BIOPHYSICS AND BIOCHEMISTRY

Effect of Oxidized Fibrinogen on Aggregation of Activated Platelets and Neutrophils

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The effect of oxidized fibrinogen on platelet-neutrophil complex formation was evaluated by studying the platelet aggregation (changes in light transmission and turbidimetric assay). Activation of cells by thrombin (0.015 U/ml) in the presence of oxidized fibrinogen was accompanied by the formation of larger intermolecular aggregates of platelets and leukocytes as compared to those detected in experiments with non-oxidized fibrinogen. Addition of thrombin (0.2 U/ml) in the presence of oxidized fibrinogen was followed by the formation of more stable complexes of platelets and leukocytes as compared to those revealed in experiments with non-oxidized fibrinogen. An increase in the width of aggregation curves was most pronounced in the system of 10^{-4} M Fe^{2+} and 10^{-4} M H_2O_2 with oxidized fibrinogen. Our results indicate that oxidized fibrinogen contributes to the "floating" or suspension of platelet-leukocyte complexes.

Key Words: *platelets; neutrophils; platelet-neutrophil complexes; free radicals; oxidized fibrinogen*

Cardiovascular diseases are often accompanied by the formation of platelet-neutrophil complexes [8-10], which has an adverse effect on microcirculation. Microcirculatory disturbances are observed in various diseases of the heart and peripheral vessels and have a major role in the progression and complication of these disorders. Patients with unstable angina pectoris have a considerable number of blood complexes, which serves as a criterion for high risk of stroke and infarction [10]. A relationship exists between the development of complications after surgeries on heart vessels (restenosis) and platelet-neutrophil complex formation [8]. Studying the mechanism of complex formation is an urgent problem.

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Published data show that P selectin is a granular integral protein expressed on the surface of activated platelets. This protein is responsible for stage I of the interaction between platelets and polymorphonuclear neutrophils (PMN) [6]. P selectin recognizes surface ligands on PMN, including PSGL-1 [6]. Stage II is mediated by the 2β-integrin-dependent mechanism, which results in the formation of stable intercellular complexes. β-Integrins are localized on the surface of PMN and bind to unknown ligands on the surface of platelets [6]. Fibrinogen also promotes the formation of these complexes. Previous experiments on the isolated guinea pig heart revealed that c7E3Fab (chimeric Fab fibrinogen-like fragment) blocks the fibrinogen receptor on platelets (IIb/IIIa), decreases the total number of complexes, and reduces the complications of vascular ischemia [5].

Much attention is paid to the involvement of fibrinogen in complex formation between PMN and

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platelets. Fibrinogen is an independent risk factor for atherosclerosis, infarction, and stroke. The plasma protein fibrinogen is most sensitive to oxidative modification. Oxidized fibrinogen increases the degree of hemostatic disorders. For example, oxidized fibrinogen has a potentiating effect on induced platelet aggregation and increases the production of free radicals by activated leukocytes [1]. However, the role of oxidized fibrinogen in platelet-PMN complex formation is poorly understood.

Here we studied the effect of oxidized fibrinogen on platelet-neutrophil complex formation under conditions of continuous mixing. Platelet aggregation was studied by recording of changes in light transmission (CLT) [2] and turbidimetric assay.

MATERIALS AND METHODS

The cells were obtained by differential centrifugation in a density gradient of 1077 g/cm^3. Erythrocyte hemolysis was induced by osmotic shock [4]. Polymorphonuclear leukocytes (PML) were isolated. The blood (7.5 ml) was stabilized with sodium citrate (citrate/blood ratio 1:5), mixed with 1.5 ml 5% dextran 500, and incubated at 37°C for 30 min. The supernatant of leukocytes was layered on Histopaque (density 1077, 4:3 v/v; Sigma). Centrifugation was performed at 250*g* for 20 min. The remaining erythrocytes were hemolyzed by addition of 4 ml distilled water to the pellet of cells. Tonicity of the medium was restored by addition of Hanks buffer (4 ml, double concentration) not containing Ca^{2+} and Mg^{2+}. Centrifugation was performed at 100*g* for 15 min. The pellet of neutrophils was resuspended in 1 ml Hanks buffer. The cells were counted in a Goryaev chamber. The cell suspension was stored in a refrigerator at 2-8°C.

Platelets were isolated using a washing solution with high concentration of citrate [14]. The blood was stabilized with sodium citrate (4.5%, pH 6.5; citrate/blood ratio 1:5) and centrifuged at 100*g* for 15 min to obtain platelet-rich plasma (PRP). PRP (2-3 ml) was centrifuged at 600*g* for 4 min. The pellet of platelets was resuspended in 1 ml washing buffer of 33 mM sodium citrate, 1 mM citric acid, 103 mM NaCl, 5 mM KCl, and 0.8 mM $MgCl_2 \times 6H_2O$ (pH 6.5) for 45 min. The platelet suspension was brought to a final concentration with Ca^{2+}-free Hanks buffer. Platelets were stored at 37°C.

PML were stained with a vital dye hydroethidine (HE, Leiden). The matrix solution was prepared by dissolution of 8 mg HE in 1 ml dimethylsulfoxide (DMSO). The solution was stored in a freezing chamber at -20°C. For the staining procedure, 2.5 ml HE was added to 1 ml isolated neutrophils (5×10^7 cells/ml). The final concentration of HE was 20 mg/ml. Incubation was performed at 4°C for 30 min.

The cells were washed by centrifugation at 100*g* for 5 min. The supernatant was removed. HEPES-Tyrode buffer (1 ml) was added to the pellet of cells. Twofold washing was performed by centrifugation at 100*g* for 5 min.

Platelets were stained with a vital dye BCECF (Sigma). BCECF (1 mg) was dissolved in DMSO (1 ml). The solution was stored at -20°C for 1 month. PRP (1 ml) was incubated with 2 ml BCECF at 37°C for 30 min. Platelets were isolated by the standard method.

Fluorescent images were obtained using a Nikon ECLIPSE E-800 fluorescence microscope equipped with a confocal module C1.

Fibrinogen was oxidized by the following three methods: (1) treatment with 10^{-4} M Fe^{2+}; (2) treatment with 10^{-4} M Fe^{2+} and 10^{-4} M H_2O_2; and (3) autooxidation.

During Fe^{2+}-induced oxidation, 2 ml fibrinogen (3 mg/ml, Sigma) were incubated in phosphate buffered saline (pH 7.4, Sigma) with 10^{-4} M $FeSO_4\times7H_2O$ or 10^{-4} M $FeSO4\times7H_2O$ and H_2O_2 (10^{-4} M). Incubation was performed in a thermostat at 37°C for 1 h. Fe^{2+} ions were bound by addition of deferoxamine mesylate (Sigma) to a concentration of 1 mM. Oxidized protein was dialyzed against 3 l phosphate buffered saline (0.15 M NaCl and 5 mM NaH_2PO_4, pH 7.4) for 15 h.

Autoxidized fibrinogen was prepared by incubation of fibrinogen (3 mg/ml) in phosphate buffered saline (Sigma). Incubation was performed in a thermostat at 37°C (1 h) and room temperature (15 h).

The suspension of isolated platelets was mixed with isolated neutrophils (neutrophil/platelet ratio 1:30). The mean radius of aggregates and intensity of light transmission were recorded on a NPF Biola device. The device was calibrated. Light transmission of the platelet-neutrophil suspension and water was taken as 0 and 100%, respectively. Thrombin (RENAM) was added after 30-sec recording of curves. The study was conducted in cylindrical quartz cuvettes under constant agitation (800 rpm, 37°C).

The data were analyzed by Student's *t* test. The results of three independent experiments are expressed as mean values and error of the mean.

RESULTS

The optimal concentrations of thrombin were selected to study the coaggregation of platelets and leukocytes. The aggregation of isolated platelets (without leukocytes) in the presence of fibrinogen was assayed at a thrombin concentration of 0.00375-

0.2 U/ml (Fig. 1, *a*, *b*). Platelet aggregation was moderate at low concentrations of thrombin (0.00375 and 0.0075 U/ml). It was manifested in a small increase in light transmission and low value of the mean radius of aggregates. High-intensity aggregation of platelets was observed in the presence of thrombin at concentrations of 0.015 and 0.2 U/ml. We revealed an increase in light trans-

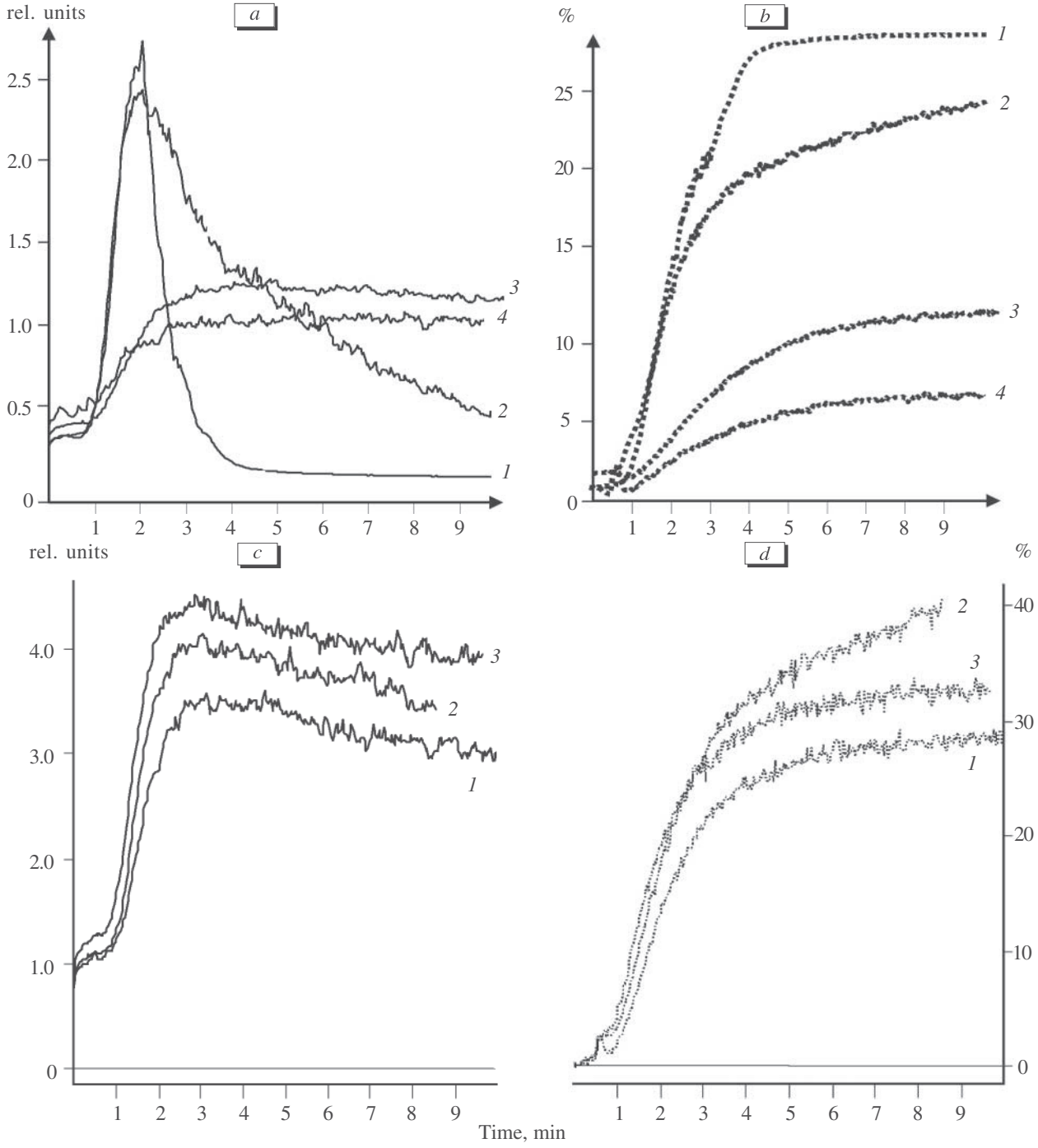

Fig. 1. Kinetic curves for aggregation of isolated platelets and platelets-and-leukocytes in the presence of oxidized or non-oxidized fibrinogen. (*a*, *b*) Aggregation of isolated platelets in the presence of 1 mg/ml fibrinogen (30,000 platelets/ml) and various concentrations of thrombin (addition of thrombin after 30-sec recording of the curve): mean radius of aggregates (*a*) and light transmission (*b*). Thrombin concentrations: 0.2 (*1*), 0.015 (*2*), 0.0075 (*3*), and 0.00375 U/ml (*4*). (*c*, *d*) Aggregation of thrombin-activated platelets (0.015 U/ml) and neutrophils in the presence of non-oxidized or oxidized fibrinogen: mean radius of aggregates (*c*) and light transmission (*d*). Platelets and leukocytes in the absence of fibrinogen (*1*); platelets and leukocytes in the presence of 1 mg/ml non-oxidized fibrinogen (*2*); and platelets and leukocytes in the presence of fibrinogen oxidized by 10^{-4} M Fe^{2+} and 10^{-4} M H_2O_2 (*3*).

mission and maximum value on the curve for the mean radius of aggregates (Fig. 1, *a*, *b*). Further experiments were performed with thrombin at the concentrations, which resulted in high-intensity aggregation of platelets.

Coaggregation of thrombin-activated platelets and neutrophils was studied in the presence of non-oxidized and oxidized fibrinogen (Fig. 1, *c*, *d*). Studying the mean radius of aggregation after addition of thrombin (0.015 U/ml) in the presence of leukocytes showed that aggregation curves are characterized by irreversible aggregation (as differentiated from platelet aggregation curves in the presence of fibrinogen). Curves for the mean radius of aggregates rapidly reached the maximum value, but declined progressively in the follow-up period. These results illustrate the stability of platelet-leukocyte complexes.

Oxidized fibrinogen had the maximum-amplitude curves for the mean radius of aggregates (as compared to non-oxidized fibrinogen). According to the CLT method, these data indicate that oxidized fibrinogen contributes to the formation of larger intermolecular complexes of platelets and leukocytes.

Light transmission curves reached a plateau (without fibrinogen and in the presence of oxidized fibrinogen) or increased progressively (in the presence of non-oxidized fibrinogen). We conclude that non-oxidized fibrinogen promotes the formation of fibrin. Our results are consistent with published data on the reduced polymerization of oxidized fibrin monomers [3].

Besides studying the aggregation of platelets and leukocytes, the formation of platelet-leukocyte complexes was evaluated by means of confocal fluorescence microscopy. This series should confirm or disprove the formation of intercellular aggregates in our system. Vitally stained platelets (green dye BCECF) and leukocytes (nuclear dye HE) were activated with thrombin. The experimental conditions were similar to those in studying the aggregation of platelets and leukocytes. One minute after addition of thrombin, the cells were fixed with 1% paraformaldehyde for a further microscopic study (Fig. 2).

Fig. 2 shows the images of fixed samples of platelets and leukocytes, which illustrates the coaggregation of thrombin-activated platelets and leukocytes: moderate aggregation of platelets in the absence of fibrinogen (Fig. 2, *a*); leukocytes-induced formation of individual platelet complexes in the absence of fibrinogen (Fig. 2, *b*); strong coaggregation of platelets and leukocytes in the presence of non-oxidized fibrinogen (Fig. 2, *c*, *d*) that confirms the formation of platelet-leukocyte complexes (study of aggregation curves); and formation of the largest complexes in the presence of oxidized fibrinogen (Fig. 2, *e*).

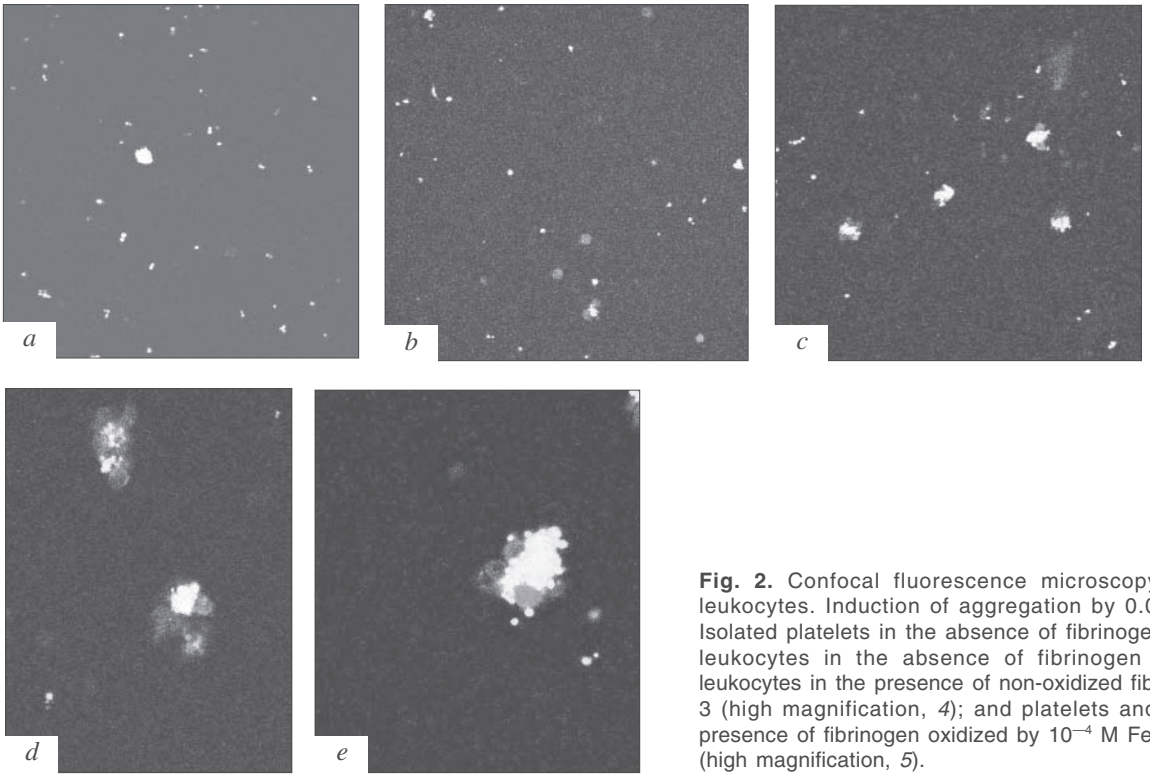

Fig. 2. Confocal fluorescence microscopy of platelets and leukocytes. Induction of aggregation by 0.015 U/ml thrombin. Isolated platelets in the absence of fibrinogen (*1*); platelets and leukocytes in the absence of fibrinogen (*2*); platelets and leukocytes in the presence of non-oxidized fibrinogen (*3*); sample 3 (high magnification, *4*); and platelets and leukocytes in the presence of fibrinogen oxidized by 10^{-4} M Fe^{2+} and 10^{-4} M H_2O_2 (high magnification, *5*).

The results of confocal fluorescence microscopy confirm the formation of intercellular aggregates of platelets and leukocytes under these experimental conditions (study of coaggregation between platelets and leukocytes in the presence of non-oxidized or oxidized fibrinogen). Considerable amounts of intercellular aggregates were formed in the presence of oxidized fibrinogen.

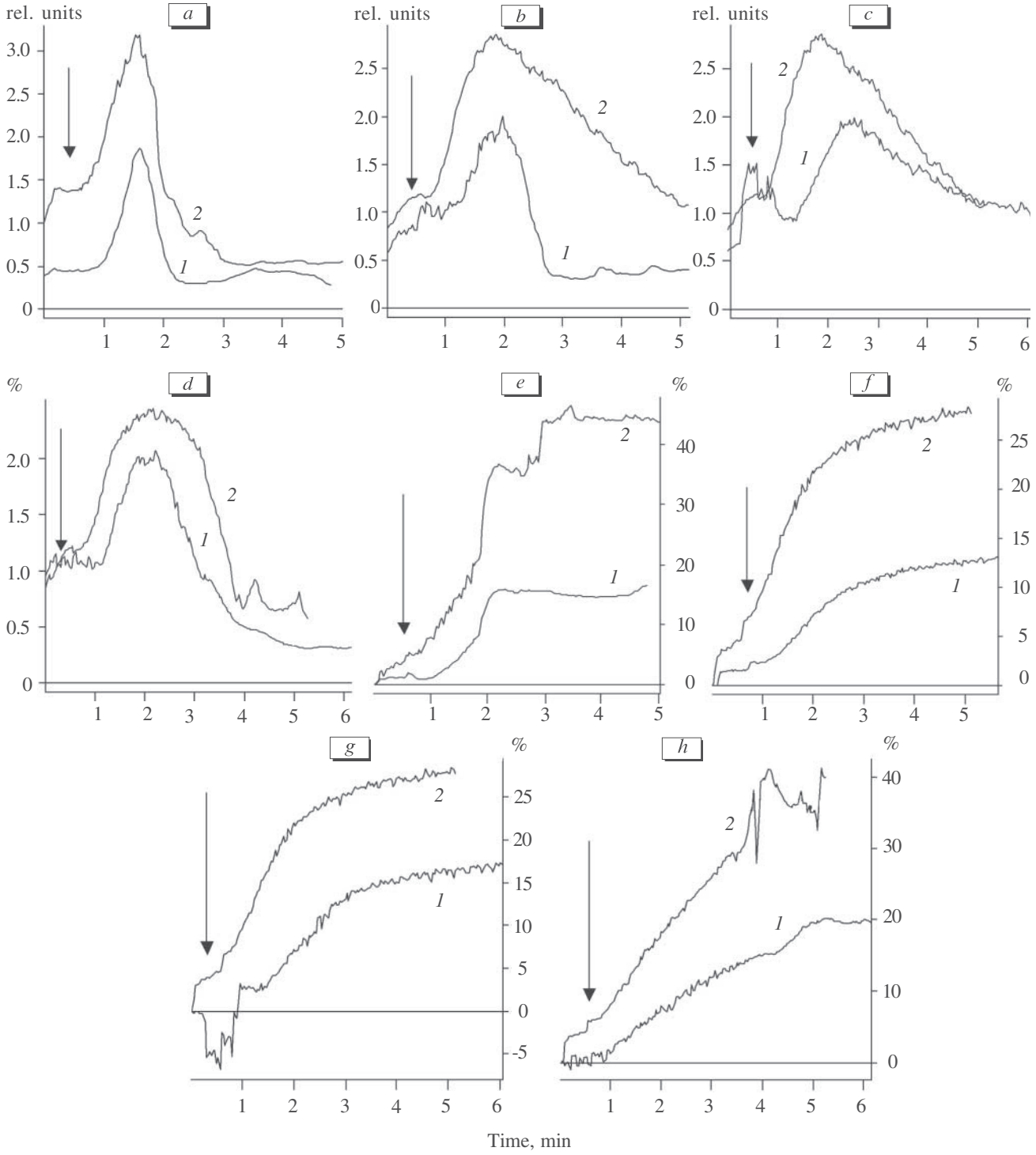

Fig. 3. Kinetic curves for thrombin-induced aggregation (0.2 U/ml) of isolated platelets (curve *1*) and isolated platelets and neutrophils (curve *2*) in the presence of differently oxidized fibrinogen (final concentration 1 mg/ml). Arrows: administration of thrombin. (*a-d*) Mean radius of aggregates; (*e-h*) light transmission. (*a*, *e*) Native (non-oxidized) fibrinogen; (*b*, *f*) 10^{-4} M Fe^{2+}; (*c*, *g*) 10^{-4} M Fe^{2+} and 10^{-4} M H_2O_2; and (*d*, *h*) autoxidation.

TABLE 1. Relative Changes in the Mean Radius of Aggregates and Light Transmission during Aggregation of Thrombin-Activated Platelets and Neutrophils

Parameter	Fibrinogen			
	non-oxidized	oxidized by 10^{-4} M Fe^{2+}	oxidized by 10^{-4} M Fe^{2+} and 10^{-4} M H_2O_2	autoxidation
Mean radius of aggregates after 4 min/maximum amplitude, rel. units	21±7	54±10	58±10	29±8
Light transmission 1.5 min after addition of thrombin/light transmission 4 min after addition of thrombin, %	200±22	50±7	42±6	175±20

In the next series, we studied the effect of oxidatively modified fibrinogen on platelet-neutrophil complex formation at high concentration of thrombin (0.2 U/ml). Fig. 3 shows the kinetic curves for variations in the mean radius of aggregates and light transmission of thrombin-activated (0.2 U/ml) platelets and leukocytes in the presence of differently oxidized fibrinogen.

Strong aggregation of platelets was observed at a thrombin concentration of 0.2 U/ml in the presence of non-oxidized fibrinogen (Fig. 1, Fig. 3, *a*). Curves for variations in the mean radius of aggregates are shown in Fig. 1. The aggregation curve rapidly reached the maximum value over 1 min after addition of non-oxidized fibrinogen. A rapid decline in the aggregation curve was found in the follow-up period. As differentiated from non-oxidized fibrinogen, the mean radius of aggregates decreased progressively in the presence of oxidized fibrinogen (Fig. 3, *b-d*). This conclusion was derived from an increase in the width of aggregation curves. Increasing the degree of fibrinogen oxidation was accompanied by a greater increase in the width.

Previous studies showed that the initial segment of aggregation curves illustrates the formation of intercellular aggregates from platelets and leukocytes in the presence of oxidized or non-oxidized fibrinogen. A decrease in the mean radius of aggregates reflects the degradation of intercellular aggregates.

In our experiments, the mean radius of aggregates decreased below the baseline value. These cells passed beyond the operational limits of an aggregometer laser beam. It was probably associated with precipitation of aggregated cells or agglutination of cells with fibrin. Platelet aggregation is accompanied by the formation of fibrin, which results in the clotting and adhesion of cells to the wall of a cuvette or stirrer. The latter process seems to be most probable, since the use of non-oxidized fibrinogen is always followed by the appearance of cell agglomerates.

The mean radius decreased slowly in the presence of oxidized fibrinogen (widening of aggregation curves), which reflects the stability of these complexes.

The data on aggregation curves (mean radius of aggregates) were analyzed. The ratio of the mean radius of aggregates (4th minute after addition of thrombin) to the maximum amplitude served as a quantitative parameter for the widening of kinetic curves (Table 1).

An increase in the width of aggregation curves for the mean radius of aggregates was proportional to the oxidation degree of fibrinogen. An increase in the width of aggregation curves was most pronounced in the presence of fibrinogen oxidized by 10^{-4} M Fe^{2+} and 10^{-4} M H_2O_2 (Table 1).

The aggregation curve for light transmission was analyzed after treatment with non-oxidized fibrinogen. It should be emphasized that all curves for an increase in oxidized fibrinogen reached the maximum value. By contrast, the curve for an increase in light transmission of non-oxidized fibrinogen had a sharp rise 1.5-2 min after addition of thrombin. A sharp rise in light transmission curves is probably related to the fact that fibrin formation accompanies platelet aggregation. First, fibrinogen is converted to fibrin after 1.5-2 min. These changes are accompanied by variations in light transmission. And second, fibrin contributes to platelet clotting. A clot of platelets adheres to the wall of a cuvette or stirrer.

These data indicate that oxidized fibrinogen is much more potent in maintaining the “floating” or suspension of platelet-leukocyte complexes.

Thrombosis is one of the most serous complications of cardiovascular diseases. Studying the mechanisms for abnormalities in vascular hemostasis during atherosclerosis is an urgent problem.

Oxidative stress has a major role in atherosclerosis. For example, this process is involved in cell adhesion. Previous studies showed that adhe-

sion of leukocytes to endothelial cells is induced by free radicals [13]. Oxidant-induced adhesion of leukocytes to endothelial cells is inhibited by hydroxyl radical-trapping agents or Fe^{2+}-chelating compounds. Some mechanisms of these processes were discussed. Treatment of endothelial cells with free radicals is followed by phosphorylation of tyrosine kinase $pp125_{FAK}$, which has a role in free radical-induced adhesion [12,15].

Studying the adhesion of platelets and leukocytes and confocal fluorescence microscopy showed that activation of cells with 0.015 U/ml thrombin in the presence of oxidized fibrinogen is accompanied by the formation of larger intermolecular aggregates of platelets and leukocytes (as compared to those detected in the presence of non-oxidized fibrinogen).

Addition of thrombin (0.2 U/ml) in the presence of oxidized fibrinogen is followed by the formation of more stable complexes of platelets and leukocytes as compared to those revealed in experiments with non-oxidized fibrinogen.

Our results indicate that oxidized fibrinogen is much more potent in maintaining the "floating" or suspension of platelet-leukocyte complexes. It contributes to the spread of complexes over the vascular system and increases the risk of blood vessel occlusion.

REFERENCES

1. O. A. Azizova, A. V. Aseychev, A. P. Piryazev, *et al*., *Byull. Eksp. Biol. Med*., **144**, Suppl. 2, 397-407 (2007).
2. Z. A. Gabbasov, E. G. Popov, I. Yu. Gavrilov, *et al*., *Lab. Delo*, No. 10, 15-18 (1989).
3. E. V. Roitman, O. A. Azizova, Yu. A. Morozov, and A. V. Aseychev, *Byull. Eksp. Biol. Med*., **138**, No. 9, 277-279 (2004).
4. A. Boyum, *Scand. J. Immunol*., Suppl. 5, 9-15 (1976).
5. C. Kupatt, H. Habazettl, P. Hanusch, *et al*., *Arteriosclerosis, Thromb. Vascular Biol*., **20**, 2226 (2000).
6. V. Evangelista, S. Manarini, S. Rotondo, *et al*., *Blood*, **88**, 4183-4194 (1996).
7. P. H. Kuijper, Gallardo, H. I. Tores, *et al*., *Blood*, **89**, No. 1, 166-175 (1997).
8. J. K. Mickelson, V. M. Lakkis, G. Villarreal-Levy, *et al*., *J. Am. Coll. Cardiol.*, **28**, No. 2, 345-353 (1996).
9. K. Okajima, W. P. Yang, H. Okabe, *et al*., *Am. J. Hematol*., **36**, No. 4, 265-271 (1991).
10. I. Ott, F.-J. Neumann, M. Gawaz, *et al*., *Circulation*, **94**, 1239-1246 (1996).
11. D. Sako, X. J. Chang, K. M. Barone, *et al*., *Cell*, **75**, No. 6, 1179-1186 (1993).
12. M. D. Schaller, C. A. Borgman, B. S. Cobb, *et al*., *Proc. Natl. Acad. Sci. USA*, **89**, No. 11, 5192-5196 (1992).
13. H. Sellak, E. Franzini, J. Hakim, and C. Pasquer, *Blood*, **83**, No. 9, 2669-2677 (1994).
14. T. Shimizu, K. Shibata, and S. Kora, *Vox. Sang*., **64**, No. 1, 19-23 (1993).
15. S. Roy, C. K. Sen, A. Gozin, *et al*., *Antioxidants and Redox Regulation of Genes*, Eds. K. Sen *et al*., San Diego, CA (2000), pp. 265-295.