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Ability of Different Glycoprotein IIb-IIIa Ligands to Support Platelet Aggregation Induced by Activating Antibody CRC54

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Abstract—The ability of different ligands of glycoprotein (GP) IIb-IIIa (αIIb/β3-integrin) to support platelet aggregation stimulated by activating anti-GP IIb-IIIa monoclonal antibody (monoAB) CRC54 has been investigated. Antibody CRC54 stimulated aggregation of washed platelets not only in the presence of fibrinogen, the main GP IIb-IIIa ligand, but also in the presence of von Willebrand factor (vWF). Unlike these ligands, fibronectin failed to support CRC54-induced aggregation. Fibrinogen and vWF dependent platelet aggregation was completely suppressed by GP IIb-IIIa antagonists—preparations Monafram ($F(ab')_2$ fragments of monoAB that blocked GP IIb-IIIa receptor activity) and aggrastat (RGD-like peptidomimetic). However, aggregation stimulated in the presence of vWF was also completely inhibited by monoAB AK2 directed against GP Ib and capable of blocking its binding with vWF. CRC54-induced aggregation of platelets from patient with GP Ib deficiency in the presence of vWF was significantly lower than aggregation of platelets from normal donors and was not inhibited by anti-GP Ib antibody but still blocked by GP IIb-IIIa antagonist Monafram. Monafram also suppressed CRC54-stimulated platelet adhesion to plastic-adsorbed fibrinogen, vWF, and fibronectin. Unlike CRC54-induced platelet aggregation supported by fluid phase vWF, CRC54-induced adhesion to adsorbed vWF was not affected by anti-GP Ib antibody. Aggregation induced by CRC54 in the presence of fibrinogen and vWF was only partially suppressed by prostaglandin E1, an inhibitor of platelet activation, and was associated with serotonin release from platelet granules only when Ca^{2+} concentration was decreased from 1 mM (physiological level) to 0.1 mM. The data indicate that vWF supports CRC54-induced platelet aggregation via interaction with two receptors—GP IIb-IIIa and GP Ib. Aggregation induced by CRC54 in the presence of vWF or fibrinogen is only partially dependent on platelet activation and is accompanied with granule secretion only at low Ca^{2+} concentrations.

Key words: platelets, glycoprotein IIb-IIIa, glycoprotein Ib, von Willebrand factor, fibrinogen, fibronectin, monoclonal antibodies

Platelet membrane glycoprotein (GP) IIb-IIIa complex serves as receptor for fibrinogen and several other adhesive proteins, including von Willebrand factor (vWF) and fibronectin, which contain RGD (Arg-Gly-Asp) amino acid sequence. In fibrinogen, the main physiological GP IIb-IIIa ligand, not only RGD, but also a sequence located in the C-terminal part of the γ-chain (residues 400-411) plays an important role in its interaction with GP IIb-IIIa. The dependence of GP IIb-IIIa activity on platelet activation state is a unique property of this receptor. On the surface of non-activated platelets, GP IIb-IIIa is unable to bind high molecular weight ligands. However, activation of platelets by such agonists as ADP, thrombin, collagen, and others leads to the changes of its conformation and exposure of the ligand binding site. Subsequent binding of fibrinogen with activated GP IIb-IIIa results in platelet aggregation (see reviews [1, 2]).

In the absence of fibrinogen, aggregation of activated platelets might be also mediated by the interaction of GP IIb-IIIa with vWF [3-5]. Although vWF is able to bind with GP IIb-IIIa, another protein, GP Ib, serves as its main specific receptor on the platelet surface. Interaction of vWF immobilized on sub-endothelial vascular structures with GP Ib plays an essential role in platelet adhesion to the sites of the vessel wall injury and initiation of thrombogenesis [6, 7].

Binding of plasma vWF with GP Ib and subsequent platelet aggregation is stimulated by changes of hemodynamic conditions—disturbance of the blood laminar flow and high shear rate of different fluid layers towards each other. Platelet aggregation induced by increased shear rate is mediated not only by vWF interaction with GP Ib. Development of activating signal initiated by this interac-

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tion leads to GP IIb-IIIa activation and binding of vWF with this receptor. Binding of vWF with GP Ib under low shear rate conditions could be stimulated by modifications of this ligand—specific mutations increasing its binding ability (von Willebrand disease type 2b), desialation, treatment with antibiotic ristocetin or component of snake venom botrocetin (see reviews [6, 7]). One more GP IIb-IIIa ligand, fibronectin, in the same way as vWF, binds on the platelet surface not only with activated GP IIb-IIIa but also with another receptor, α5/β1-integrin. Adhesion of non-activated platelets to fibronectin is mediated first of all by this receptor [8].

It is known that GP IIb-IIIa can be activated not only through platelet activation by different agonists but also by direct action on this receptor of some monoclonal antibodies (monoABs) directed against so called LIBS (ligand-induced binding site) epitopes. Such antibodies interact preferentially with ligand occupied GP IIb-IIIa, and some of them are able to stimulate activity of this receptor and subsequent platelet aggregation in platelet rich plasma and in suspension of washed platelets in the presence of fibrinogen [9-11]. In the present study we were trying to clarify whether other GP IIb-IIIa ligands, namely vWF and fibronectin, were able (in the same way as fibrinogen) to support platelet aggregation induced by one of the activating antibodies directed against GP IIb-IIIa LIBS epitope. It is shown that vWF is able to maintain that type of aggregation and that development of this aggregation response requires the interaction of this ligand not only with activated GP IIb-IIIa but with GP Ib as well.

MATERIALS AND METHODS

Reagents and antibodies. Bovine serum albumin (BSA) was purchased from Serva (Germany); ADP, human thrombin, and prostaglandin E1 (PGE1) from Sigma (USA); [^{14}C]serotonin (5-hydroxy-2-[^{14}C]-tryptamine creatinine sulfate) from Amersham (USA); human fibrinogen from Calbiochem (USA); human fibronectin from Imtek (Moscow, Russia); 4-nitrophenol phosphate (disodium salt) from Merck (Germany), and GP IIb-IIIa antagonist preparation aggrastat (tirofiban) from Merck & Co (USA). vWF purified from human plasma as described earlier [12] and anti-GP Ib monoAB AK2, which blocked GP Ib interaction with vWF [12], were kindly provided by Dr. Michael C. Berndt (Monach University, Melbourne, Australia). MonoAB VM16d is also directed against GP Ib, but it does not inhibit the interaction of GP Ib with vWF [13]. Anti-GP IIb-IIIa monoAB FraMon (earlier used name CRC64) inhibits GP IIb-IIIa interaction with fibrinogen and other ligands and GP IIb-IIIa-dependent platelet aggregation induced by different agonists [14]. Preparation Monafram® (earlier used name FRAMON) is an F(ab′)$_2$ fragment of monoAB FraMon which effectively inhibits platelet aggregation in the same way as antibody FraMon [15-17]. MonoAB CRC54 is directed against LIBS epitope in GP IIIa [9, 18]. Previously it was demonstrated that this antibody is able to stimulate GP IIb-IIIa binding with fibrinogen and fibronectin and platelet aggregation in platelet rich plasma and washed platelet suspension in the presence of fibrinogen [9, 19].

Platelets. Washed platelets were prepared from the blood of healthy donors as described elsewhere [20] and finally resuspended in Tyrode/Hepes solution (137 mM NaCl, 2.7 mM KCl, 0.36 mM NaH_2PO_4, 0.1% dextrose, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.35) supplemented with 0.35% BSA. After resuspension, $CaCl_2$ was added to the washed platelets at final concentration of 1 mM and in some experiments at 0.1 mM. In experiments where serotonin secretion was evaluated, platelets were labeled with [^{14}C]serotonin as described earlier [21]. Washed platelets were also obtained from the blood of a previously characterized patient with hereditary deficiency of GP Ib (Bernard–Soulier syndrome) [22].

Platelet aggregation and serotonin secretion. Platelet aggregation and serotonin secretion were studied in washed platelet suspension at platelet concentration of $3 \cdot 10^8$/ml. Platelet suspension in Tyrode/Hepes solution containing 0.35% BSA and 1 or 0.1 mM (in some experiments) $CaCl_2$ was supplemented with fibrinogen, vWF, or fibronectin and 300 μl of suspension were placed into the cuvette of the aggregometer (BIOLA Ltd, Russia). Platelet aggregation was registered by evaluation of changes of light transmission (T, %) at 37°C and stirring at 800 rpm. Inhibitors of platelet aggregation (antibodies AK2 and VM16d, preparations Monafram and aggrastat, and PGE1) were added to the aggregation cuvette not less then 2 min before aggregation inducers. Inducers (antibody CRC54 and thrombin) were added to the aggregometer cuvette 30 sec after the beginning of the light transmission registration. MonoAB CRC54 was added at the final concentration of 450 μg/ml. In preliminary experiments, it was shown that CRC54 at this concentration caused maximal aggregation response (data not shown). After addition of CRC54 aggregation was followed for at least 8.5 min and after addition of thrombin (0.1 or 0.5 U/ml) for 4.5 min. [^{14}C]Serotonin-labeled platelets were used when serotonin secretion from platelet granules was investigated. Serotonin release was measured as described previously [19, 21] under the same conditions as platelet aggregation and expressed as percent of total serotonin incorporated into platelets. Only platelet preparations in which thrombin at low dose (0.1 U/ml) induced well expressed aggregation and secretion (maximal level of aggregation $\geqslant$ 40 T (%) and of [^{14}C]serotonin secretion $\geqslant$ 30% of incorporated label) were used for further experiments.

Platelet adhesion. Fibrinogen, vWF, and fibronectin were added into the wells of 24-well polystyrene culture

plates (Costar, USA) at the concentration of 10 μg/ml in 500 μl of phosphate buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate, pH 7.35) and incubated for 1 h at 37°C. Unbound proteins were washed off with PBS and the plastic was blocked with 2% BSA in PBS (500 μl per well) for 1 h at 37°C. Control wells were incubated with BSA but without prior addition of fibrinogen, vWF, or fibronectin. Washed platelets were added to the wells at the concentration of $3 \cdot 10^8$/ml in 250 μl of Tyrode/Hepes solution containing 1 mM $CaCl_2$. The samples were incubated for 30 min at 37°C without any additions or in the presence of monoAB CRC54, Monafram, or antibody AK2, which were added into the medium before incubation. Non-adherent platelets were washed off with Tyrode/Hepes and the level of platelet adhesion was evaluated by measuring endogenous acid phosphatase activity according to the method described by Suzuki et al. [23]. Phosphatase substrate, 4-nitrophenol phosphate, was added into the wells at 4 mg/ml in 250 μl of 50 mM citrate buffer, pH 5.5, containing 0.1% Triton X-100. Plates were incubated for 2-5 min at room temperature and the reaction was stopped by addition of 1 M NaOH (100 μl per well). A 100-μl sample of substrate solution from each well was transferred into the wells of 96-well plates, and optical density was measured at 405 nm (A_{405}). In each experiment, the level of platelet adhesion to fibrinogen in the absence of any additions was considered as 100%.

RESULTS

It was previously demonstrated the antibody CRC54 directed against the LIBS epitope in GP IIb-IIIa was able to stimulate activity of this receptor and induce platelet aggregation in platelet rich plasma or suspension of washed platelets in the presence of fibrinogen—the main GP IIb-IIIa ligand [9, 19]. As shown in Fig. 1, antibody CRC54 could cause aggregation of washed platelets not only in the presence of fibrinogen, but also in the presence of vWF—another GP IIb-IIIa ligand. However, under the same conditions we failed to register platelet aggregation in the presence of fibronectin, one more protein able to bind with activated GP IIb-IIIa (Fig. 1a). CRC54-induced aggregation was reproducibly registered already in the presence of 10 μg/ml vWF and maximal aggregation response was achieved at vWF concentration of about 80 μg/ml. Addition of vWF without CRC54 did not cause aggregate formation (Fig. 1c). In the presence of fibrinogen, aggregation was reproducibly detected at its concentration of 50 μg/ml, and saturation was achieved within the range of concentrations from 0.5 to 2.0 mg/ml (Fig. 2b). The maximal level of CRC54-induced aggregation in the presence of a saturating concentration of vWF (80 μg/ml) was approximately 2 times higher than in the presence of saturating concentration of fibrinogen (1 mg/ml)—35.2 ± 4.7 and 17.5 ± 1.9 T (%), respectively (mean ± standard errors, $n \geqslant 9$, $p < 0.01$, t-test for means).

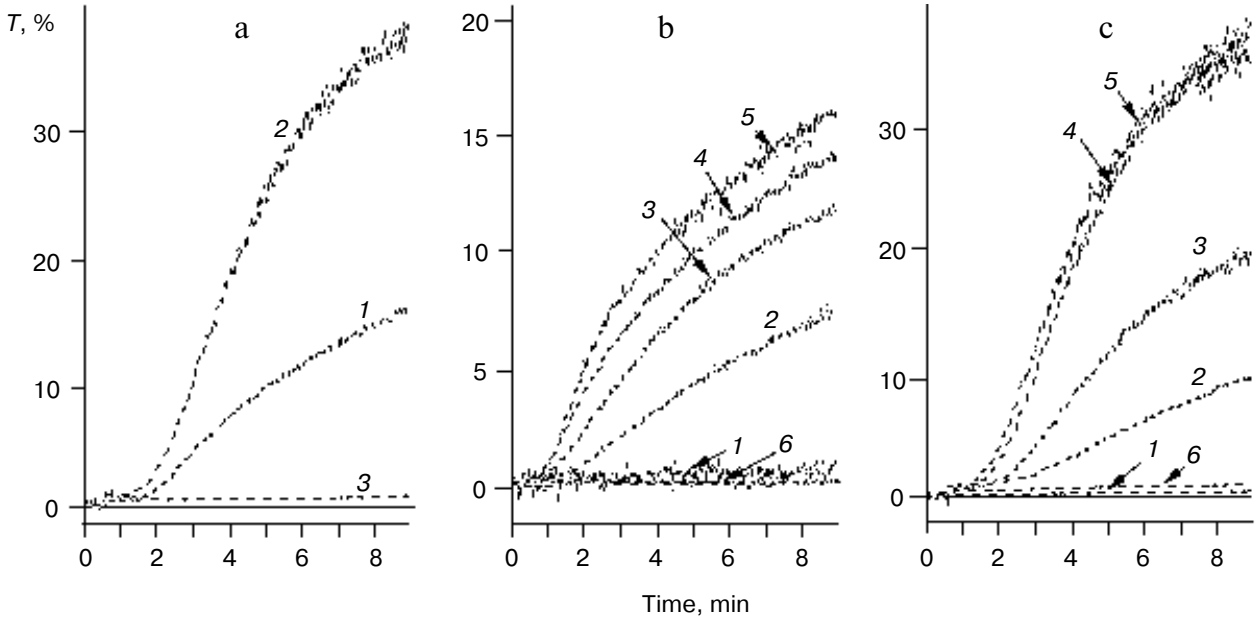

Fig. 1. CRC54-induced platelet aggregation in the presence of different GP IIb-IIIa ligands. CRC54 was added to platelets at the concentration of 450 μg/ml. Aggregation was registered: a) in the presence of 1 mg/ml fibrinogen (curve *1*), 80 μg/ml vWF (curve *2*), and 200 μg/ml fibronectin (curve *3*); b) in the presence of 0, 0.05, 0.1, 0.5, and 2.0 mg/ml fibrinogen (curves *1-5*, respectively) or in the presence of 2 mg/ml fibrinogen without CRC54 (curve *6*); c) in the presence of 0, 10, 40, 80, and 160 μg/ml vWF (curves *1-5*, respectively) or in the presence of 160 μg/ml vWF without CRC54 (curve *6*). All experiments were performed at Ca^{2+} concentration of 1 mM.

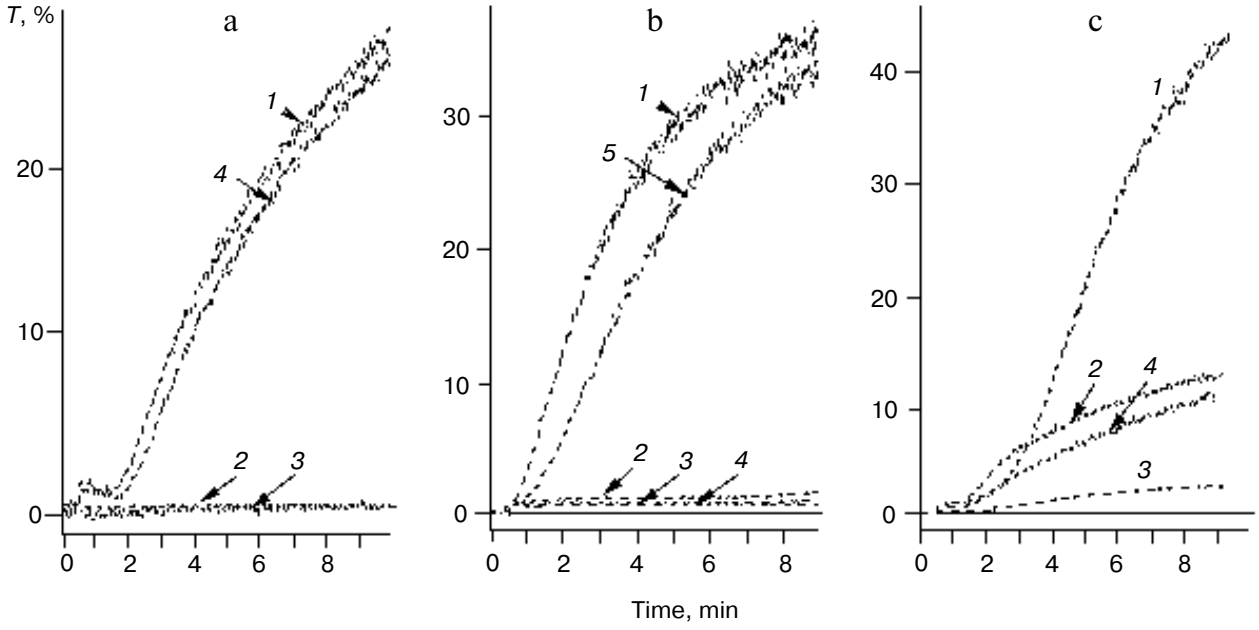

Fig. 2. Inhibition of CRC54-induced platelet aggregation by GP IIb-IIIa and GP Ib antagonists. In all experiments, aggregation was stimulated by 450 μg/ml CRC54. a) Suspension of platelets from a healthy donor was supplemented with 1 mg/ml fibrinogen and incubated without antagonists (curve *1*), or in the presence of 20 μg/ml Monafram (curve *2*), 1 μg/ml aggrastat (curve *3*), and 20 μg/ml monoAB AK2 (curve *4*). b) Suspension of platelets from healthy donor was supplemented with 80 μg/ml vWF and incubated without antagonists (curve *1*), or in the presence of 20 μg/ml Monafram (curve *2*), 1 μg/ml aggrastat (curve *3*), 20 μg/ml monoAB AK2 (curve *4*), and 20 μg/ml monoAB VM16d (curve *5*). c) Suspension of platelets from healthy donor was supplemented with 80 μg/ml vWF and incubated without antagonists (curve *1*). Suspension of platelets from the patient with GP Ib deficiency was supplemented with 80 μg/ml vWF and incubated without antagonists (curve *2*), in the presence of 20 μg/ml Monafram (curve *3*) or 20 μg/ml monoAB AK2 (curve *4*). All experiments were performed at Ca^{2+} concentration of 1 mM.

CRC54-induced aggregation, both in the presence of vWF and fibrinogen, was completely inhibited by GP IIb-IIIa antagonists—preparation Monafram representing $F(ab')_2$ fragments of antibody FraMon which blocked GP IIb-IIIa receptor activity [15-17], and low molecular weight RGD-like peptidomimetic aggrastat [1] (Fig. 2, a and b). At the same time vWF supported aggregation was also completely suppressed by antibody AK2, which blocked the interaction of this protein with another platelet receptor, GP Ib [12], but was not inhibited by monoAB VM16d which was also directed against GP Ib but did not affect its interaction with vWF [13] (Fig. 2b). CRC54-induced fibrinogen dependent aggregation was not influenced by monoAB AK2 (Fig. 2a). These results indicated that platelet aggregation induced by CRC54 in the presence of vWF required its interaction not only with GP IIb-IIIa but also with GP Ib. In order to confirm this suggestion the same experiments were performed with platelets obtained from a patient with hereditary GP Ib deficiency (Bernard–Soulier syndrome). The level of CRC54-induced aggregation of the patient's platelets in the presence of vWF was considerably lower than the level of aggregation of normal platelets. It should be also mentioned that this lowly expressed aggregation response of the patient's platelets was not suppressed by anti-GP Ib antibody AK2, but still completely blocked by GP IIb-IIIa antagonist, preparation Monafram (Fig. 2c).

Studies of platelet adhesion to GP IIb-IIIa ligands adsorbed to plastic have shown that in the absence of CRC54 platelets efficiently attached to fibrinogen and fibronectin while the level of adhesion to vWF did not exceed the level of nonspecific adhesion to the BSA treated surface. Platelet attachment to all three substrates was significantly increased in the presence of CRC54 (Fig. 3). Basal as well as CRC54-stimulated adhesion to fibrinogen was completely inhibited by GP IIb-IIIa antagonist Monafram (Fig. 3a). In case of fibronectin, Monafram inhibited only CRC54-induced adhesion but did not affect the attachment of platelets that were not treated with CRC54 (Fig. 3c). This adhesion was apparently mediated by the interaction of fibronectin with another receptor, α5/β1 integrin [8]. CRC54-induced adhesion to vWF as well as to fibrinogen and fibronectin was also completely inhibited by Monafram. However, unlike vWF-dependent platelet aggregation stimulated by CRC54, platelet adhesion to vWF was not inhibited by anti-GP Ib antibody AK2 (Fig. 3b). Also, this antibody did not influence the basal as well as the CRC54-stimulated adhesion to fibrinogen (Fig. 3a). Thus, unlike CRC54-induced platelet binding with fluid phase vWF, binding with

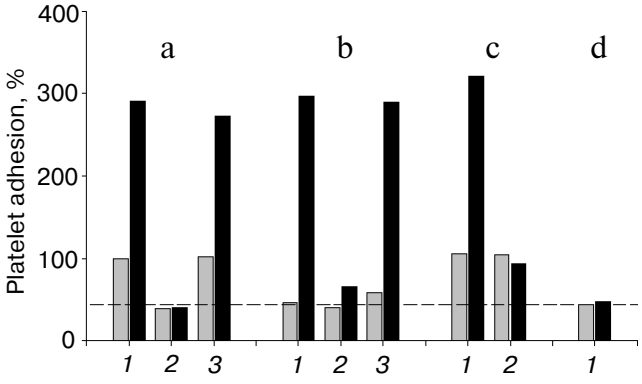

Fig. 3. Platelet adhesion to plastic adsorbed fibrinogen (a), vWF (b), fibronectin (c), and BSA (d). Platelets were incubated in medium not supplemented (gray columns) or supplemented with 450 μg/ml CRC54 (black columns) without GP IIb-IIIa and GP Ib antagonists (*1*) and in the presence of 20 μg/ml Monafram (*2*) or 20 μg/ml monoAB AK2 (*3*). All experiments were performed at Ca^{2+} concentration of 1 mM. In each experiment platelet adhesion to fibrinogen without CRC54 and GP IIb-IIIa and GP Ib antagonists was considered as 100%. Means of three experiments are presented.

immobilized protein, i.e., under conditions excluding its participation in inter-platelet interactions, was mediated only by GP IIb-IIIa without the involvement of GP Ib.

PGE1, a powerful inhibitor of platelet activation, was capable of blocking platelet aggregation induced by the strongest platelet agonist—thrombin—at high concentration (0.5 U/ml) (Fig. 4a). However, it only partially, by 50-60%, inhibited CRC54-induced aggregation both in the presence of vWF and fibrinogen (Figs. 4b and 4c, respectively). This inhibition was presumably not caused by suppression of secretion from platelet granules of biologically active compounds, which potentiate platelet aggregation (ADP, serotonin, and others). In the standard incubation medium containing 1 mM Ca^{2+} antibody CRC54 both in the presence of fibrinogen or vWF did not stimulate at all or only slightly stimulated serotonin secretion (Fig. 5). However, decrease in Ca^{2+} concentration from 1 to 0.1 mM in the same platelet preparation led to significant increase in secretion (Fig. 5). The level of CRC54-induced secretion strongly differed in different platelet preparations. However, the level of [^{14}C]serotonin secretion exceeding 5% of platelet-incorporated label was registered in six out of 10 experiments in the presence of fibrinogen (level of secretion from 6 to 30%) and in four out of seven experiments in the presence of vWF (level of secretion from 5 to 20%). Thus, at least at low Ca^{2+} concentration interaction of fibrinogen and vWF with platelets induced by CRC54 was able to stimulate platelet activation associated with granule secretion. In the presence of both ligands, CRC54-induced platelet aggregation was also higher at low Ca^{2+} concentration (table). However, these differences could not be explained only by the action of agonists that were secreted from platelets and potentiated platelet aggregation. The level of aggregation

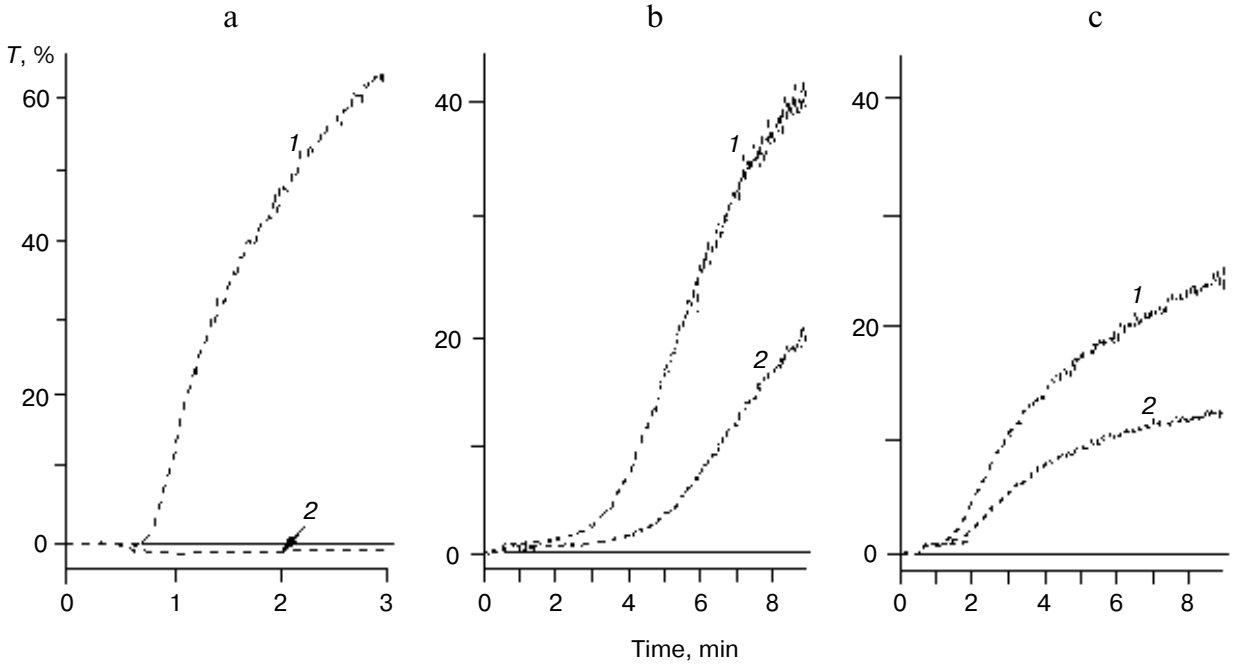

Fig. 4. Inhibition of platelet aggregation by PGE1. Aggregation was stimulated by 0.5 U/ml thrombin (a), 450 μg/ml CRC54 in the presence of 1 mg/ml fibrinogen (b), or 80 μg/ml vWF (c). Before addition of aggregation inducers platelets were preincubated without (curves *1*) or in the presence of 3 μg/ml PGE1 (curves *2*). All experiments were performed at Ca^{2+} concentration of 1 mM.

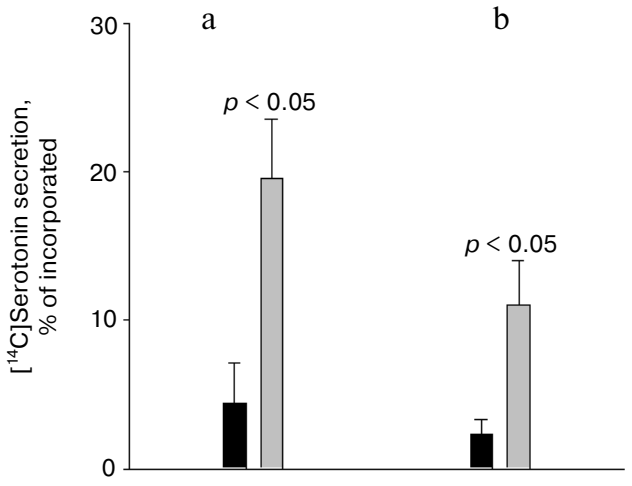

Fig. 5. CRC54-induced serotonin secretion. Secretion of [^{14}C]serotonin was stimulated by 450 μg/ml CRC54 in the presence of 1 mg/ml fibrinogen (a) or 80 μg/ml vWF (b) in medium containing 1.0 (black columns) or 0.1 mM Ca^{2+} (gray columns). Means ± standard errors are presented for six out of 10 experiments in the presence of fibrinogen and for four out of seven experiments in the presence of vWF in which serotonin secretion at 0.1 mM Ca^{2+} exceeded 5% of the incorporated label. $p < 0.05$, significance of differences between 1.0 and 0.1 mM Ca^{2+} (*t*-test for pairs).

at low Ca^{2+} concentration was higher even in these experiments where aggregation was not associated with serotonin secretion (table). Control measurements of platelet aggregation and secretion induced by 0.1 U/ml thrombin were performed in each experiment where CRC54 effects were studied. Parameters of thrombin-induced aggregation and serotonin secretion did not differ in incubation media with different Ca^{2+} concentrations—maximal aggregation level was 58.2 ± 3.5 and 56.1 ± 2.6 T (%), and secretion level—59.5 ± 6.9 and 58.3 ± 6.2% of incorporated [^{14}C]serotonin at 1.0 and 0.1 mM Ca^{2+}, respectively (means ± standard errors, $n \geqslant 7$, $p > 0.05$, *t*-test for pairs).

DISCUSSION

It is known that some monoABs directed against conformation-dependent LIBS epitopes in GP IIb-IIIa are able to activate this receptor and stimulate platelet aggregation in platelet rich plasma and in washed platelet suspension in the presence of fibrinogen, the main GP IIb-IIIa ligand [9-11]. In the present study using one of these antibodies, CRC54 [9, 18, 19], it was demonstrated that this aggregation could be stimulated not only in the presence of fibrinogen but also in the presence of vWF. Upon addition of saturating concentration of these ligands, which were considerably lower for vWF than for fibrinogen (80 μg/ml or ~15 nM and 1 mg/ml or ~3 μM, respectively) the level of platelet aggregation in the presence of vWF was even higher (by ~2 times) than in the presence of fibrinogen. At the same time, addition to incubation medium of fibronectin, another GP IIb-IIIa ligand, did not cause stimulation of platelet aggregation, although as shown earlier [9] and confirmed in this study CRC54 was able to stimulate platelet adhesion to fibronectin adsorbed to plastic surface.

It is well known that vWF is able to interact with two receptors on the platelet surface—GP Ib and GP IIb-IIIa. Interaction of vWF immobilized on sub-endothelium with GP Ib under blood flow conditions mediates platelet adhesion at sites of the vessel wall injury. At high shear rate GP Ib is able to interact also with fluid phase vWF. This reaction stimulates platelet activation, binding of vWF with activated GP IIb-IIIa, and subsequent aggregation (see reviews [6, 7]). At low shear rate binding of fluid phase vWF with GP Ib and subsequent platelet aggregation could be stimulated by artificial modifications of this ligand (desialation, treatment with antibiotic ristocetin or component of snake venom botrocetin) or as a result of specific mutations increasing the ability of vWF to interact with this receptor (see reviews [6, 7]). Binding of vWF with GP IIb-IIIa at low shear rate and in the absence of fibrinogen is stimulated upon platelet activation with different ligands leading to the induction of the functional activity of this receptor [3-5].

CRC54-induced platelet aggregation at different Ca^{2+} concentrations and at different levels of serotonin secretion

Ligand (secretion)	Maximal aggregation level (T, %)	
	1 mM Ca^{2+}	0.1 mM Ca^{2+}
Fibrinogen (+)	14.2 ± 2.6	36.6 ± 7.5 ($p = 0.029$)
Fibrinogen (–)	17.6 ± 3.7	30.6 ± 5.4 ($p = 0.035$)
vWF (+)	38 ± 6.9	47.8 ± 7 ($p = 0.038$)
vWF (–)	24.8	42.0

Notes: Results of experiments in which parallel measurements of platelet aggregation and serotonin secretion were performed at both Ca^{2+} concentrations (n = 3-6) are presented. Secretion (+) and (–) designate experiments in which the level of [^{14}C]serotonin secretion at 0.1 mM Ca^{2+} was respectively higher and lower than 5% of incorporated label. For $n \geqslant 4$ means ± standard errors are presented and in brackets significance (p) of the differences between parameters registered at different Ca^{2+} concentrations (*t*-test for pairs). For $n < 4$ means only are presented. Differences between groups secretion (+) and secretion (–) calculated for experiments with fibrinogen are not significant (*t*-test for means).

In the present study using activating anti-GP IIb-IIIa monoAB CRC54 we have shown for the first time the ability of native vWF to stimulate platelet aggregation at low shear rate (standard aggregation conditions of Born's method) via its interaction with both CRC54-activated GP IIb-IIIa and GP Ib as well. Involvement of both receptors was confirmed by inhibitory analysis of this reaction. It appeared that, unlike aggregation in the presence of fibrinogen, vWF dependent aggregation was suppressed not only by GP IIb-IIIa antagonists but also by antibody that blocked interaction of vWF with GP Ib. Moreover, vWF dependent aggregation of platelets from a patient with hereditary deficiency of GP Ib was significantly lower than aggregation of platelets from normal donors. This low aggregation response was still inhibited by GP IIb-IIIa antagonist but was not affected by antibody that blocked GP Ib activity.

These results proved that at least full scale aggregation induced by CRC54 in the presence of vWF required interaction of the ligand with two receptors—activated GP IIb-IIIa and GP Ib. Registration of aggregation response in a patient with GP Ib deficiency, which should depend only on vWF interaction with GP IIb-IIIa, might probably be explained by the increased sensitivity of the method because of the giant size of the patient's platelets, typical for this pathology. Thus we suggest that initial vWF interaction with CRC54-activated GP IIb-IIIa is not sufficient for the development of well pronounced aggregation response but creates conditions for further binding of GP IIb-IIIa-associated vWF with another receptor, GP Ib, located on the surface of adjacent platelets. According to this suggestion heterogenic interaction of vWF with GP IIb-IIIa and GP Ib mediates formation of the majority of inter-platelet molecular bridges and supports efficient platelet aggregation.

Studies of platelet adhesion have shown that under static conditions used in these experiments basal adhesion to vWF adsorbed to plastic did not exceed the level of nonspecific adhesion to BSA. However, antibody CRC54 was able to stimulate platelet attachment to vWF and in accordance with previously obtained data [9] to fibrinogen and fibronectin as well. CRC54-induced adhesion to all three substrates was suppressed by GP IIb-IIIa antagonists, but unlike vWF-dependent aggregation, adhesion to immobilized vWF was not inhibited by antibody AK2. Thus the data indicate that under conditions in which adhesive ligands were unable to support formation of inter-platelet bridges, CRC54-induced interaction of platelets with surface-immobilized vWF was mediated by its binding only with GP IIb-IIIa without the involvement of GP Ib. Adhesion experiments also completely excluded the possibility of any influence of anti-GP Ib antibody AK2 used in this study on the interaction of vWF with GP IIb-IIIa.

The ability of vWF to support platelet aggregation induced by another anti-LIBS monoAB, D3GP3, directed against an epitope in GP IIIa different from that of CRC54 [9, 18], was demonstrated by Mondoro et al. [24]. However, these authors did not perform any inhibitory analysis of this reaction. The ability of vWF to support aggregation of platelets activated by physiological agonists was previously studied in platelet rich plasma of patients with afibrinogenemia [3] or in suspension of washed platelets supplemented with exogenous vWF [4, 5]. In these papers, it was demonstrated that, in the absence of fibrinogen, vWF was able to maintain aggregation induced by ADP or several other inducers. Aggregation was less pronounced than in the presence of fibrinogen [3, 4] and in the same way as fibrinogen dependent aggregation was completely suppressed by GP IIb-IIIa antagonists [3-5]. However, as shown by Goto et al. [5], aggregation induced by the mixture of ADP and epinephrine in the presence of vWF was not changed by anti-GP Ib blocking antibody, which evidently distinguished this type of aggregation from that induced by monoAB CRC54. But it should be taken into account that Goto et al. used another anti-GP Ib antibody and did not perform experiments with GP Ib deficient platelets.

Platelet aggregation induced by CRC54 in the presence of vWF was only partially dependent on platelet activation. One of the most powerful inhibitors of platelet activation, PGE1, only partially inhibited this type of aggregation. As demonstrated in this and an earlier paper [9], analogous results could be observed upon stimulation of aggregation in the presence of fibrinogen. These data indicated that the primary interaction of platelets with both ligands and initiation of aggregation did not require platelet activation. However, binding of ligands with GP IIb-IIIa or in the case of vWF with GP IIb-IIIa and GP Ib as well could switch on activating pathways that potentiate platelet aggregation. Under experimental conditions used in this study, the development of an activation-dependent component of CRC54-induced aggregation was evidently not mediated by secretion from platelet granules of proaggregatory compounds. At physiological Ca^{2+} concentration (1 mM) serotonin secretion was either extremely low or, as in most experiments, was not detected at all. However, when Ca^{2+} concentration was decreased from 1 to 0.1 mM, well expressed serotonin secretion (>5% of incorporated label, and on average >10%) was registered in more than 50% of experiments. This observation suggested that at least under those conditions interaction of both ligands (vWF and fibrinogen) with platelet receptors could stimulate secretion reactions.

Results obtained in the serotonin secretion studies are in good accordance with earlier data showing that CRC54 [19] as well as some other activating anti-LIBS monoABs [10] are able to stimulate secretion from granules in platelet rich plasma anticoagulated with citrate, i.e., at low Ca^{2+} concentration. The observed dependence of secretion on Ca^{2+} concentration was rather expectable.

In early studies, it was shown that upon platelet activation with weak or moderate physiological agonists (like ADP), secretion from platelet granules was stimulated only at low Ca^{2+} concentrations both in platelet rich plasma [25] and in suspension of washed platelets supplemented with fibrinogen or vWF [4].

As shown in this study, changes in Ca^{2+} concentration did not affect the level of platelet aggregation and secretion upon their stimulation with thrombin, a more powerful inducer although used at quite low concentration (0.1 U/ml). It is also interesting that higher level of platelet aggregation stimulated by CRC54 at low Ca^{2+} cannot be explain only by the action of compounds released from platelet granules. In several experiments where secretory response was not detected at both low and high Ca^{2+} concentrations, platelet aggregation was still higher in 0.1 mM compared with that at 1.0 mM Ca^{2+}. The reason for such differences remains unclear. One of the possible explanations is increase in the affinity of GP IIb-IIIa interaction with monoAB CRC54 and/or increase in receptor activating ability upon decrease in Ca^{2+} concentration. At least one fact is in line with this suggestion. As we have shown earlier [18], binding of CRC54 with platelets was dramatically increased upon chelation of bivalent cations with EDTA.

Thus, in the present paper it has been demonstrated for the first time that vWF can support platelet aggregation by heterogenic interaction with both platelet receptors—activated GP IIb-IIIa and GP Ib. Initiation of aggregation did not require platelet activation although its full scale development was associated with the switching of activation pathways inhibited by PGE1 and at low Ca^{2+} concentration with secretion from platelet granules. However, it should be taken into account that all these reactions were registered under quite artificial experimental conditions, upon GP IIb-IIIa activation with specific monoAB, and elucidation of their physiological significant requires further investigation.

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