Effect of Radiation on Prothrombin Interactions with Cell Membrane Fragments

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 134, No. 11, pp. 515-518, November, 2002
Original article submitted June 13, 2002

Binding of ^{125}I-labeled prothrombin to normal and radiation-damaged (1 and 3.5 Gy) fragments of cell membranes was studied. Irradiation significantly increased adsorption capacity of cell membranes for ^{125}I-prothrombin. It was concluded that phase rearrangement of phospholipids serves as the molecular basis for increased thrombogenicity of cell membranes.

Key Words: *prothrombin; cell membrane fragments; radiation exposure*

Postirradiation disturbances in the blood coagulation system result from excessive blood clotting during the first hours after exposure, which later leads to procoagulant deficiency due to their enhanced consumption in the course of disseminated intravascular coagulation. Molecular mechanisms triggering this process are poorly understood. Basing on our previous studies [4,6] we proposed a functional concept of blood clotting initiation by cell membranes (CM) [2]. However, physical changes in native bilamellar CM structure responsible for its thrombogenicity remain unclear. Here we evaluated these mechanisms by studying specificity of binding of vitamin K-dependent clotting factors (*e.g.* prothrombin) to damaged CM.

MATERIALS AND METHODS

Prothrombin was isolated from porcine citrate plasma as described previously [4] and labeled with [^{125}I]Na without carrier (^{125}I-chloramine method [9]).

Cell membrane fragments were isolated from the inner layer of the thoracic aorta wall obtained from irradiated and intact pigs. Nine pigs aged 4-5 months (25-30 kg) were used: 3 served as the controls and others were exposed to single γ-irradiation in doses of 1 and 3.5 Gy (3 animals per group) on a Puma γ-device (^{137}Cs).

Material for analysis was collected on day 3 after irradiation. The aortas were thoroughly washed from blood, dried with filter paper, and the inner layer of the vascular wall was separated. The preparation was weighed and suspended (4 mg/ml) in 0.05 M Tris-HCl buffer (pH 7.4) containing 0.1 M NaCl and 0.005 M $CaCl_2$ in a Potter—Elveheim homogenizer at 1-2°C. CM were precipitated by 15-min centrifugation at 3000*g*. Radiolabeled prothrombin binding was carried out in an incubation mixture containing 3.6 mg/ml CM fragments (this concentration provided optimal prothrombin activation in the Quick test) and 1% serum albumin in 0.05 M Tris-HCl buffer with 0.1 M NaCl and 0.005 M $CaCl_2$ (pH 7.4). Prothrombin was used in concentrations of 1.35×10^{-9}-7×10^{-6} M. A total of 35 concentrations of prothrombin were used (in duplicates). Labeled prothrombin in the same buffer as CM (0.1 ml) was added to CM suspension (0.9 ml). The mixtures were incubated for 1 h at 20°C and centrifuged for 30 min at 8000*g*. Radioactivity of the supernatant and precipitate was measured in a Minigamma scintillation counter (LKB Wallac). The contents of free and bound protein were determined using Scatchard analysis.

Linear dependence was determined by the method of least squares for each of the two detected binding types separately. The calculations were performed using special software [5] and were verified by the method of differences [1].

RESULTS

Prothrombin binding within the test concentration range was described by concave Scatchard curves (Fig. 1, *a*,

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0007-4888/02/1345-0442$27.00

b), which attested to the presence of two types of binding centers: high affinity (HA; $K_{d1}=6.3\times10^{-8}$ M) and low affinity (LA; $K_{d2}=1.6\times10^{-6}$ M) binding centers. The number of LA centers (Q_2) 15-fold surpassed the number of HA centers (Q_1); the total percentage of prothrombin binding reflects the total result of changes in K_d and Q (Table 1). Since $K_{d1}<<K_{d2}$ and $Q_2>>Q_1$ (Table 1), the percentage of total binding characterizes binding in two types of centers separately. The relative contribution of HA centers to total prothrombin binding is always higher than for LA centers. Irradiation 7-10-fold increased affinity for both HA and LA binding centers for prothrombin. At the same time, the number of HA centers increased 2-3-fold, while the number of LA centers decreased 2-fold. This led to a 4-fold increase in total prothrombin binding to CM. Irradiation modified binding curves at low prothrombin concentrations (<1% of its plasma content). Scatchard curves formed a cupola (Fig. 1, *c*). This attested to positive cooperative effects during prothrombin binding to HA centers.

In previous studies prothrombin binding was investigated primarily on model phospholipid vesicles carrying only one type of binding centers with K_d of 2×10^{-7}-2×10^{-6} M depending on the content of phosphatidylserine (PS) [7,11]. HA binding centers for vitamin K-dependent clotting factors were detected on stimulated platelets, leukocytes, vascular endotheliocytes, and resident macrophages. These centers are clotting cofactor proteins behaving as receptors synthesized by cells or bound from outside. The membrane of intact and unstimulated cells is athrombogenic and bind minimum K vitamin-dependent clotting factors. This interaction is triggered by the appearance of PS on the membrane [8] in response to some physical, chemical, and bacterial factors on vascular cells (endotheliocytes, platelets, and monocytes) and nonvascular cells (fibroblasts, pericytes, and muscle cells).

Actual coincidence of K_d for binding centers on phospholipid vesicles and LA centers of CM fragments suggests that these centers are fragments of the phospholipid surface including molecules of both acid and neutral phospholipids. HA center seems to represent a local interphase loosening of the membrane resulting from its damage and characterized by the appearance of phospholipid structures organized in hexagonal lyotropic mesophases [3]. Prothrombin binding is effected first via distant electrostatic interactions between Ca^{2+} and PS molecules and then via hydrophobic interactions [14] ensuring more strong binding of the protein molecule to interphase loosening of the membrane. This hypothesis is confirmed by changes in adsorption capacity of CM exposed to ionizing radiation. Increased affinity of binding centers for prothrombin after irradiation can be due to more pronounced destruction of CM as a result of lipid peroxidation [12]. The appearance of hydroperoxides of polyunsaturated acyl chains of phospholipids disturbs their packing in the bilayer and leads to the formation of numerous defects in the membrane structure. Degradation of hydroperoxides leads to the formation of lysophospholipids. Similarly to phosphatidylethanolamine, they are cone-shaped molecules destabilizing lipid bilayer. Lysophospholipids accumulate in the plasma membrane and form inverted mesophases still more transforming its structure. Moreover, free radicals and MDA can oxidize free SH-groups of aminophospholipid translocase essential for its activity. This impairs maintenance of membrane phospholipid asymmetry and leads to the appearance of PS in the outer leaflet and formation of a heterophasic phospholipid structure. Thus, these destructive changes increase not only prothrombin affinity for binding centers, but also the number of HA binding sites. Similar results were obtained in the study of annexin V binding to irradiated human cells and mouse endothelial cells [10,13]. The number of LA binding sites decreased, presumably

TABLE 1. Parameters of Prothrombin Binding with CM Fragments (*M*±*m*)

Parameter	Control	Irradiation dose, Gy	
		3.5	1
K_{d1}, nM	63.0±28.4	9.32±2.95	6.32±1.36^{+}
K_{d2}, nM	1640±290*	178±24*	170±29*$^{+}$
Q_1, nM	17.5±7.9	40.1±13.8	33.3±7.7^{+}
Q_2, nM	278±50*	122±20*	120±24*$^{+}$
Total percent of binding with			
HA centers	15.7±3.2	57.5±20.4	62.2±20.3
LA centers	10.2±4.1	14.2±4.1*	13.8±4.6

Note. $p<0.05$: *compared to HA centers, $^{+}$compared to irradiation in a dose of 3.5 Gy.

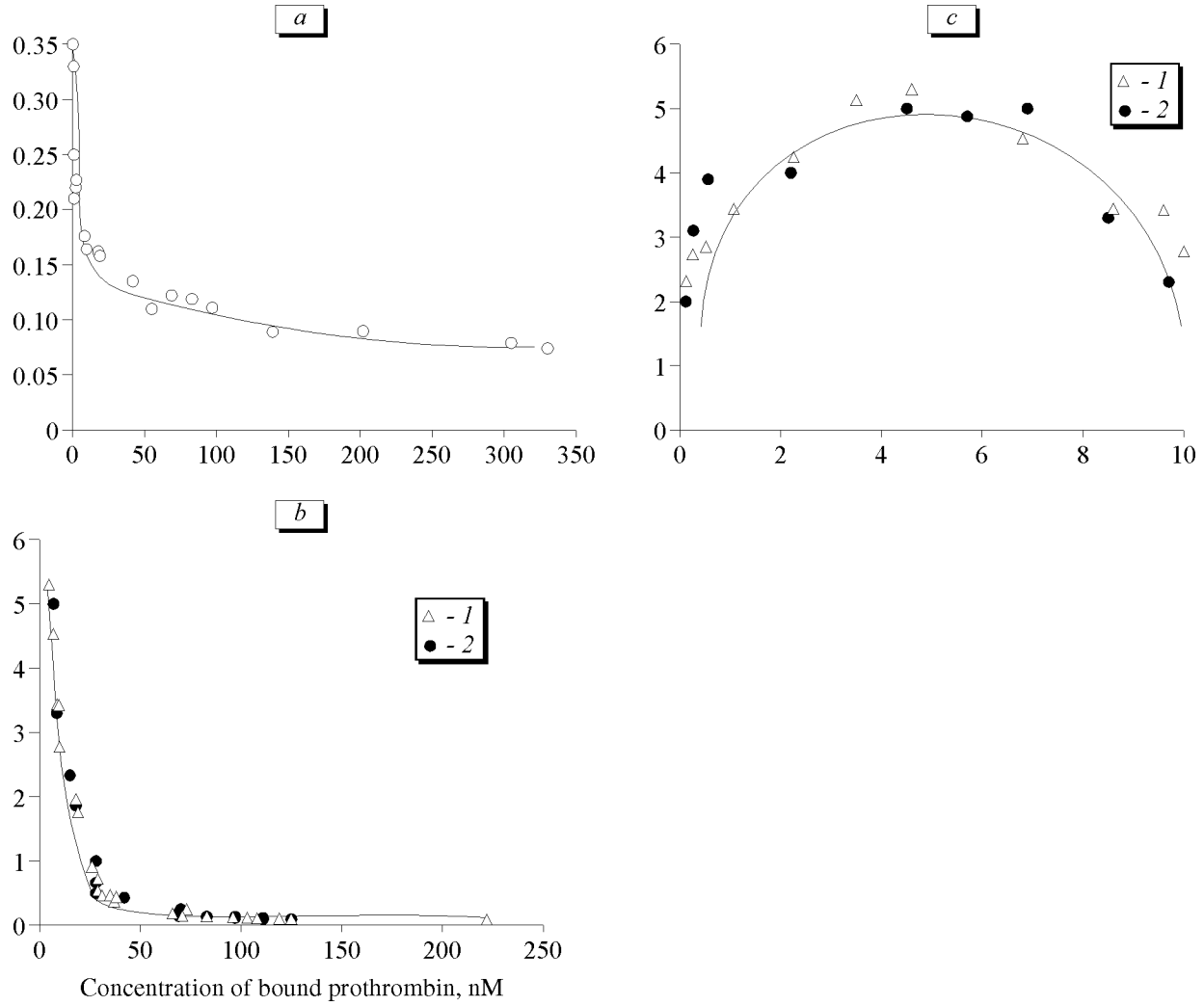

Fig. 1. Scatchard plots for prothrombin binding with fragments of intact cell membranes (*a*) and membranes irradiated (*b*, *c*) in doses of 3.5 (*1*) and 1 Gy (*2*). Ordinates: ratio of bound to free prothrombin concentrations.

because of PS clustering and organization of numerous HA centers. Positive cooperation of this binding is determined by summation of different interactions during prothrombin binding to HA centers and more pronounced destruction of the membrane induced by irradiation.

The observed changes in CM sorption capacity for prothrombin are physiologically significant. They show the molecular mechanism of primary hypercoagulation induced by ionizing radiation and prove the role of these changes in initiation of the hemostasis system.

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