Oxidized Forms of Fibrinogen Induce Expression of Cell Adhesion Molecules by Cultured Endothelial Cells from Human Blood Vessels

O. N. Shcheglovitova, O. A. Azizova*, Yu. A. Romanov, A. V. Aseichev*, M. M. Litvina, E. R. Polosukhina, and E. V. Mironchenkova

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Oxidized forms of fibrinogen similarly to initial non-oxidized fibrinogen induced expression of P-selectin and ICAM-1 cell adhesion molecules in the cultured endothelial cells derived from human umbilical vein. The effect of oxidized fibrinogen on the expression of adhesion molecules was more pronounced. These data attest to more active participation of oxidized forms of fibrinogen into inflammation in the vascular wall, the first stage of atherogenesis.

Key Words: *oxidized forms of fibrinogen; cell adhesion molecules; P-selectin; ICAM-1; vascular endothelial culture*

Oxidatively modified LDL play a key role in the pathogenesis of atherosclerosis. These oxidized LDL activate blood cells and trigger the processes leading to disturbances in the vascular wall cells. Blood protein fibrinogen participates in both blood clotting reactions and pathogenesis of atherosclerosis. Increased fibrinogen content is an independent risk factor for the development of atherosclerosis with its complications [5-7,14]. Moreover, fibrinogen is characterized by higher vulnerability to free radicals compared to other blood proteins [11].

The above peculiarities can explain active participation of oxidatively-modified fibrinogen in atherogenesis *in vivo*.

We showed that oxidative-modified fibrinogen triggers platelet aggregation, and potentiates ADP-induced platelet aggregation and production of ROS in zymosan-stimulated leukocytes [2]. In primary culture of endothelial cells (EC) derived from human umbilical vein, fibrinogen induces the production of chemokine IL-8 involved in the development of inflammation in human vascular wall. It is important that the oxidized fibrinogen is more active in induction of this chemokine [1].

Chronic inflammation in blood vessels is a factor of atherogenesis. The mechanism of this inflammation relates to enhanced adhesion of blood leukocytes to the vascular endothelium via binding of complementary cell adhesion molecules (CAM) to the membranes of these cells. Interaction of P- and E-selectins on the surface of vascular endothelial cells with hydrocarbon ligands on the surface of leukocytes is the first stage of the contact between these cells [8]. More close contact between the interacting cells leading to leukocyte migration through the endothelium is determined by complementary CAM β_2 on leukocytes and ICAM-1 on EC [12]. The dynamic changes on CAM expression on EC underlie recruitment of neutrophils and then other populations of leukocytes from the blood. Activation of CAM expression is mediated by cyto-

N. F. Gamaleya Institute of Epidemiology and Microbiology, Russian Academy of Medical Sciences; *Research Institute of Physicochemical Medicine, Russian Ministry of Health, Moscow

kines and chemokines produced by activated leukocytes and activated EC [9].

In this paper, we examined expression of CAM (P-selectin and ICAM-1) on the surface of EC of human blood vessels under the action of oxidative-modified fibrinogen.

MATERIALS AND METHODS

EC were isolated from human umbilical vein [10]. The cells were grown in flasks in medium 199 supplemented with 20 mM HEPES, 10% FCS, 2 mM glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (all the chemicals from Gibco), and 50 μg/ml endothelial growth factor derived from human neural tissue. The culture medium was refreshed every other day. The monolayer of primary EC culture was trypsinized (0.05% trypsin with 0.02% EDTA, Gibco), passed onto the 6-well plates, and used on day 4.

Oxidized forms of fibrinogen were obtained by UV-irradiation of fibrinogen in FEK-56PM apparatus and by the process of self-oxidation. Fibrinogen (12 mg, Sigma) was dissolved in 2 ml phosphate buffer to a final concentration of 6 mg/ml, and irradiated for 17 min in a well of a 24-well plate. The solution was stirred with a magnetic stirrer at a rate producing no foam. The degree of oxidative modification was assessed by a decrease in fluorescence of aromatic amino acids [2]. Self-oxidized fibrinogen was obtained by exposure of fibrinogen solution (12 mg in 2 ml phosphate buffer) at 37°C for 1 h and then at 4°C for 18 h.

The monolayer EC cultures after the contact with fibrinogen were washed with phosphate buffer at 37°C and dispersed with a mixture of trypsin and EDTA. The cell suspension was analyzed in a FACS-Calibur flow cytometer (Becton Dickinson) with argon laser at λ=488 nm; the following 4 parameters were determined: forward (small) angle of light scattering (FSC), lateral scattering (SSC), green fluorescence (FITC at λ=530 nm), and orange fluorescence (phycoerythrin [FE] at λ=585 nm). Gating of examined cell population was performed in FSC and SSC coordinates. Then the examined cell population was analyzed for fluorescence in various coordinates (histogram). High-resolution (1024 channels) computer-assisted procedures of data sampling and analysis were used.

The data were analyzed statistically using STATN software.

TABLE 1. Expression of P-Selectin by Human Cultured Vascular Endothelial Cells Activated with Fibrinogen (%, $M\pm m$)

Index	Time, h	
	4	6
Cell control	34.83±4.93	16.2±1.2
Fibrinogen control	47.48±1.20 (p=0.06)*	22.80±0.81 (p<0.01)*
Oxidized fibrinogen	50.67±0.75 (p=0.05)+	48.37±2.30 (p<0.004)+

Note. Here and in Table 2: significance was calculated by Student's t test in comparison with *cell control and +fibrinogen control.

TABLE 2. Expression of ICAM-1 by Human Cultured Vascular Endothelial Cells Activated with Fibrinogen (%, $M\pm m$)

Index	Time, h	
	4	6
Cell control	18.63±1.67	18.0±0.2
Fibrinogen control	45.50±1.07 (p<0.0004)*	85.10±0.98 (p<0.0004)*
Oxidized fibrinogen	55.3±0.97 (p<0.001)+	90.25±0.25 (p=0.05)+

RESULTS

The experiments were carried out according to the following scheme: initial and oxidized fibrinogen samples in phosphate buffer were diluted 2-fold with serum-free growth medium (final concentration 3 mg/ml) and added to 4-day monolayer EC culture (2 ml per well of a 6-well plate; 2 replicates). Expression of CAM on EC surface was analyzed after 4-, 5-, and 6-h incubation at 37°C.

The tests of P-selectin expressed on EC surface and statistical data characterizing individual parameters are shown (Fig. 1, Table 1). After 4- and 6-h exposure with EC, initial fibrinogen activated P-selectin expression on the cell surface. In this case, the percent of activated cells was higher than the percent of the control cells expressing the same CAM, the difference were significant after 6-h exposure. The number of EC expressing P-selectin after 4- and 6-h incubation with oxidized fibrinogen surpassed the number of EC exposed to initial fibrinogen during the same time. In this case, the difference was near-significant after 4-h exposure (p=0.05) and significant after 6-h exposure (p<0.004).

The statistical data on ICAM-1 testing (another CAM expressed on EC surface) are presented in Fig. 2 and Table 2. Initial fibrinogen activated expression of ICAM-1 on the surface of a larger number of EC in comparison with the expression of this CAM on control cells. The difference between the

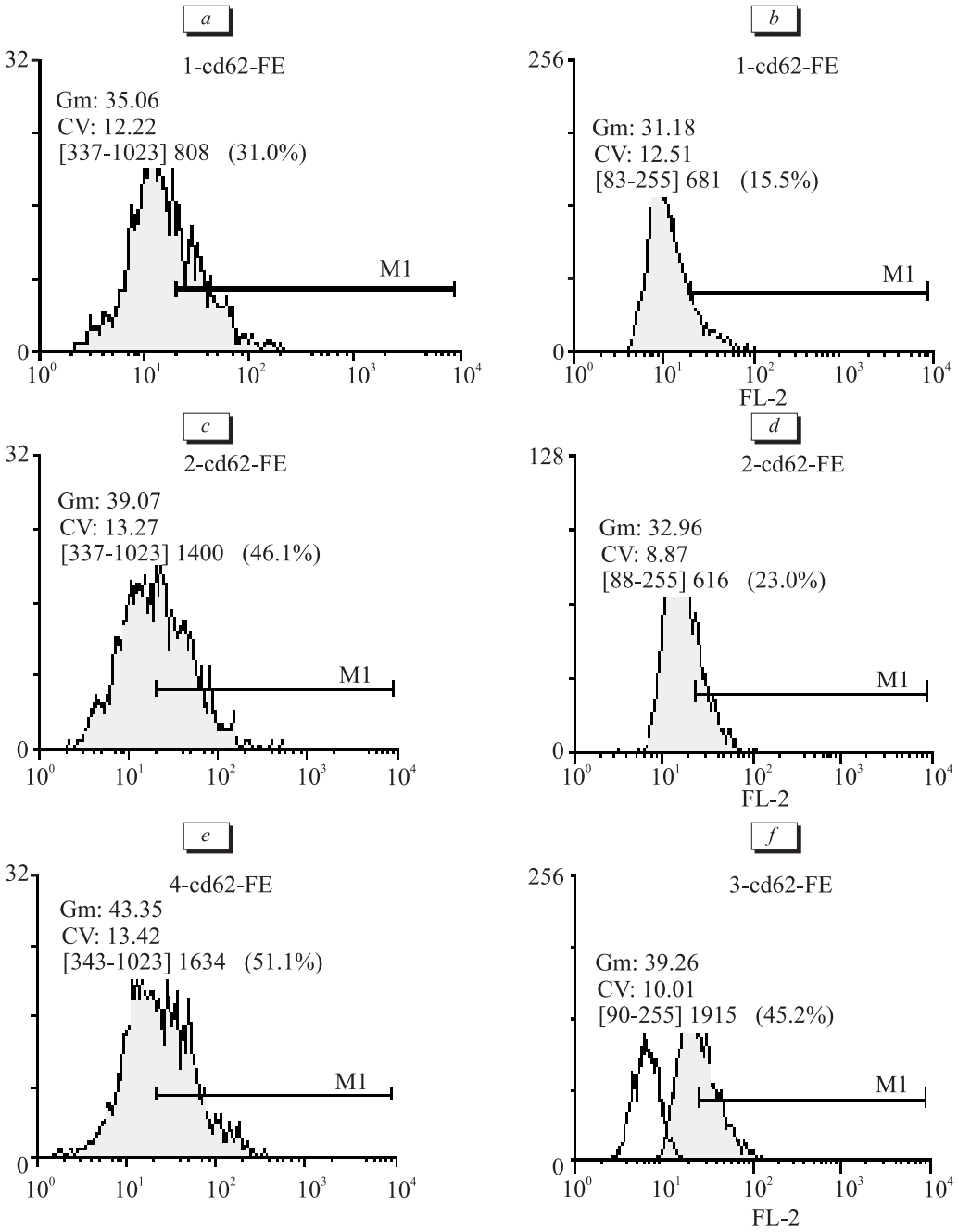

Fig. 1. Expression of P-Selectin by cultured EC activated with fibrinogen during 4 h (*a*, *c*, *e*) and 6 h (*b*, *d*, *f*). Here and in Fig. 2: *a*, *b*) cell control; *c*, *d*) fibrinogen control; *e*, *f*) oxidized fibrinogen.

percents of fibrinogen-activated and control cells was significant after 5- and 6-h exposure ($p<0.0004$). The percent of cells expressing ICAM-1 was higher after incubation with oxidized fibrinogen. In this case, the difference was significant after 5-h exposure ($p<0.001$), and was near-significant after 6-h exposure ($p=0.05$).

The data suggest that similarly to oxidatively modified LDL, oxidized fibrinogen is involved in triggering the initial stages of atherogenesis leading to endothelial dysfunction manifesting in up-regulation of CAM expression on EC surface.

Leukocytes adhered to the endothelium can interact with blood leukocytes via E-selectins thus promoting recruitment of leukocytes to the inflammation focus [13]. In hemorrhage regions, leukocytes interact with platelets also expressing P-selectin [3]. The monocytes recruited by this mechanism can enhance the production of fibrin, probably, by releasing some tissue factor [4].

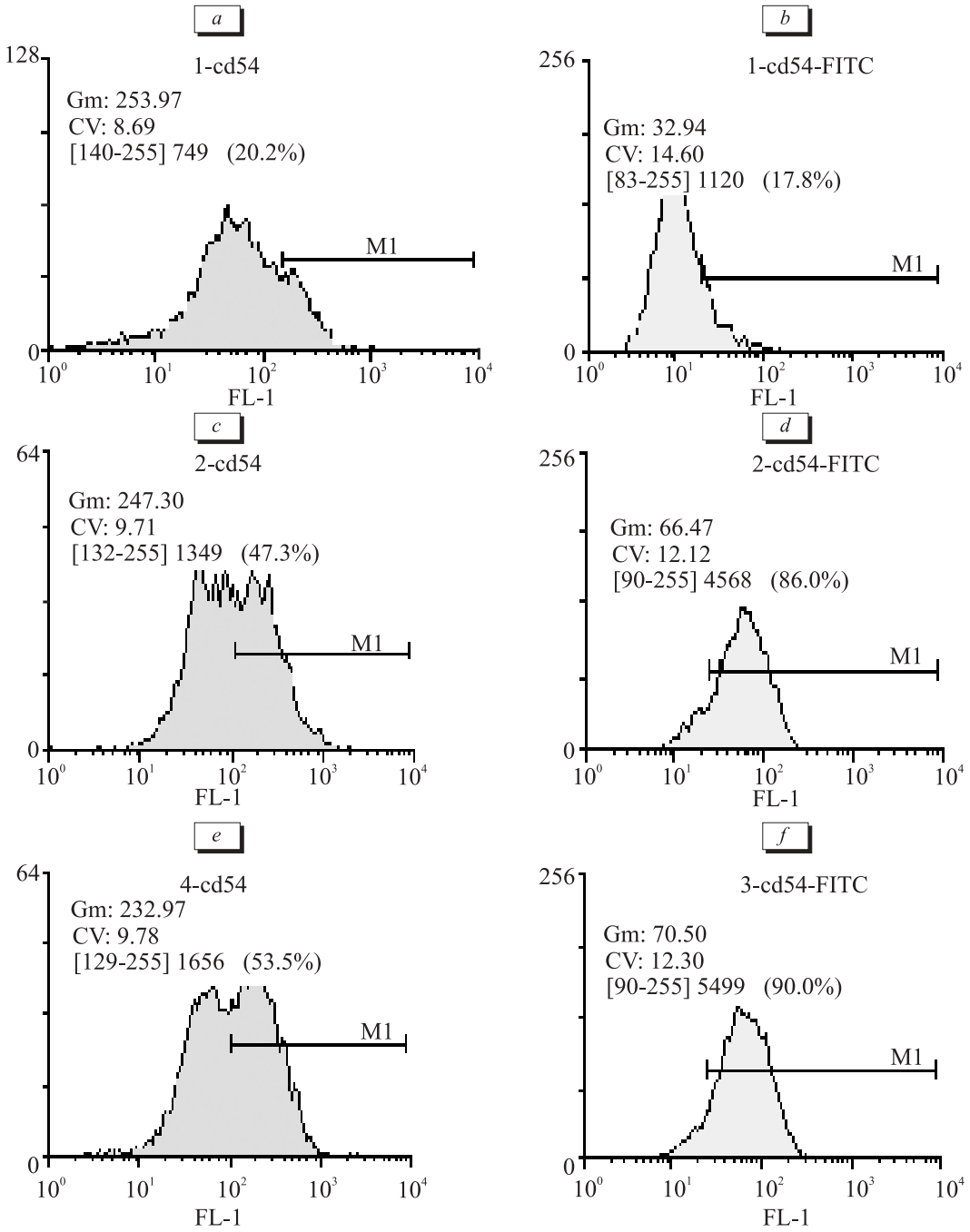

Fig. 2. Expression of ICAM-1 by cultured EC activated with fibrinogen during 5 h (*a*, *c*, *e*) and 6 h (*b*, *d*, *f*).

Thus, our experiments showed that oxidatively modified fibrinogen can participate in the development of atherosclerosis.

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