Effects of Oxidized Fibrinogen on the Functions of Blood Cells, Blood Clotting, and Rheology

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Oxidatively-modified fibrinogen induces platelet aggregation and potentiates ADP-induced platelet aggregation and production of active oxygen forms in zymosan-stimulated leukocytes. Fibrinogen induces IL-8 production in primary culture of endothelial cells from human umbilical vein; the oxidized form of fibrinogen is more active, similarly as during induction of the expression cell adhesion molecules (P-selectin and ICAM-1). Oxidized fibrinogen (10 and 20% oxidation degree) impairs microrheological properties of the blood, sharply reduces erythrocyte deformability, modifies blood viscosity, and reduces suspension stability of the blood. Oxidized fibrinogen modified blood clotting parameters and ADP-, ristocetin-, and collagen-induced platelet aggregation in whole blood. Oxidized fibrinogen disordered the formation of fibrin clot and blood clotting process. Platelet aggregation was activated in response to ADP, but not to ristocetin and collagen, the degree of activation increased in direct proportion to the degree of fibrinogen oxidation. This indicates the “dysregulatory” effect of oxidized fibrinogen on platelets. The formation of platelet complexes with polymorphonuclear leukocytes was intensified in the presence of oxidized fibrinogen; polymorphonuclear leukocyte luminol-dependent fluorescence intensity in the presence of platelets increased after incubation with oxidized fibrinogen in comparison with native fibrinogen. Hence, oxidized fibrinogen plays an important role in the development of atherosclerosis and its complications (thromboses).

Key Words: *oxidized fibrinogen; blood cells; vascular endothelium culture; blood rheology; blood clotting*

Numerous studies showed that the development of many pathological states (ischemia, inflammation) and diseases (atherosclerosis, coronary disease, diabetes mellitus, hypertension, cancer) is associated with activation and intensification of free-radical processes [4]. A perspective trend of research all over the world is the study of various modifications (including oxidative) of plasma proteins, such as serum albumin, fibrinogen (FG), and lipoproteins (LP) and the role of these processes in the development of atherosclerosis.

Our studies carried out during the recent 15 years showed that oxidized LP (oLP) play the key role in the development of vascular atherosclerosis. Oxidized LP were detected in the plasma and vascular walls of cardiovascular patients and diabetics. Numerous studies at the cellular level and on animals have shown that oLP initiate and are involved in the development of atherosclerosis at all stages: they modify the permeability of the endothelium, expression of cell adhesion molecules interacting with monocytes and platelets, they promote the

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formation of foamy cells, which leads to the formation of chronic inflammation, synthesis of IL-1 and IL-6 cytokines, formation of atherosclerotic plaques and their subsequent rupture. In addition, oLP activate thrombogenesis with subsequent development of stroke and infarction [11].

The blood contains other proteins, participating in the development of atherosclerosis. For example, FG is an independent risk factor for atherosclerosis and its complications, stroke and infarction [15]. Fibrinogen causes thickening of the vascular intima and its high plasma content is associated with increased thrombogenesis and risk of stroke and infarction, as well as with the risk of complications after cardiosurgery [19]. Importantly, that FG is a protein most sensitive to oxidative stress [22]. As atherosclerosis development is closely linked with free-radical reactions of lipid, protein, and nucleic acid oxidation, we hypothesized that oxidized FG (oFG), along with oLP, can appear in the plasma and promote pathological processes observed in atherosclerosis.

Based on this hypothesis, starting from 2001 we have studied the effects of oFG on the functions of blood cells and endothelial cells (EC), on blood clotting and rheology.

Effects of oFG on platelet aggregation and neutrophil functions

In addition to its role in the clotting cascade, FG stimulates migration and proliferation of smooth-muscle cells, promotes platelet aggregation (PA), and increases blood viscosity [15]. Elevated (*vs.* the reference values) plasma level of this protein is an independent risk factor for the development of thrombotic complications in various inflammatory processes [20,24]. It has been found that plasma FG is easily oxidized, and presumably, as a result of free-radical processes activation, oFG accumulates in the blood and stimulates thrombogenesis processes.

Oxidative modification of FG was attained by using UV exposure (mercuric lamp) of FG solution. Oxidative modification was monitored by recording the decrease in the protein tryptophan fluorescence (measurements were carried out on a Perkin Elmer spectrofluorometer at λ=280 nm excitation and λ=340 nm emission wavelengths).

In order to evaluate the effect of oFG on PA, solution of UV-irradiated FG was mixed in 1:1 volume ratio with platelet-rich donor plasma (PRP). Directly after mixing FG solution with PRP the PA curve was recorded (changes in the mean radius of aggregations) on an automated PA analyzer (Biola) in the mode for analysis of fluctuations in light transmission [5].

Reversible PA was recorded after addition of oFG to PRP; it manifested by a slow increase in aggregation rate 30 sec after the beginning of aggregation curve registration and reached the maximum 1 min after the beginning of registration (Fig. 1), after which aggregation was increasing slowly. Addition of native FG to PRP did not lead to this effect.

The relationship between the degree of FG oxidation and intensity of reversible PA presented as a funnel-shaped curve (Fig. 1). The maximum PA was observed with FG oxidized to the degree when tryptophan fluorescence intensity decreased by about 24% (oFG range from 12.7 to 63.3% oxidized tryptophan residues was studied). Greater oxidation of FG was associated with a drop of PA intensity. If more than 50% FG tryptophan residues were oxidized, no PA was observed in some cases.

The next step was the study of the relationship between oFG concentration and degree of PA. The amplitude of PA increased with increasing the concentration of FG (pre-exposed to UV radiation) from 0.1 to 1 mg/ml and the plateau was attained as a concentration of about 1 mg/ml. The rate of PA virtually did not depend on FG concentration. Spontaneous PA observed after addition of native FG was significantly lower than aggregation after addition of oFG. Some authors observed intensification of PA under the effect of oFG, while others noted a decrease of PA under its effect. These disagreements with our results can be explained by differences in the methods of FG oxidation and in the degree of FG oxidation. We detected platelet activa-

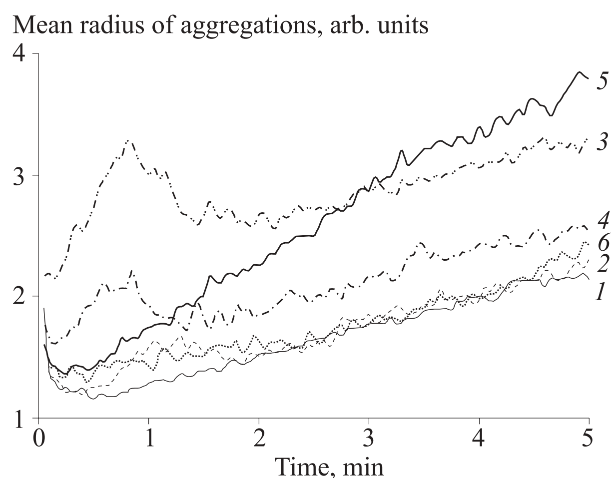

Fig. 1. Platelet aggregation curves in a typical experiment, with PRP to which UV-exposed FG of different oxidation degree was added; PA was evaluated by the decrease in tryptophan fluorescence. *1*) native FG; *2-6*) oFG oxidized by: *2*) 12.7%; *3*) 24%; *4*) 38.2%; *5*) 46.2%; *6*) 63.3%.

tion only in the persence of low-oxidized FG. Hence, we revealed that oFG can act as a PA inductor [3].

The next step of the study was evaluation of oFG effect on ADP-induced PA in PRP. Platelet aggregation was studied by the kinetic turbidimetry on an automated PA analyzer (Biola). Working solution was prepared by mixing PRP with FG solution in a concentration of 2 mg/ml (1:1 volume ratio), the sample was warmed to 37°C, and the aggregation curves were recorded after 2 min. Aggregation inductor ADP (5 μmol/liter) was used. The results of 15 independent experiments indicate that oFG activates ADP-induced PA by 1.33±0.22 times in comparison with native FG (light transmission was measured 3.5 min after ADP addition).

In order to clear out the mechanism of the activating effect of oFG on ADP-induced PA in PRP, we used inhibitors of enzymes involved in intracellular signaling processes: acetylsalicylic acid (cycloxygenase inhibitor); H7 (protein kinase C inhibitor), genistein (tyrosine kinase inhibitor); α-tocopherol (antioxidant and lipoxygenase inhibitor); and U73122 (phospholipase C inhibitor).

The effects of inhibitors on ADP-induced PA in PRP with oFG or native FG were studied. Platelet aggregation was evaluated after preincubation of PRP with the inhibitor at 37°C. The parameter for analysis was derived from the kinetic aggregation curves: light transmission value 3.5 min after addition of ADP.

Arachidonic acid is metabolized in platelets by means of cycloxygenase and lipoxygenase. Cycloxygenase metabolites are involved in the regulation of platelet function. For example, thromboxane A_2, an important proaggregation agent, is synthesized from arachidonic acid by the cycloxygenase pathway. Blocking of cycloxygenase, for example, with indomethacin, causes suppression of PA phase 2 and of secretion of compact granules induced by ADP and other "weak agonists". The findings of our experiments with acetylsalicylic acid confirm the suppression of PA phase 2 by this inhibitor. Addition of oFG to PRP leads to a less pronounced suppression of PA by the inhibitor than addition of native FG. At acetylsalicylic acid concentrations of 1.5, 5, and 150 μmol/liter light transmission in the presence of native FG decreased 1.41, 2.22, and 4.25 times in comparison with the control, respectively. In the presence of oFG light transmission decreased to a lesser extent: by 1.16, 1.49, and 2.65 times *vs.* the control, respectively. These data suggest that oFG activates cycloxygenase.

This result is practically important, because acetylsalicylic acid is a widely used antiaggregant. The development of oxidative stress during exacerbation of coronary disease and further oxidation of FG can significantly reduce the therapeutic effect of acetylsalicylic acid, which should be taken into consideration when evaluating the degree of FG oxidation in the blood.

Proteinkinase C plays an important role in platelet activation. This enzyme is activated by diacylglycerol and its activity depends on the presence of calcium. Activity of proteinkinase C in the platelets manifests by many effects: it modifies receptors (for example, α_1-adrenergic receptor), the signal transduction in platelets by modulating the agonist effects on adenylate cyclase and PIP_2 hydrolysis, activities of Ca^{2+} channels.

Our results are in line with international findings indicating that proteinkinase C inhibition with H7 leads to a significant suppression of all PA parameters: mean radius of aggregation and parameters of light transmission kinetic curves. Importantly, this effect manifests during PA phases 1 and 2. Similarly as with acetylsalicylic acid, oFG reduces the effect of the inhibitor. A significant effect is observed at very high concentrations of H7, starting from 200 μmol/liter. In the presence of native FG, light transmission decreased by 5.07 times under the effect of H7 (200 μmol/liter) compared to the control. In the presence of oFG light transmission decreased less markedly in comparison with the control (2.38 times).

This can indicate a certain activating effect of oFG on protein kinase C, which suggested investigating the inhibitor effects on another important modulator of intracellular signaling processes, tyrosine kinase. Our data indicate that genistein causes a moderate suppression of ADP-induced PA. This effect is directed largely to light transmission and less so to the mean radius of aggregation. Oxidized FG slightly reduced the inhibitor effect. Light transmission at genistein concentration of 240 μmol/liter decreased 2.08 times in the presence of native FG in comparison with the control, while in the presence of oFG it decreased 1.83 times *vs.* the control.

We hypothesized that the mechanism of oFG effect on platelets is LPO activation, which could modify the intracellular regulation pathways in which free-radical oxidation was involved. In order to investigate this possibility, we studied the effect of oil-soluble antioxidant (α-tocopherol) on ADP-induced PA. α-Tocopherol acetate was used in concentrations of 50 to 300 μmol/liter. Preincubation of PRP with α-tocopherol acetate did not suppress ADP-induced PA either with native or oxidized FG.

Phosphoinositide-specific phospholipase C is the key enzyme in the intracellular signaling pro-

cesses. One product of its activity is inositol triphosphate releasing Ca^{2+} from intracellular depots. Another product, diacylglycerol, together with Ca^{2+} activates protein kinase C. Phospholipase C inhibitor U73122 largely suppressed ADP-induced PA, which manifested equally in the presence of native and oxidized FG.

Hence, inositol triphosphate-dependent elevation of intracellular Ca^{2+} concentration is important for manifestation of the effect of oFG on platelets. Our data indicate that oFG stimulates the most important PA pathways leading to intensification of their ADP-induced aggregation [2].

Effect of oFG on blood rheology

It is known that FG is one of the major participants in the blood clotting cascade. It can be hypothesized that oFG modifies the clotting parameters and blood microrheology. The role of FG in erythrocyte aggregation and formation of blood rheology in general is also well known. We studied the effects of oFG on blood clotting and rheology.

Two solutions of UV-irradiated oFG were used (10 and 20% oxidation).

oFG (2 mg/ml) was added into tubes with 10 ml citrate blood, mixed, and analyzed immediately. Donor blood served as the control.

The samples were analyzed by clotting methods for studies of the blood clotting system (activated partial thromboplastin time, prothrombin time, thrombin time, FG concentration) and by thromboelastogram parameters; platelet count and PA in response to aggregation inductors (ADP, ristocetin, and collagen), and content of soluble fibrin-monomer complexes were evaluated.

Macrorheological studies included measurements of viscosity at shear stress of 5-300 sec^{-1} on an AKR-2 rotation viscosimeter, evaluation of suspension stability, and estimation of Caisson viscosity and threshold fluidity τ_0.

Erythrocyte aggregation was studied on an ADE-5 automated erythroaggregometer by recording the intensity of back-scattering (λ=630 nm) from 1-mm-thick Couette bloodflow, due to which the following parameters of the process were evaluated: time of linear aggregation formation (T_1, sec); time of three-dimensional aggregation formation (T_2, sec); characteristics of final size of aggregations (Ampl); total hydrodynamic strength of aggregations (β, sec^{-1}); and index of largest aggregations strength ($I_a^{2.5}$, %).

Erythrocyte deformation was studied by rigidometry. Methods for tentative evaluation of erythrocyte aggregation and deformability [9] were used.

The degree of changes in blood rheological values was evaluated in percent of control (rheological parameters of control samples served as the checkpoint).

It was found that addition of 0.5 ml oFG solution to the blood virtually did not change its concentration in the plasma and the main physicochemical parameters of the blood sample (primarily pH); nor did it reduce hematocrit and platelet count.

Reaction to addition of 10% oxidized FG manifested by moderate activation of the internal blood clotting pathway with simultaneous suppression of the external pathway (Fig. 2, *a*). The response of blood clotting system to 20% oxidized FG was different, manifesting by suppression of both blood clotting pathways ($p<0.05$). Inhibition of thrombin generation and hence, of the clot growth and condensation were detected in both cases.

Increase in the content of soluble fibrin-monomer complexes was recorded simultaneously in both cases, presumably, depending on the oxidation degree of FG added into the sample. The concentration of soluble fibrin-monomer complexes after addition of 10% oxidized FG increased by one-third in comparison with control, while after addition of 20% oxidized FG it increased 1.5 times ($p<0.05$).

It seems that oFG suppresses the blood clotting cascade factors with, primarily, the phospholipid structure (mainly the external pathway factors), due to which they are easily oxidized.

Prolongation of thrombin time, in turn, indicates that oFG effect is predominantly linked with the final stage of clotting, because addition of oFG led to accumulation of soluble fibrin-monomer complexes in the plasma, decrease in thromboelastogram amplitude ($p<0.05$), and decrease in the clot elasticity and compactness ($p<0.05$). The formation of normal fibrin clot was disordered.

Similar results were demonstrated previously [17]. However, the changes observed in our study indicate that, first, oFG is less accessible for thrombin and, second, these changes are paralleled by inhibition of fibrin-monomer polymerization. We see that the degree of these effects also directly depends on the degree of FG oxidation.

Study of fibrinolytic activity revealed no appreciable changes after addition of oFG.

Contrary to this, oFG exhibited a manifest effect on PA. Platelet aggregation with ristocetin and collagen inductors was suppressed (Fig. 2, *b*). The degree of this effect was somewhat more pronounced in the presence of 10% oxidized FG ($p<0.05$). Activation of PA was observed in response to ADP, its degree increasing in direct proportion to the degree of FG oxidation. It was 35% higher in com-

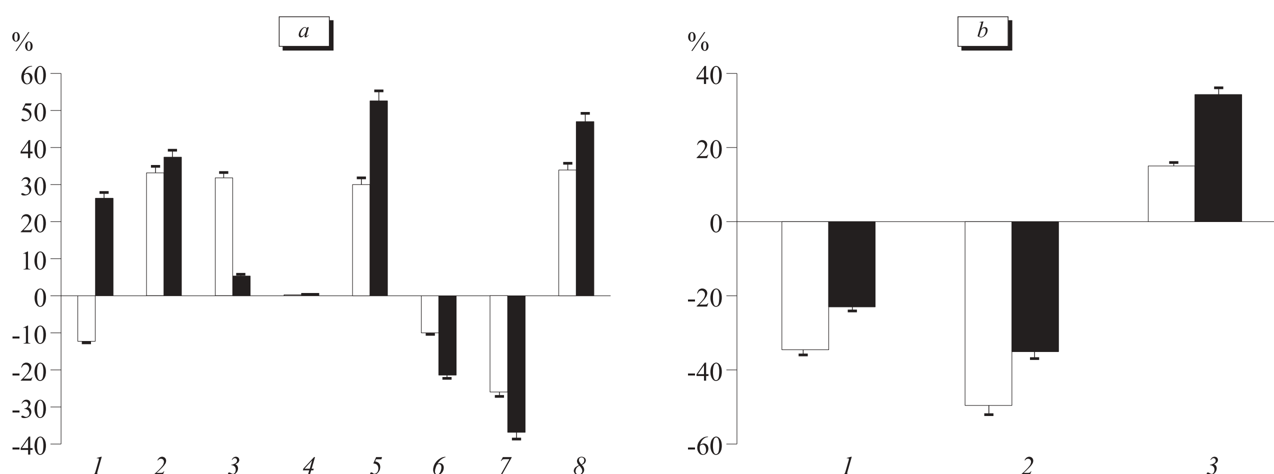

Fig. 2. Changes in blood clotting parameters (*a*) and PA activity (*b*) in the presence of FG oxidized by 10% (light bars) and by 20% (dark bars). *a*: *1*) activated partial thromboplastin time; *2*) thrombin time; *3*) International normalized ratio; *4*) time of reaction on thromboelastogram (TEG); *5*) time from start to end of the clot formation on TEG; *6*) maximum amplitude on TEG; *7*) clot elasticity; *8*) soluble fibrin-monomer complexes. *b*: *1*) ristocetin-induced PA; *2*) collagen-induced PA; *3*) ADP-induced PA.

parison with the control in the presence of 20% oxidized FG and virtually 2-fold higher than in the samples with 10% oxidized FG.

Hence, we can speak about opposite effects of oFG on platelets. On the one hand, oFG suppresses adhesion mediated by collagen receptors and aggregation associated with von Willebrand factor. But the fact of more pronounced effects of less oxidized FG (10%) seems paradoxical and deserves further investigation. On the other hand, a drastic increase of PA in response to ADP suggests a significant intensification of their reactive release (platelet degranulation). This confirms a "dysregulatory" effect of oFG on platelets.

We conclude that oFG effect on the blood clotting system is mainly determined by the degree of its oxidation. It seems that this characteristic is the most important for the development of the above changes, as changes in FG concentrations in the samples were the minimum. Similarity between the detected effects with the effects of oxidized LDL and VLDL on the hemostasis system is obvious.

The effects of 10% oxidized FG are of different nature and seem to be partially compensated for by the pool of natural antioxidants. By contrast, highly oxidized FG "hits" all blood clotting mechanisms. Changes in the hemostasis parameters in the presence of highly oxidized FG are observed in situations with pathologically high activity of disseminated intravascular blood clotting [12].

Study of oFG effect on blood rheology also showed significant changes in the parameters. The effect of 10% oxidized FG manifested by a significant ($p<0.05$) reduction of erythrocyte deformability, presumably resultant from changes in physicochemical structure of the membranes: increased viscosity of the lipid phase, decreased elasticity and mechanical strength of the membrane.

This was paralleled by changes in the erythrocyte aggregation/disaggregation. The time of linear and 3D aggregations was prolonged, the size of cell aggregations decreased significantly ($p<0.05$), their total hydrodynamic stability decreasing, while the largest cell formations acquired greater resistance to shear destruction. Presumably, the pool of natural antioxidants partially leveled the effects of oFG.

The effects of 20% oxidized FG differed from those observed in experiments with 10% oxidized FG and were more pronounced. To begin with, erythrocyte deformability was sharply deteriorated (by 75%; $p<0.05$). The time of linear aggregation formation was also prolonged (by 37%; $p<0.05$), while the time of formation of three-dimensional aggregations was shorter. In parallel, total hydrodynamic stability of cell conglomerations increased, while the largest cell formations were less resistant to the shift destruction ($p<0.05$). The results of this experiment indicate a negative impact of highly oxidized FG forms on blood microrheology in general, manifesting by more rapid formation of small hydrodynamically stable 3D aggregations. These changes *in vivo* often promote disorders in the capillary bloodflow because of obstruction of the capillaries by these cell aggregations.

Addition of 10% oxidized FG just negligibly changed plasma and blood viscosity at all shear stress values; 20% oxidized FG also did not increase plasma viscosity, but led to an increase in the blood viscosity at all shear stress values ($p<0.05$). A decrease in the suspension stability of the blood in response to oFG was observed in both cases.

It is noteworthy that oFG virtually did not change Caisson viscosity, but significantly increased the difference between this parameter and asymptotic viscosity ($p<0.05$).

This result indicates that erythrocyte membranes are the main "targets" for oFG, and in this respect its effect on blood rheology is similar to that of ovidized LDL and VLDL. However, the reduction of suspension stability of the blood was the predominant effect, which was confirmed by the total reduction of threshold fluidity of the blood in both experiments.

The results indicate a notable dysregulatory effect of oFG on blood clotting and rheology [7,8].

Effect of oFG on the production of active oxygen forms by blood leukocytes

The system of reactive oxygen species (ROS) generation by blood phagocytic cells (neutrophilic granulocytes and monocytes) is one of the main systems of prooxidant generation, which fact renders special interest to studies of oFG effect on neutrophils. Due to their high oxidative potential, ROS damage proteins, nucleic acids, cell membranes, initiate LPO in LDL. Oxidative modification can involve other proteins, for example plasma FG, as FG are proteins the most sensitive to oxidative stress. On the other hand, it is important to evaluate the proinflammatory potential of oFG by its effect on the neutrophils. We therefore studied the effects of oFG on ROS production by leukocytes, stimulated by barium sulfate crystals.

The capacity of whole blood and isolated leukocytes to generate ROS was detected by luminol-dependent chemiluminescence (LDCL), stimulated by barium sulfate crystals and opsonized zymosan. Before LDCL measurements whole blood or leukocyte suspension in Hanks solution (0.4 ml) was incubated with 0.1 ml FG solution during up to 90 min at 37°C. The final concentration of FG was 0.2 mg/ml. The amplitude of LDCL flash was analyzed. Fibrinogen was modified by UV exposure.

The effects of oxidized and native FG on LDCL of leukocyte suspension stimulated by barium sulfate crystals were studied after incubation of different duration. Prolongation of incubation resulted in lesser intensity of LDCL. The intensity of LDCL in the presence of native FG increased by about 80% at the beginning of incubation and by 110% in the presence of oFG in comparison with the control (without FG). The greatest differences in LDCL intensity were observed after 30-min incubation: it increased by 30% in the presence of native FG and by 100% *vs.* control in the presence of oFG.

These data indicate that oFG enhances ROS generation by leukocytes stimulated by barium sulfate crystals. The intensity of leukocyte LDCL increased with higher degree of FG oxidation.

Similar data were obtained with leukocyte activation by opsonized zymosan: both oxidized and native FG stimulated the intensity of leukocyte LDCL, the intensity of LDCL being higher with oFG than with native FG. The intensity of LDCL in the studied system decreased with prolongation of incubation.

The next step of the study was evaluation of the relationship between the intensity of cell LDCL and FG concentration. The intensity of LDCL increased with increase of FG content in the reaction medium, reaching the maximum at FG concentration of 600 μg/ml and decreasing with further increase of FG concentration.

The next step was study of the effects of FG of different degree of oxidation on the intensity of leukocyte LDCL. The intensity of leukocyte LDCL increased with higher oxidation of FG, reaching the maximum in the presence of FG with 30% of oxidized tryptophan residues. Further increase of oxidation degree led to reduction of the activation effect.

Hence, oFG enhances the production of ROS by stimulated leukocytes [6]. As we know, the production of ROS by leukocytes is increased in cardiovascular patients. Our data suggest that leukocyte activation by oFG is one of the causes of this phenomenon.

Effect of oFG on endothelial cells

One of the pathogenetic factors of atherosclerosis is the development of chronic inflammation in the blood vessel wall, which attracts special interest to this problem. The mechanism of inflammation is associated with activation of blood leukocyte adhesion to the vascular endothelium by means of connection of the complementary cell adhesion molecules (CAM) on the membrane of these cell populations. Reactions between selectins (P and E) on the surface of vascular EC and carbohydrate ligands on leukocyte surface determine the primary contact of cells [16]. More close contact of reacting cells leading later to leukocyte migration through the endothelial layer is realized by the complementary CAM-β_2 on the leukocytes and ICAM-1 on EC [23]. Dynamic changes in CAM expression on EC determines recruiting of neutrophils and then other blood leukocyte populations. Activation of CAM expression is mediated by cytokines and chemokines produced by activated leukocytes and EC [21].

Two main tasks in this study were as follows: 1) to study the capacity of oFG to induce the production of IL-8 (a chemokine recruiting neutrophils and platelets) in human blood vessel EC and 2) to study the expression of P-selectin and ICAM-1 on the surface of human blood vessel EC under the effect of oFG.

The first step was to detect the relationship between the level of IL-8 production in EC culture and FG concentration. The maximum production of the cytokine after 6 h of incubation from the moment of induction was observed at FG concentration of 1.4 mg/ml, after 24 h of incubation at FG concentration of 3 mg/ml.

Then we studied oFG capacity to induce the production of IL-8 in EC. Fibrinogen was oxidized by UV exposure during different periods. The final concentrations of irradiated preparation, corresponding to its concentrations causing the maximum production of IL-8, were used: 1.4 and 3 mg/ml.

The dynamics of the cytokine production was studied after induction by the native FG and after its irradiation. The production of IL-8 24 h after EC induction with FG irradiated under conditions when about 10% aromatic amino acid residues undergo oxidative modification (5-min irradiation) was 10-30% higher than after induction by the native FG. The production of IL-8 was more active during the earlier periods (6 h after the beginning of induction).

The next step was study of FCS effect on the production of IL-8 by EC. Fibrinogen was exposed to UV light for 5-17 min and added into EC culture medium with and without FCS; the samples were collected after 6-h induction. Under these conditions 10% amino acid residues underwent oxidative modification after 5-min UV exposure, 22% after 10-min, and 40% after 17-min UV irradiation. Increase in the degree of FG oxidation led to an increase in IL-8 production: the production of IL-8 increased by 200% in the presence of 40% oxidized FG. More active production of IL-8 was observed in culture medium without FCS. In addition, a longer exposure of FG activated its capacity to induce IL-8 production. In our experiments the final concentration of FG was 3 mg/ml. In lower concentrations oFG less actively stimulated IL-8 production.

Fibrinogen is an independent risk factor for atherosclerosis and its complications. The mechanisms of processes with FG participation, causing the development of atherosclerosis, are little studied. It is known that one of the initial stages of atherosclerosis development is monocyte adhesion to vascular endothelial cells, which eventually leads to thickening of the intima [11].

We showed for the first time that FG induced the production of IL-8 (chemoattractant recruiting monocytes) by EC [1]. These data suggest that FG contributes to the development of atherosclerosis also at the expense of IL-8 synthesis induction in EC. In addition, it was shown that IL-8 production by EC increased 1.5-2.0 times in the presence of oFG. These data demonstrate possible contribution of oFG into the development of atherosclerosis.

As was shown above, monocytes adhere to the endothelium due to interactions between complementary CAM: ICAM-1 and MCP-1, and IL-8 chemokine. The next step of our study was evaluation of CAM (P-selectin and ICAM-1) expression on the surface of human blood vessel EC under the effect of oFG.

Experiments were carried out according to the same protocol. Specimens of the native FG and oFG in PBS were 2-fold diluted with EC growth medium without serum (final concentration 3 mg/ml) and applied onto a monolayer of 4-day EC culture (2 ml/well of a 6-well plate); each experiment was repeated twice. After 4, 5, and 6 h of incubation in a thermostat at 37°C the expression of CAM on EC surface was measured by analyzing specimens of cell suspensions in a FACS-Calibur flow cytometer (Becton Dickinson) with argon laser (λ=488 nm).

Native FG activated the expression of P-selectin on the surface of EC after their co-incubation for 4 and 6 h (Table 1), the percentage of activated cells being higher in comparison with the percentage of control cells expressing this CAM (the difference was significant after 6-h incubation). The percentage of EC expressing P-selectin after 4- and 6-h incubation with oFG was higher than after EC contact with native FG under the same conditions; the difference was threshold significant after 4-h exposure (p=0.05) and significant after 6-h exposure (p<0.004).

Native FG activated ICAM-1 expression on the surface of many EC in comparison with the expression of this CAM by control cells (Table 2), the difference between the percentage of activated and control cells being statistically significant for 5- and 6-h exposure (p<0.0004). The expression of ICAM-1 by EC increased significantly due to oFG in comparison with EC incubated with native FG, the difference being statistically significant for 5-h exposure (p<0.001) and was threshold significant for 6-h exposure (p=0.05).

As we noted above, oxidized LDL play the key role in the development of atherosclerosis, participate in all stages of this process, causing endothelial dysfunction, formation of foamy cells, mig-

ration of smooth-muscle cells and their transformation into foamy cells. One of the primary stages of atherosclerosis is the development of inflammatory process in the vascular wall under the effect of oxidized LDL, which is associated with expression of CAM by EC, with which blood leukocytes and monocytes react.

We showed that oFG induces the expression of CAM (P-selectin and ICAM-1) on the surface of cells in primary culture of human vascular EC [10]. The expression under the effect of oFG is higher than under the effect of native FG. These data indicate that oFG, similarly as oxidized LDL, participates in the initiation of the first stages of atherogenesis — endothelial dysfunctions manifesting by increased expression of CAM on the surface of EC.

Studies of the recent decade showed that the development of some cardiovascular diseases (strokes and infarctions) is associated with the formation of platelet-neutrophil complexes [14]. These complexes were detected also during cardiosurgical operations [13]. The formation of these complexes has a negative impact for microcirculation, disorders in which are of priority importance in many diseases of the heart and peripheral vessels. It was shown that platelet-neutrophil complexes decreased vascular permeability, increased blood viscosity, and promoted tissue hypoxia.

Receptors for FG are present not only on platelets, but also on the neutrophils (MAC-1) [13]. Hence, study of FG and oFG role in the formation of platelet-neutrophil complexes is an important problem.

TABLE 1. Expression of P-Selectin by Human Vascular EC (% of Cells) after Activation by FG

Group	Time of incubation, h	
	4	6
Control	34.83±4.93	16.20±1.20
FG	47.48±1.20	22.80±0.81*
oFG	50.67±0.75^{+}	48.37±2.30^{++}

Note. *p<0.01 compared to the control; ^{+}p=0.05, ^{++}p<0.01 compared to FG.

TABLE 2. Expression of ICAM-1 by Human Vascular EC (% of Cells) after Activation by FG

Group	Time of incubation, h	
	4	6
Control	18.63±1.67	18.0±0.2
FG	45.50±1.07*	85.10±0.98*
oFG	55.30±0.97^{+}	90.25±0.25^{++}

Note. *p<0.001 compared to the control; ^{+}p=0.05, ^{++}p<0.001 compared to FG.

Effect of oFG on the formation of platelet-neutrophil complexes

Suspension of isolated platelets was mixed with isolated neutrophils (1:30 neutrophil:platelet ratio). Changes in the mean radius of aggregations and light transmission were registered on a device manufactured by the Biola Company. The device was calibrated as follows: light transmission of the platelet and neutrophil suspension was taken for 0%, light transmission of water for 100%. Thrombin (RENAM) with the initial activity of 9 U/ml was added during sec 30 after the beginning of recording the curves. The experiment was carried out in cylindrical quartz cuvettes during constant mixing (800 rpm) at 37°C.

The first step was to evaluate the possibility of using PA and neutrophil aggregation parameters for the study of complex formation between these cells. Changes in the radiuses and light transmission in PA in the presence of neutrophils were studied without thrombin. Evaluation of the time course of the mean radius of aggregations in test samples without thrombin showed that the maximum mean radius of platelet aggregations with neutrophils was 1.58±0.30 arb. units, which was 1.5 times more than the mean size of aggregations from neutrophils alone and 6-fold more than the mean size of aggregations from platelets alone. Study of the light transmission parameter under the same conditions showed that it was greater in the neutrophil+platelet system than in aggregations of platelets or neutrophils alone.

The formation of platelet+neutrophil complexes was studied under static and dynamic conditions (during cell mixing) [13]. The formation of complexes under dynamic conditions was registered by cell fixation at a certain moment. However, the available methods do not show the entire kinetics of complex formation. In addition, the studies are usually carried out on fixed (non-viable cells). We studied the formation of platelet complexes with neutrophils on living cells, which better corresponded to *in vivo* conditions. Moreover, the method proposed in our study showed a dynamic picture of complex formation.

The next step was to select thrombin concentration optimal for studies of PA kinetics; it was selected in the range of concentrations from 0.0075 to 0.3000 U/ml. Thrombin concentration of 0.015 U/ml was chosen, at which moderate aggregation of platelets and leukocytes was recorded.

Thrombin is a potent platelet agonist, causing the expression of P-selectin (responsible for the main adhesive mechanism between platelets and polymorphonuclear neutrophils) on the surface of platelets [14]. It was expected that platelet complexes with neutrophils would form in the presence of thrombin, which, in turn, would result in PA intensification and formation of mixed leukocyte-platelet aggregations. Study of the kinetics of complex formation between thrombin-activated platelets and intact neutrophils by evaluating the fluctuations in light transmission and by the turbidimetrical method helped to evaluate online the mean size of aggregations. It was found that activation by throm-

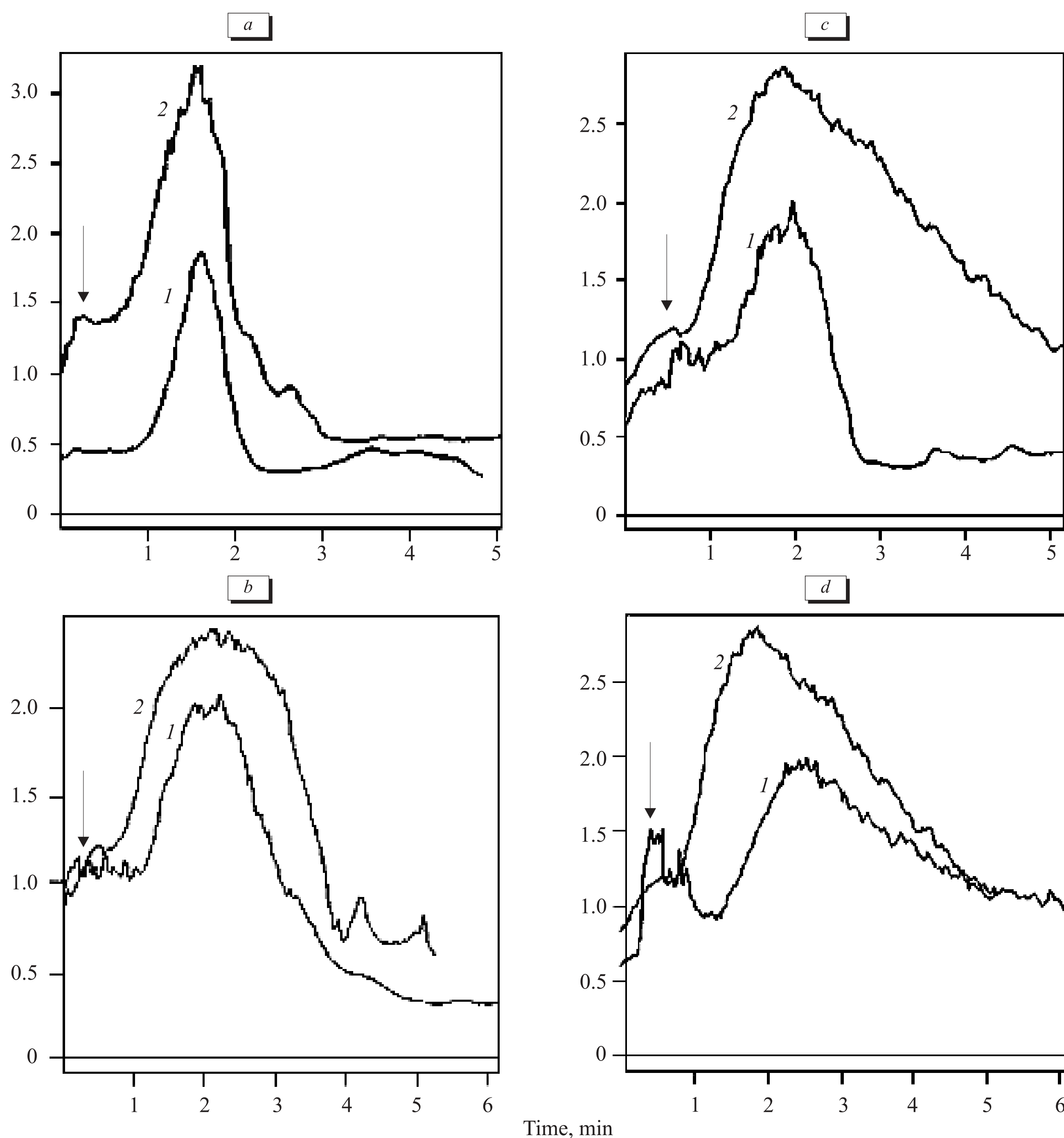

Fig. 3. Kinetic curves of thrombin-induced aggregation (0.2 U/ml) of isolated platelets (*1*) and isolated platelets with neutrophils (*2*) in the presence of FG (final concentration 1 mg/ml), oxidized by different methods. *a*) native FG; *b*) autooxidized FG (prepared by exposure of FG solution at ambient temperature for 24 h); *c*) FG oxidized by 10^{-4} M Fe^{2+}; *d*) FG oxidized by 10^{-4} M Fe^{2+} and 10^{-4} M H_2O_2. *c*, *d*) FG oxidized during 1 h at 37°C with subsequent dialysis of FG solution against PBS for 24 h. Ordinates: mean radius of aggregations, arb. units. Arrow shows thrombin addition.

bin in a concentration of 0.015 U/ml the mean radius of aggregations and light transmission in the platelet-neutrophil system increased 2.5 times in comparison with aggregation of platelets alone.

Further evaluation of light transmission fluctuations, turbidimetry, and fluorescent confocal microscopy showed intensification of platelets—polymorphonuclear leukocytes complex formation in the presence of FG and even more so oFG (Fig. 3). Fibrinogen for these experiments was oxidized by 10^{-4} M Fe^{2+}, 10^{-4} M Fe^{2+} and 10^{-4} M H_2O_2 or autooxidized. The effect was observed after thrombin activation of leukocytes and platelets under conditions of constant mixing. The effect was also observed under conditions of constant mixing during interactions of physiologically active leukocytes with platelets, activated by thrombin and fixed by paraformaldehyde. Platelet fixation results in the formation of leukocyte complexes with solitary platelets. On the other hand, interactions between nonfixed platelets with leukocytes result in the formation of complexes of platelet aggregations with leukocytes. These oFG effects can have great consequences for microcirculation under conditions of elevated FG concentrations in the blood and FG oxidation.

Our next task was to evaluate the neutrophil capacity to produce ROS during the formation of platelet—neutrophil complexes in the presence of oFG. To this end, we studied the effect of oFG on interactions between platelets and polymorphonuclear leukocytes during phagocytosis of opsonized zymosan by the latter cells. The LDCL intensity of polymorphonuclear leukocytes, stimulated by opsonized zymosan, increased 2-3-fold in the presence of platelets. After incubation with oFG the intensity of polymorphonuclear leukocyte LDCL increased 1.5-2 times in comparison with native FG. It was found for the first time that thrombin caused generation of polymorphonuclear leukocyte LDCL flash in the presence of platelets. These results indicate an important role of interactions between polymorphonuclear leukocytes and platelets in activation of oxidative stress and effect of oFG on this process.

Hence, our results indicate that FG and particularly oFG can make an important contribution to the development of atherosclerosis. We showed that oFG caused PA [3] and increased ADP-induced PA [2] and ROS production in zymosan-stimulated leukocytes [6]. For the first time we showed that FG induced the production of IL-8 chemokine in primary culture of human umbilical vein EC; this chemokine is essential for the development of inflammation in human blood vessel walls; oFG was more active in inducing its synthesis [1]. Oxidized FG much more actively than native FG induced the expression of CAM (P-selectin and ICAM-1) in human umbilical vein EC [18]. These data indicate more active involvement of oFG (in comparison with native FG) in the process of inflammation in the vascular wall, the first stage of atherogenesis.

In addition, we found that 10% and 20% oxidized FG disordered blood microrheology, reduced erythrocyte deformability, modified blood viscosity, and reduced suspension stability of the blood [8]. Treatment with oFG led to changes in the following blood clotting parameters: activated partial thromboplastin time, prothrombin time, thrombin time, FG concentration, concentrations of soluble fibrin-monomer complexes; thromboelastogram parameters, platelet count and induced aggregation with ADP, ristocetin, and collagen. Fibrinogen oxidized by 10% moderately activated the internal blood clotting pathway and simultaneously suppressed the external pathway. Fibrinogen oxidized by 20% suppressed both external and internal blood clotting pathways. Oxidized FG disordered the formation of fibrin clot. Study of PA showed suppressed adhesion reaction, mediated by collagen receptors, and aggregation associated with von Willebrand factor. Activation of PA in response to ADP inductor was observed; the degree of this activation increased in direct proportion to the degree of FG oxidation. Hence, in general we can speak about dysregulatory effect of oFG on platelets.

Our studies showed that the formation of platelet complexes with polymorphonuclear leukocytes increased in the presence of oFG and that the intensity of polymorphonuclear leukocyte LDCL in the presence of platelets increased by 1.5-2 times after incubation with oFG in comparison with incubation with native FG.

Summing up the data presented in this review, we conclude that oFG plays an important role in the development of atherosclerosis and its complications (thromboses).

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