Clinical Hemorheology and Microcirculation 57 (2014) 291–302
DOI 10.3233/CH-131774
IOS Press

An effect of glycoprotein IIb/IIIa inhibitors on the kinetics of red blood cells aggregation

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Abstract. The reversible aggregation of red blood cells (RBCs) continues to be of the basic science and clinical interest. Recently it has been reported about a specific binding between fibrinogen and unknown erythrocyte glycoprotein receptors. The aim of this study was to investigate whether the red blood cell aggregation (RBCA) include the cell-cell interaction using the membrane receptors that bind such ligands as fibrinogen or fibronectin. To test this hypothesis the RBCs were incubated with monafram - the drug of the monoclonal antibodies against glycoprotein (GP) IIb/IIIa, with the GPIIb-IIIa receptor antagonist tirofiban, epifibatide and with the fibrinogen inhibiting peptide. It has been found that the RBC incubation with monafram resulted in a marked RBCA decrease mainly in persons with high level of aggregation. Another research session has shown that RBC incubation with fibronectin was accompanied by a significant RBCA rise. The monafram addition to red cell incubation medium resulted in a significant RBCA lowering. The cell incubation with tirofiban and epifibatide issued in RBCA decrease. The similar results were obtained when RBCs were incubated with the fibrinogen inhibiting peptide. Although monafram, tirofiban, eptifibatide and the fibrinogen inhibiting peptide were related to fibrinogen function they didn't inhibit RBCA completely. Therefore, under moderate and low red blood cell aggregation the cell binding is probably related to nonspecific mode. It seems evident that the specific and nonspecific modes of red blood cell aggregate formation could co-exist. Additional theoretical and experimental investigations in this area are needed.

Keywords: Red blood cells, aggregation, bridging model, glycoprotein (GP) IIb/IIIa receptors, fibrinogen, fibronectin, monafram, eptifibatide, tirofiban

1. Introduction

The elevated red blood cell aggregation (RBCA) has been observed in many physiological and pathophysiological states [3, 4, 14, 16, 21, 22, 25, 26, 32–40]. The reversible aggregation of red blood cells into linear and three-dimensional structures continues to be of the basic science and clinical interest: RBC aggregation affects low shear blood viscosity and microcirculation and can be markedly enhanced in several clinical states [18, 21, 26, 33, 38, 39]. Moreover the red blood cell aggregation at low flow

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rates increases venous vascular resistance [4, 11, 17, 39]. However the process of aggregate formation is not well understood [5, 8, 25, 29]. Although there are now several reports dealing with plasmatic and cellular factors affecting red blood cell aggregation many questions remain to be answered [24, 25, 27, 29, 30]. At present, there are two co-existing hypotheses for the red blood cell aggregate formation: bridging model and depletion one [29]. According to the bridging model, red cell aggregation is proposed to occur when the bridging forces due to adsorption of macromolecules onto adjacent cell surfaces exceed disaggregating forces due to electrostatic repulsion and mechanical shearing [3, 5]. For erythrocyte aggregation, the fibrinogen is considered to be the main macromolecule [6, 8, 21, 22, 31]. Several studies support the concept that fibrinogen interacts with RBC membrane and this binding, may be a trigger for RBC hyperaggregation in inflammation due to non-specific and specific mechanisms [12, 20, 31]. F.A. Carvalho et al. [8, 9] have recently reported a specific binding between fibrinogen and an erythrocyte membrane integrin receptor. The existence of a single molecule interaction between fibrinogen and an unknown receptor on the erythrocyte membrane has been demonstrated by force spectroscopy measurements using an atomic force microscope [8]. The results obtained for a Glanzmann thrombastenia (a rare hereditary bleeding disease caused by alpha-IIb/beta-3 deficiency) showed an impaired fibrinogen-erythrocyte binding [1]. Correlation with genetic sequencing data demonstrates that one of the units of the fibrinogen receptor on the erythrocyte membrane is a product of the expression of the beta (3) gene, found to be mutated in this patient [8]. Thus there are some pieces of evidence of the specific red blood cell binding during the aggregation process under the interaction the cellular membrane and the aggregating ligands. However an additional research to clear up the main mechanism of this cell-cell communication is necessary to accomplish. This study was designed to investigate the possibility of the cell-cell interaction using some membrane receptors that can bind the fibrinogen or fibronectin.

2. Materials and methods

2.1. Preparation of blood samples

Venous blood samples were obtained from healthy donors ($n = 78$; age range 22–25 years) via withdrawal into sterile vacuum tubes containing heparin (1.5 mg/ml). The study was approved by the local ethic committee at the Faculty of Basic Medicine, Lomonosov Moscow State University, and an informed consent of all the subjects were obtained according to the recommendations of the Declaration of Helsinki [The International Response to Helsinki VI, The WMA's Declaration of Helsinki on Ethical Principles for Medical Research Involving Human Subjects, as adopted by the 52nd WMA General Assembly, Edinburgh, October, 2000].

Red blood cells (RBCs) were separated from the blood by centrifugation at 1,400 g for 20 min and washed 3 times with 10 mM phosphate buffered saline (PBS) (pH = 7.4). The washed RBCs were then resuspended in PBS at a hematocrit of approximately 40%.

To initiate RBC aggregation cells were suspended:

1) in native plasma with mean fibrinogen concentration – 2.60 ± 0.12 g/L (plasma1) and fibrinogen concentration – 3.10 ± 0.15 g/L (plasma2), in both cases Hct = 40%;
2) in 3% Dextran 70 containing fibronectin (5.0 μM).
3) in native plasma with pure platelets and with normal platelet concentration.

2.2. Protocols for in vitro aggregation studies

In each of the *in vitro* research sessions RBC suspensions were divided into several aliquots and exposed to: 1) Monafram – the monoclonal antibodies (monoAB CRC64) against glycoprotein (GP) IIb/IIIa – the receptor for fibrinogen or fibronectin (5.0 μM; 10.0 μM; 50 μM); 2) Tirofiban – the nonpeptide GP IIb/IIIa receptor activity inhibitor (5.0 μM); 3) Eptifibatide – the synthetic peptide inhibitor of GPIIb/IIIa receptor activity (4.0 μM); 4) Fibrinogen-inhibiting peptide (5.0 μM); 5) Fibronectin – the ligand, that can be bound to GP IIb/IIIa membrane receptors (5.0 μM); 6) monoAB PS10 against P-selectin of platelets and endothelial cells (5 μg/ml; control to monoAB CRC64). In these sessions cell incubation was performed at 37°C for 30 min. The control was red blood cells incubated in the phosphate buffered saline without any drugs. Stock solutions of drugs were prepared in DMSO or in water. After the cell incubation the aggregation of red blood cells were estimated at the appointed time. The analyses were completed within 1.5–2 h after the blood collection. The reagents were purchased from Sigma, except monafram (monoAB CRC64) and monoAB PS10 that were received in Russian Cardiological, Scientific and Productive Company.

2.3. Red blood cell microrheology measurement

Red blood cell aggregation in native plasma was assessed by the Myrenne Aggregometer, which provides an index of RBC aggregation facilitated by low shear. In brief, the suspension was subjected to a short period of high shear to disrupt pre-existing aggregates. After that the shear was abruptly reduced to $3\,s^{-1}$ and light transmission through the suspension was integrated for five seconds; the resulting index, termed « M_5 » by the manufacturer and « RBCA » herein, increased with enhanced RBC aggregation. A direct microscopic method with computer image analysis was used to work out the red cell aggregation process in detail (termed as aggregation index "AI") [24]. Kinetics of red blood cell aggregation-disaggregation was studied by light backscattering technique [14] using laser-assisted optical rotational erythrocyte aggregometer LADE (rheological gap: 0.9 mm, $\lambda = 650$ nm; OOO ReoMedLab, Russia). The time of the spontaneous red cell aggregation and the hydrodynamic RBC aggregate stability (in the range of shear rates from $\sim 2.5\,s^{-1}$ to $\sim 130\,s^{-1}$) were estimated by this method [19]. Briefly, the light intensity-time curves and the light intensity – shear rate ones were linearly approximated in the semi-logarithmic coordinates and the slopes of these lines were used to characterize the time of RBCA and the RBC aggregate stability respectively.

2.4. Optical traps for red cell aggregation study

In order to study force interaction between separated RBCs of the cell couple, two independent optical traps (method of the laser tweezers) were used [34]. The traps were formed by orthogonally polarized continuous-wave laser beams from two neodymium-doped yttrium aluminium garnet (Nd-YAG) lasers with the wavelength of 1064 nm, the output power of 100 mW and TEM_{00} mode structure. Large numerical aperture (N.A. 1.3) 100× oil immersion objective (Olympus, ∞-corrected Plan Semi-Apochromat) was used to focus the laser beams and form the optical traps. Laser power in each trap varied in the range between 6 and 20 mW. Two beam expanders provided the maximal optical field gradient at the beam waist for the most effective RBC trapping. The positions of the traps inside the sample were controlled within the focal plane of the focusing objective by the beam shifters consisting of two convex lenses placed in the confocal configuration. Step-motor translation of L1 and L2 lens perpendicular to the optical axis

allowed movement of the trap with translation accuracy of about 30 nm per step. Visual control of the trapped objects was realized in transmission configuration using the CCD camera.

2.5. Miscellaneous techniques

The whole blood and red cell suspension hematocrites were determinated via the microhematocrit methods (i.e., 12,000× g for 7 minutes). Plasma fibrinogen concentration was estimated by Claus method.

2.6. Statistics and data presentation

Wherever applicable, a Student *t* test was carried out to analyze the differences between samples. The difference was considered statistically significant when $p < 0.05$.

3. Results

3.1. The alteration of the red blood cell aggregation (RBCA) under monoAB CRC64

It was found that in the whole population of healthy volunteers ($n = 78$) three groups of people could be distinguished: with high (15%), low (13%) and middle level (78%) of red blood cell aggregation (Fig. 1). The difference of RBCA equal to the double standard deviation from the mean data for the whole population was the basis of the division into three subgroups. The double standard deviation from the mean values of RBCA characteristics was used for the division of the whole population into these subgroups. The aggregate properties of the red blood cells of the individuals with highRBCA were modified by monoAB against GPIIb/IIIa (monafram). It was found that the red blood cell incubation with monafram resulted in RBC aggregation decrease by 23% ($p < 0.05$) under mean plasma fibrinogen concentration – 2.60 ± 0.12 g/L. Aggregation lowering was about 45% under mean plasma fibrinogen concentration 3.10 ± 0.15 g/L in the incubation medium (Fig. 2). Importantly the positive RBCA lowering effect has been shown under different monafram concentrations (Fig. 3).

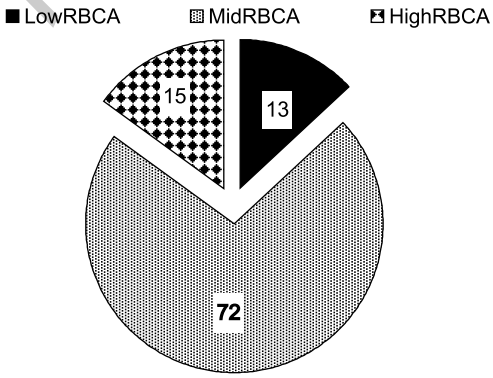

Fig. 1. The apportionment of the degree of red blood cell aggregation (RBCA) among the human population of the healthy volunteers ($n = 78$). Abbreviations: LowRBCA – low red blood cell aggregation; MidRBCA – middle values of red blood cell aggregation; HighRBCA – high red blood cell aggregation.

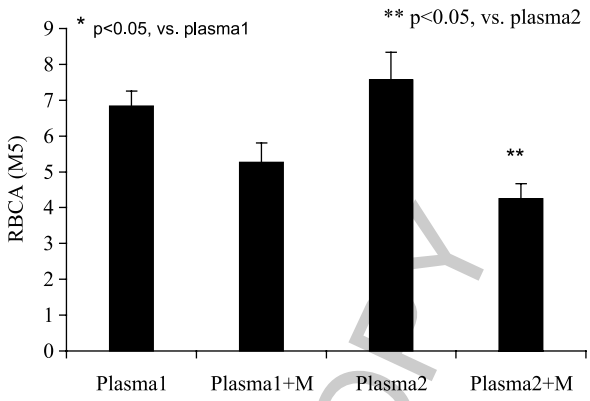

Fig. 2. The alteration of red blood cell aggregation under RBC incubation in autologous plasma containing 2.60 ± 0.12 (plasma 1) and 3.10 ± 0.15 g/L (plasma 2) of fibrinogen with and without monafram. Abbreviations: Plasma1 – mean plasma fibrinogen concentration – 2.60 ± 0.12 g/L; Plasma2 – mean plasma fibrinogen concentration 3.10 ± 0.15 g/L; M – monafram (10.0 μM).

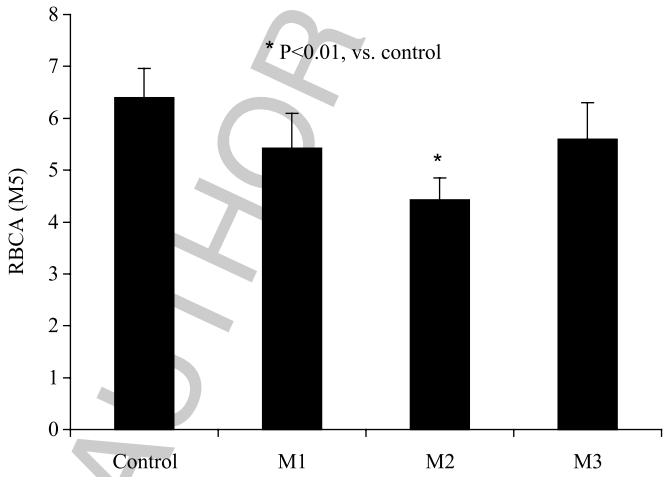

Fig. 3. The alteration of red blood cell aggregation under their incubation with the different monafram concentrations. Abbreviations: M1 – monafram concentration – 5.0 μM; M2 – 10.0 μM; M3 – 50.0 μM.

In another research session, it has been shown that the cell incubation with fibronectin was accompanied by their aggregation rise by 14% (Fig. 4). On the other hand the monafram added to red cell incubation medium resulted in a significant RBCA lowering by 50% (vs. fibronectin; $p < 0.01$). The alteration of the time of RBC aggregate formation under cell incubation with monoAB against GP IIb/IIIa (monafram) and monoAB against platelet P-selectin was analysed. The obtained results showed that the rate of RBCA formation was decelerated in 71% of the cases after monafram while PS10 did not affect this process (Fig. 5).

It has been shown that the aggregate stability in subgroup with highRBCA was markedly decreased after the cell incubation with monafram ($p < 0.05$; Fig. 6). On the other hand no significant difference in this aggregation characteristic after cell incubation with PS10 and monafram was found in the subgroup of individuals with MidRBCA.

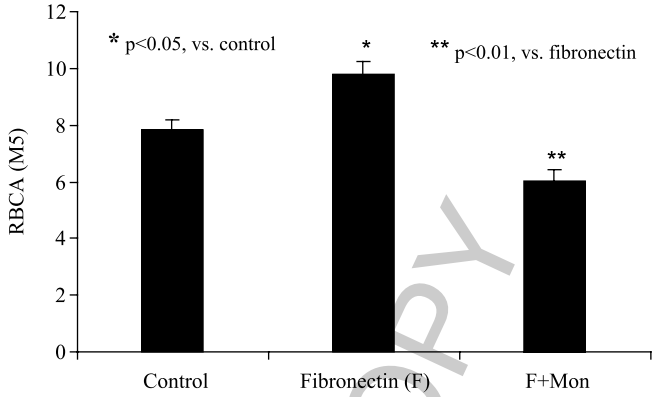

Fig. 4. Alteration of RBC aggregation after the cell preincubation with monafram. Abbreviations: Control – RBCs in 3% Dextran 70 without fibronectin; Fibronectin (F) – RBCs in 3% Dextran 70 containing fibronectin (5 μM); F + Mon – RBCs in 3% Dextran 70 containing fibronectin (5 μM) and monafram (10.0 μM).

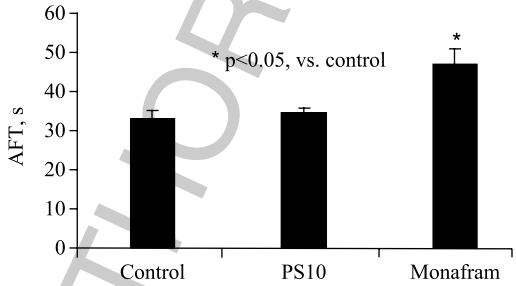

Fig. 5. The alteration of the aggregate formation time (AFT) after cell incubation with PS10 (5 μg/ml) and monafram (10.0 μM). Abbreviations: AFT – aggregate formation time; PS10 – antibodies against platelet P-selectin.

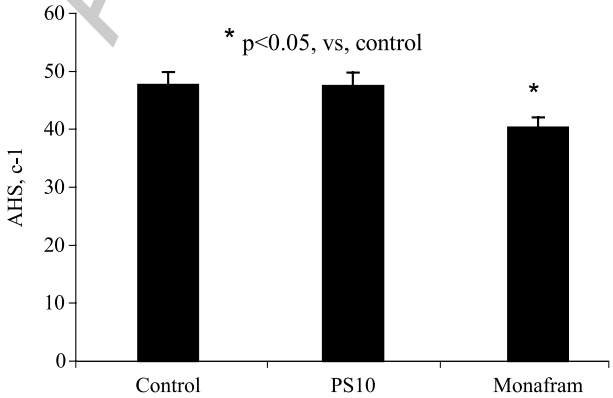

Fig. 6. The hydrodynamic red cell aggregate stability (AHS) in individuals with high RBCA (HighRBCA) under cell incubation with PS10 (5 μg/ml) and Monafram (10 μM). For abbreviations see Fig. 5.

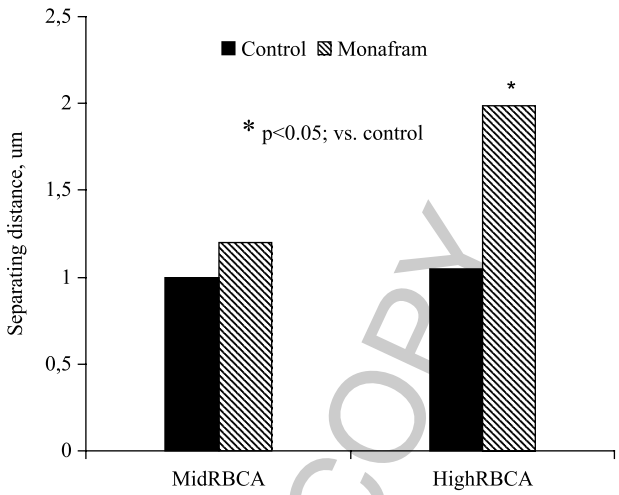

Fig. 7. The alteration of the distance between the centers of two RBCs under an increase of the separating force from 6 pN to 8 pN: the dependence on cell incubation with monafram (10 μM) and an initial level of RBCA (MidRBCA and HighRBCA).

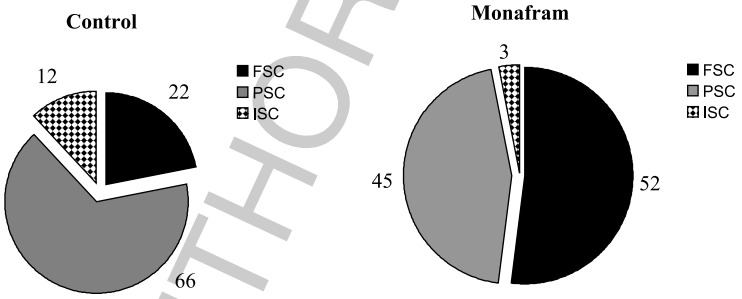

Fig. 8. Increase of couples of RBCs fully separated with laser tweezers after cell incubation with monafram. Abbreviations: FSC – fully separated cells; PSC – partly separated cells; ISC inseparable cells.

There are some more pieces of evidence of the monafram effect on red cell aggregation. We have employed method of the laser tweezers using RBCs suspension in pure platelet plasma ($\sim 5 \cdot 10^3 \mu l^{-1}$ RBCs in a sample).

The separation facilitation of the couples of red blood cells after monafram was more significant in HighRBCA (Fig. 7). Indeed under an increase of the separating forces from 6.0 pN to 8.0 pN (laser tweezers) the distance between the centers of two RBCs was extended by 2.05 ± 1.50 μm ($p < 0.05$) in comparison with control (without monafram), where distance alteration was 1.14 ± 0.78 μm only. Besides in individuals with HighRBCA the part of fully separated couples of RBCs was significantly higher ($p < 0.02$) in the samples with monafram then in the control ones (Fig. 8).

3.2. Characteristics of RBC aggregation under the action of tirofiban, fibrinogen-inhibiting peptide and eptifibatide

Another line of proofs of the possible receptor-binding of the RBCs during aggregation has also been obtained. The cell incubation with the GP IIb/IIIa receptor activity inhibitor tirofiban resulted in

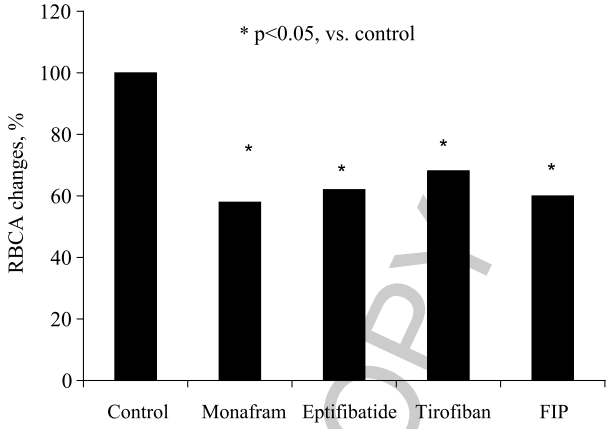

Fig. 9. Relative changes (%) of red blood cell aggregation after incubation with monafram (Mon, 10.0 μM), with IIb/IIIa receptor inhibitors - tirofiban (5.0 μM), eptifibatide (4.0 μM) and with the fibrinogen-inhibiting peptide (FIP, 5.0 μM).

RBCA lowering by 33% (from 6.80 ± 0.22 to 4.53 ± 0.34). Fibrinogen inhibiting peptide (FIP) decreased RBCA by 48% (from 6.60 ± 0.44 to 3.43 ± 0.18; $p < 0.05$). It is known that this chemical strongly inhibits fibrinogen, fibronectin and von Willebrand factor binding with the platelet membrane receptors [34]. We have shown that the red cell aggregation was decreased significantly after RBC treatment with fibrinogen-binding peptide (Fig. 9). This chemical inhibits platelet aggregation and adhesion to fibrinogen and vitronectin by competing with the GPIIb/IIIa receptor [34]. We have detected that the fibrinogen binding peptide acted similar to other RBCA lowering substances and its effect was significant. Another fibrinogen binding inhibitor eptifibatid reduced the rate of aggregate formation in 92% of experiments. The time of linear aggregate formation (AFT) was 8.6 ± 2.8 s in the control group of samples, while for the cells incubated with eptifibatid AFT was 14.1 ± 4.0 s. The difference in 64% was significant ($p < 0.01$; Fig. 10). Besides the couples of RBCs treated with eptifibatide were separated for larger distances comparing to the control samples under application of the same optical trapping force of 20.0 ± 3.0 pN (Fig. 11).

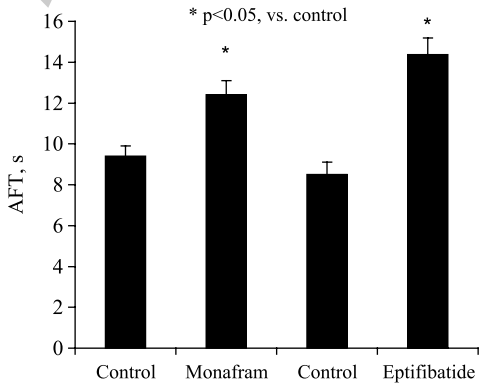

Fig. 10. The alteration of the time of aggregate formation (AFT) after cell incubation with Monafram (10 μM) and Eptifibatide (4.0 μM).

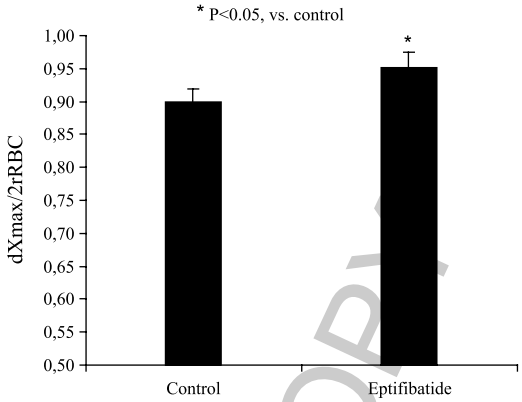

Fig. 11. The normalized maximal distance (dXmax/2rRBC) between the centers of two RBCs under the separating force about 20.0 pN after the cell incubation with Eptifibatide.

4. Discussion

It is well known that one type of the blood cells – platelets – contains a substantial internal pool of GPIIb/IIIa complexes that is exposed on the surface of activated cells [15, 28, 35]. On the other hand, there are no definitive data about the receptors of GPIIb/IIIa type on red blood cell plasmatic membrane. However a marked aggregation lowering after the cell incubation with monafram allows us to suppose that a specific fibrinogen binding under the circumstances is quite possible, at least at high level of RBCA.

By means of atomic force microscopy it has been previously demonstrated, that there is an existence of a single molecule interaction between fibrinogen and unknown receptors on the red cell membrane, with a lower but comparable affinity relative to platelet binding [8]. Our experiments with the separating of RBC couples by laser tweezers give some support of receptor hypotheses of red cell aggregation. Indeed it has been found that after cell incubation with monafram or eptifibatide RBCs could be moved by greater distances than intact cells.

Recently D. Lominadze and W. Dean, [20] showed that an integrin receptor antagonist peptide Arg-Gly-Asp-Ser (RGDS) inhibited the labeled fibrinogen binding to red cell membrane. This peptide also inhibited an aggregation of rat erythrocytes that had been initiated by the human fibrinogen. Fibronectin is a component of subendothelial matrices and is abundant in plasma. It is also considered as a ligand to the glycoprotein (GP) IIb/IIIa receptors [10]. We have found that its presence in the cell incubation medium was accompanied by a significant rise of RBCA. However this effect was completely eliminated by monafram. It is known that GP IIb/IIIa antagonists tirofiban and eptifibatide inhibit a platelet aggregation [13]. Although the monoclonal antibodies against GP IIb/IIIa receptors monafram, the GPIIb/IIIa receptor antagonists tirofiban, eptifibatide and the fibrinogen inhibiting peptide were related to fibrinogen function, they didn't inhibit RBCA completely. Therefore, it could be supposed that the red cell aggregation might be stimulated even if partly with another biopolymers, e.g. immunoglobulins [32, 40]. Taken together these data help to explain the continuing difficulty of the comprehension of the models for RBC aggregation [3, 5, 6, 19, 22, 23, 25, 29, 30]. The experiments with the disconnection of the RBCs couples by means of the laser tweezers showed the decrease of the force necessary for the cell separation after monafram or eptifibatide incubation. Following the effect demonstrated by Bronkhorst et al. [7] we observed cell-to-cell contacts that had the minimal surface contact area. In this case surface dependent osmotic force

must have the minimal value. It makes us believe that adhesion receptor concentration and focal adhesion could be a mechanism of red blood cell aggregation [34]. However, the monoclonal antibodies against GP IIb/IIIa – the receptor for fibrinogen and inhibitors of these receptors – were more active under high level of RBCA only. Therefore, under moderate and low red blood cell aggregation the cell binding is probably related to nonspecific mode.

It seems evident that the specific and non-specific modes of red blood cell aggregate formation could co-exist. Additional theoretical and experimental investigations in this area are needed.

Acknowledgments

The authors comply with the Ethical Guidelines for Publication in *Clinical Hemorheology and Microcirculation* as published on the IOS Press website and in Volume 44, 2010, pp. 1-2 of this journal.

This work was supported by grant of RFBR No. 12-04-00550-a and by FSP "Research The authors are grateful to Prof. A.V. Mazurov (Russian Cardiology Research and Production Complex, Moscow) for gifted monoclonal antibodies.

The authors would like to thank L.A. Kvartalnov (ReoMedLab, Moscow) for the technical support.

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