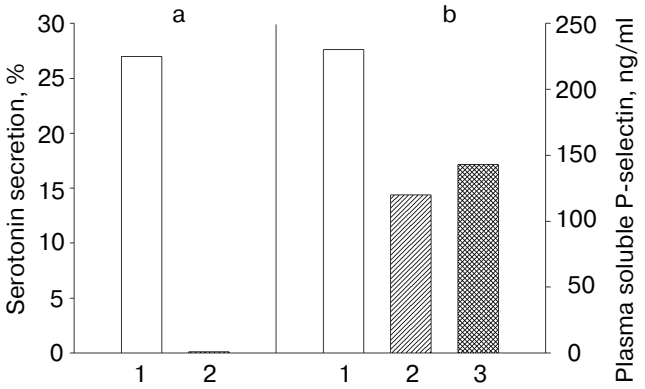

Fig. 3. Effects of aspirin on serotonin (a) and soluble P-selectin secretion (b) induced by monAB CRC54. Platelets (PRP) preincubated in the absence (*1*, *3*) or in the presence of 1 mM aspirin (*2*), were placed in the aggregometer cuvette, 450 μg/ml CRC54 (*1*, *2*) or buffer (*3*) was added to platelets, platelets were incubated for 9 min and then secretion of [^{14}C]serotonin (a) or plasma concentration of soluble P-selectin (b) were measured. Means of two experiments are presented.

from healthy donor registered upon blockade of GP IIb-IIIa receptor activity (Fig. 4). Con A-induced serotonin secretion from the patient's platelets was relatively high, 47% of incorporated, and so did not significantly differed from the mean level of secretion from platelets of healthy donors (Table 1). Thus neither occupation nor clusterization of GP IIb-IIIa is essential for the stimulation of the secretory response upon Con A-induced platelet activation.

DISCUSSION

The involvement of GP IIb-IIIa in the stimulation of secretion from dense and α-granules has been studied in the present investigation. Secretion from dense granules was registered by measuring [^{14}C]serotonin release from preliminarily labeled platelets and secretion from α-granules – by measuring the amount of the soluble from of cell adhesion molecule P-selectin in the incubation medium. Earlier it has been shown by us [15] and subsequently by other authors [21] that soluble form of P-selectin, platelet α-granule specific protein [22], is secreted upon platelet activation by different agonists and therefore this protein could be used as a marker of secre-

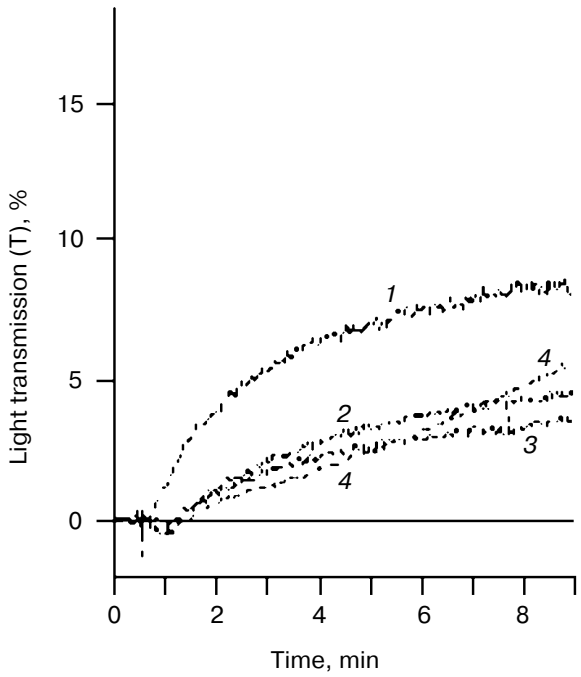

Fig. 4. Con A-induced platelet aggregation upon blockade of receptor activity and deficiency of GP IIb-IIIa. Washed platelets of a healthy donor (curves *1-3*) and a patient with Glanzmann's thrombasthenia (curve *4*) were preincubated without additions (curves *1* and *4*) or in the presence of 10 μg/ml FRAMON (curve *2*) or ReoPro (curve *3*) and then aggregation was stimulated by 100 μg/ml Con A.

tion from this type of granules. Soluble P-selectin is normally present in blood plasma [23, 24], but this does not interfere with the determination of its secretion in PRP since the amount of the protein secreted from platelets is comparable with its plasma concentration [15].

Binding of GP IIb-IIIa with fibrinogen and other ligands and platelet aggregation mediated by these interactions was inhibited by anti-GP IIb-IIIa monAB fragments—$F(ab')_2$ fragment of murine antibody FraMon [5-7] and Fab fragment of recombinant chimeric antibody c7E3 consisting of variable domains of murine monAB 7E3 and constant domains of human IgG [11, 12]. Both fragments are used as drugs in clinical practice for prevention of thrombosis—preparation FRAMON [7-9] and ReoPro [11, 12], respectively. FRAMON and ReoPro in saturating concentrations used in this study effectively and identically inhibited platelet aggregation. However, since these antibodies are directed against different epitopes in GP IIb-IIIa [9], in most experiments where their action on release reactions was studied we used both agents.

In the first part of the study we investigated the action of FRAMON and ReoPro on the secretion from granules stimulated by physiological agonists—ADP, thromboxane A_2 (using its stable analog U46619), and thrombin. All these inducers activate platelets interacting with specific receptors on platelet membrane. Signal transduction through these receptors within the cell stimulates conformational changes of GP IIb-IIIa. Due to these changes this receptor acquires the ability to bind RGD-containing ligands, the most important of which are fibrinogen and von Willebrand factor. Binding of polyvalent proteins with GP IIb-IIIa leads to the formation of molecular bridges between activated platelets and to their aggregation [1-3]. When platelets are activated in PRP, adhesive proteins in surrounding plasma are involved in the formation of platelet aggregates and when washed platelets are activated in the absence of plasma these proteins are preliminarily released from activated platelets along with other granule components [4]. Results obtained in this study demonstrated that suppression of aggregation in the presence of GP IIb-IIIa blockers might be accompanied by a decrease of secretion from platelet granules. When platelets were activated by ADP, FRAMON and ReoPro completely suppressed not only platelet aggregation but also secretion from dense and α-granules. These data indicated that upon ADP action on platelets generation of the signal that stimulates granule secretion is caused not by ADP binding to its receptor but by subsequent interaction of fibrinogen and probably other ligands with GP IIb-IIIa. When platelets were activated by thromboxane A_2 analog and by thrombin in low concentration (0.1 U/ml) GP IIb-IIIa blockers only partially inhibited secretion from dense granules. Obviously in this case for the realization of maximal secretion beside the signal transduced through the thromboxane A_2 and thrombin receptor, additional signal generated by the GP IIb-IIIa–ligand interaction is required. Secretion from α-granules induced by U46619 and by low concentration of thrombin as well as secretion from both types of granules induced by high concentration of thrombin concentration was not reduced in the presence of GP IIb-IIIa antagonists. Thus, in these cases the realization of full-scale secretion was achieved just by the action of agonists and did not required the involvement of GP IIb-IIIa.

Unlike physiological agonists used above monAB CRC54 affects GP IIb-IIIa directly and by changing its conformation stimulates fibrinogen binding and subsequent aggregation [6]. In the present study it was shown that CRC54 induced not only aggregation but also secretion from both types of platelet granules. These results pointed out that binding of fibrinogen with GP IIb-IIIa itself could be sufficient for the initiation of secretory response. Both GP IIb-IIIa antagonists inhibited by 100% secretion of serotonin and soluble P-selectin demonstrating the essential role of GP IIb-IIIa occupation in the stimulation of CRC54-induced release reactions. The ability to induce release reaction from dense granules was previously demonstrated for other anti-GP IIb-IIIa activating antibody, LIBS1 [25], whose binding site differed from CRC54 epitope [6]. Interestingly, monAB D3GP3, which is also able to stimulate fibrinogen binding with GP IIb-IIIa [26] and which interacts with an epitope different from that of CRC54 [12, 27], failed to activate platelets and stimulate secretion from platelet granules [26, 28]. The reason for such differences in the action of these antibodies remains unclear. In the present study using monAB CRC54 it was shown for the first time that antibodies that can stimulate GP IIb-IIIa activity are also able to induce secretion not only from dense but also from α-granules. CRC54-induced serotonin and soluble P-selectin secretion was suppressed in the presence of aspirin—a cyclooxygenase inhibitor that blocks synthesis of thromboxane A_2 in platelets [16]. These data are in line with the results obtained upon investigation of the effects of another activating antibody, LIBS1; inhibition of thromboxane A_2 synthesis prevented secretion from dense granules induced by this antibody [25]. These data prove that signals generated as a result of GP IIb-IIIa–fibrinogen interaction affects the process of intracellular granule exocytosis not directly but by induction of the synthesis of thromboxane A_2 that activates platelets via autocrine mechanism and consequently stimulates secretory reactions.

Our results obtained upon investigation of the effects of anti-GP-IIb-IIIa antibody fragments on the secretion from dense granules are mainly in line with the results obtained previously with the preparation ReoPro [29, 30] that was also employed in the present study as well as with other blockers of GP IIb-IIIa receptor activity—monAB 10E5 [31] and RGD-like peptidomimetic Ro 44-9883 [32]. These agents also suppressed secretion from dense

granules when platelets were activated by ADP [29-32] or low doses of more powerful inducers like thrombin, collagen, and some others [30-32]. Our investigations have shown that preparation FRAMON could also inhibit secretion from dense granules. The involvement of GP IIb-IIIa in the generation of the signal stimulating this type of secretion from dense granules was also confirmed by the data obtained in the studies of secretion in patients with Glanzmann's thrombasthenia (hereditary deficiency of GP IIb-IIIa). Platelets derived from these patients are characterized by the decreased secretion from dense granules upon their activation by ADP and some other agonists [32, 33].

The literature data on the effects of GP IIb-IIIa antagonists on the secretion from α-granules are more contradictory. According to some authors ReoPro [29, 30] and RGD-like peptidomimetics Ro 44-9883 [32] and SR121566A [30] are able to inhibit secretion from α-granules. However, according to other results GP IIb-IIIa antagonists including ReoPro either do not influence exocytosis of that type of granules [34] or even stimulate this process [35, 36]. Such inconsistency might be explained by different experimental conditions such as incubation in whole blood or PRP, the nature and the concentrations of platelet agonists used in different studies and also by application of different methods for the measurement of granule exocytosis. For example, in papers where increased exocytosis of α-granules was registered in the presence of ReoPro [35, 36] platelets were activated in whole blood and the process of exocytosis was evaluated using flow cytometry by expression on the platelet surface of the membrane form of P-selectin—the marker of α-granule membranes [22]. Under such conditions the suppression of platelet–platelet interaction in the presence of ReoPro might lead to the binding of the most activated platelets containing the highest amount of P-selectin on their surface to white blood cells and that is why these platelets would not be taken into account in analysis. According to our data ADP-induced secretion of soluble P-selectin was inhibited by 100% by FRAMON and ReoPro. Moreover, we have shown for the first time that activating antibody CRC54, which directly stimulates fibrinogen binding with GP IIb-IIIa, could also stimulate secretion from α-granules. Thus our results as well as results obtained by other authors suggest that the signal initiated by the interaction of fibrinogen with GP IIb-IIIa could be also involved in the stimulation of the secretion from platelet α-granules.

We also studied dense granule secretion upon platelet activation with Con A. This lectin activates platelets via clusterization of different platelet surface proteins including GP IIb-IIIa [17-20]. Release of P-selectin was not studied in this case since Con A itself could bind to this protein [13], and that is why it could influence the processes of its release from platelets as well as the results of ELISA. Con A added to washed platelets stimulated serotonin secretion. This result is consistent with previous data obtained in our laboratory [13] and by other authors [17]. However, clusterization of GP IIb-IIIa and its occupation by the ligands upon addition of Con A are apparently not essential for the stimulation of the secretory response. The level of Con A-induced serotonin secretion was only slightly decreased by GP IIb-IIIa blockade (by 15-20%) and was still high in a patient with hereditary GP IIb-IIIa deficiency. The data suggested that Con A could support a high level of secretion even without GP IIb-IIIa involvement—by clusterization and transduction of activating signal through other receptors. It is well known that Con A is not highly specific and could interact not only with GP IIb-IIIa, but with many other proteins on platelet surface [13]. This is also confirmed by the ability of Con A to stimulate agglutination of platelets from a patient with GP IIb-IIIa deficiency observed in this study. It is also known that, despite the absence of GP IIb-IIIa, Con A is able to stimulate formation of phosphoinositides [37] and phosphorylation of several activation proteins [19] in platelets of patients with Glanzmann's thrombasthenia. Taken together these data indicated the involvement of other, distinct from GP IIb-IIIa, membrane proteins in the formation and transduction of Con A-induced signals, which presumably support platelet activation and secretion from granules in the absence of GP IIb-IIIa or upon blockade of its receptor activity.

Thus the results obtained in this study demonstrated that binding of GP IIb-IIIa with fibrinogen and probably with other ligands leads to the stimulation of secretion from both dense and α-granules. When the level of secretion does not depend on GP IIb-IIIa interaction with its ligands or its presence in platelets full-scale secretion could be presumably supported by other signals transduced within the cell without GP IIb-IIIa participation.

In conclusion, it should be noted that GP IIb-IIIa antagonists used in this study, FRAMON and ReoPro, are antithrombotic drugs applied for the prevention and therapy of thrombotic complications in coronary angioplasty [8-11]. The fact that these agents are able to inhibit not only platelet aggregation but granule secretion as well might be clinically significant. It is known that platelets release compounds that might affect cells in the vessel wall and in particular stimulate proliferation of smooth muscle cells, a process that is significant for vessel restenosis—the main remote complication of angioplasty [29, 38].

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REFERENCES

1. Clemetson, K. J. (1995) *Thromb. Haemost.*, **74**, 111-116.
2. Shatill, S. J., Kashiwagi, H., and Pampori, N. (1998) *Blood*, **91**, 2645-2657.

3. Shatill, S. J. (1999) *Thromb. Haemost.*, **82**, 318-325.
4. Majerus, P. W. (2000) in *The Molecular Basis of Blood Diseases* (Stamatoyannopoulos, G., Majerus, P. W., Perlmutter, R. M., and Varmus, H., eds.) W. B. Sanders Company, Philadelphia-London-New York-St. Louis-Sydney-Toronto, pp. 764-790.
5. Byzova, T. V., Vlasik, T. N., and Mazurov, A. V. (1994) *Byul. Eksp. Biol. Med.*, No. 10, 402-405.
6. Mazurov, A. V., Khaspekova, S. G., Byzova, T. V., Tikhomirov, O. Yu., Berndt, M. C., Steiner, B., and Kouns, W. C. (1996) *FEBS Lett.*, **391**, 84-88.
7. Mazurov, A. V., Pevzner, D. V., Khaspekova, S. G., Dudnik, O. A., Byzova, T. V., Vlasik, T. N., Bakharev, V. N., Piscov, M. Yu., Staroverov, I. I., and Ruda, M. Ya. (2001) *Ter. Arkhiv*, No. 9, 66-73.
8. Mazurov, A. V., Pevzner, D. V., Semenov, A. V., Antonova, O. A., Dudnik, O. A., Khaspekova, S. G., Vlasik, T. N., Samko, A. N., Staroverov, I. I., and Ruda, M. Ya. (2002) *Kardiologiya*, No. 6, 8-17.
9. Mazurov, A. V., Pevzner, D. V., Khaspekova, S. G., Byzova, T. V., Vlasik, T. N., Staroverov, I. I., Ruda, M. Ya., and Chazov, E. I. (2000) *Platelets*, **11**, 356 (abstract).
10. Coller, B. S. (2001) *Thromb. Haemost.*, **86**, 427-443.
11. Topol, E. J., Byzova, T. V., and Plow, E. F. (1999) *Lancet*, **353**, 227-231.
12. Khaspekova, S. G., Vlasik, T. N., Byzova, T. V., Vinogradov, D. V., Berndt, M. C., and Mazurov, A. V. (1993) *Br. J. Haematol.*, **85**, 332-340.
13. Smirnova, I. V., Khaspekova, S. G., Ignatov, V. V., and Mazurov, A. V. (1998) *Biochemistry (Moscow)*, **63**, 710-718.
14. Agafonova, O. G., Vinogradov, D. V., Khaspekova, S. G., Lagutina, N. Ya., Labinskaya, T. A., Vasil'ev, S. A., and Mazurov, A. V. (1992) *Byul. Eksp. Biol. Med.*, No. 12, 635-637.
15. Semenov, A. V., Romanov, Yu. A., Loktionova, S. A., Tikhomirov, O. Yu., Khachikian, M. V., Vasil'ev, S. A., and Mazurov, A. V. (1999) *Biochemistry (Moscow)*, **64**, 1326-1335.
16. Patrono, C., Coller, B., Dalen, J. E., FitzGerald, G. A., Fuster, V., Gent, M., Hirsh, J., and Roth, G. (2001) *Chest*, **119** (suppl.), 39S-63S.
17. Santoro, S. A. (1983) *Biochim. Biophys. Acta*, **757**, 101-110.
18. Ramaschi, G., Torti, M., Sinigaglia, F., and Balduini, C. (1993) *Cell Biochem. Funct.*, **11**, 241-249.
19. Torti, M., Festetics, E. T., Bertoni, A., Sinigaglia, F., and Balduini, C. (1999) *Thromb. Haemost.*, **81**, 124-130.
20. Kakaiya, R. M., Kiraly, T. L., and Cable, R. G. (1988) *Thromb. Haemost.*, **59**, 281-283.
21. Kariyazono, H., Nakamura, K., Shinkawa, T., Yamaguchi, T., Sakata, R., and Yamada, K. (2001) *Thromb. Res.*, **101**, 445-453.
22. Furie, B., and Furie, B. C. (1995) *Thromb. Haemost.*, **74**, 224-227.
23. Dunlop, L. C., Skinner, M. P., Bendall, L. J., Favaloro, E. J., Castaldi, P. A., Gorman, J. J., Gamble, J. R., Vadas, M. A., and Berndt, M. C. (1992) *J. Exp. Med.*, **175**, 1147-1150.
24. Ushiyama, S., Laue, T. M., Moore, K. L., Erickson, H. P., and McEver, R. P. (1993) *J. Biol. Chem.*, **268**, 15229-15237.
25. Frelinger, A. L., III, Du, X., Plow, E. F., and Ginsberg, M. H. (1991) *J. Biol. Chem.*, **266**, 17106-17111.
26. Kouns, W. C., Wall, C. D., White, M. M., Fox, C. F., and Jennings, L. K. (1990) *J. Biol. Chem.*, **265**, 20594-20601.
27. Kouns, W. C., Newman, P. J., Puckett, K. J., Miller, A. A., Wall, C. D., Fox, C. F., Seyer, J. M., and Jennings, L. K. (1991) *Blood*, **78**, 3215-3223.
28. Kouns, W. C., and Jennings, L. K. (1991) *Thromb. Res.*, **63**, 343-354.
29. Chronos, N., Marciniak, S. J., and Nakada, M. T. (1999) *Eur. Heart J.*, Suppl. 1 (Suppl. E), E11-E17.
30. Klinkhardt, U., Kirchmaier, C. M., Westrup, D., Breddin, H. K., Mahnel, R. G., Hild, M., and Harder, S. (2000) *Thromb. Res.*, **97**, 201-207.
31. Coller, B. S., Peerschke, E. I., Scudder, L. E., and Sullivan, C. A. (1983) *J. Clin. Invest.*, **72**, 325-338.
32. Carrol, R. C., Wang, X. L., Lanza, F., Steiner, B., and Kouns, W. C. (1997) *Thromb. Res.*, **88**, 109-125.
33. Malmsten, C., Kindahl, H., Samuelsson, B., Levy-Toledano, S., Tobelem, G., and Caen, J. P. (1977) *Br. J. Haematol.*, **35**, 511-520.
34. Tsao, P. W., Forsythe, M. S., and Mousa, S. A. (1997) *Thromb. Res.*, **88**, 137-146.
35. Schneider, D. J., Taatjes, D. J., and Sobel, B. E. (2000) *Cardiovasc. Res.*, **45**, 437-446.
36. Rossi, F., Rossi, E., Pareti, F. I., Colli, S., Tremoli, E., and Gallo, L. (2001) *Hematologica*, **86**, 192-198.
37. Torti, M., Ramaschi G., Montsarrat, N., Sinigaglia, F., Balduini, C., Plantavid, M., Breton, M., Chap, H., and Mauco, G. (1995) *J. Biol. Chem.*, **270**, 13179-13185.
38. Le Breton, H., Plow, E. F., and Topol, E. J. (1996) *J. Am. Coll. Cardiol.*, **28**, 1643-1652.