ROLE OF LIPID HYPERPEROXIDATION IN DISTURBANCE OF THE STRUCTURAL ORGANIZATION OF PLATELET MEMBRANES

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Changes in the structural organization of blood cell membranes, on the one hand, and of smooth-muscle cell membranes of the vascular wall, on the other hand, play a leading role in the onset and development of coronary atherosclerosis [16]. According to modern views of the pathogenesis of atherosclerosis [4, 13], a change in the "rigidity" of platelet membranes may be one cause of their enhanced aggregation properties.

The orderliness of platelet membranes can be affected by the composition of the phospholipids, by substitution of the polyunsaturated fatty acids in their structure by saturated, and also by an increase in the molar ratio of cholesterol to phospholipids [15]. There are indications that eicosanoids (arachidonic acid metabolites) can form covalent bonds with proteins, and thus modify the structure of platelet membranes [14]. Cross reactions and aggregation of proteins in platelets due to the formation of intramolecular and intermolecular disulfide bonds have been observed during activation of lipid peroxidation (LPO) [3].

Lipid peroxidation, besides a change in concentration of membrane cholesterol, can evidently behave as regulator of the fluidity of platelet membranes [3]. However, the question of the localization of defects in the platelet membrane bilayer caused by activation of LPO has not yet been studied. Correlation between the intensity of LPO and the structural state of platelet membranes has virtually not been studied. Accordingly, the aim of this investigation was to clarify the role of hyperperoxidation in the disturbance of the structural organization of platelet membranes.

EXPERIMENTAL METHOD

Platelets with a raised level of functional activity from patients with unstable angina (UA) were used as experimental material. Previously the writers demonstrated increased aggregating activity of platelets, linked with LPO activation, in UA [9]. Platelets from healthy blood donors served as the control group.

Platelets were isolated from platelet-enriched plasma by differential centrifugation [8]. Lipids were extracted from a suspension of platelets with a mixture of chloroform and methanol (2:1). The concentration of nonesterified cholesterol (NEC) was determined by a gas-chromatographic method [12] on a gas–liquid chromatograph of "Packard" type (model 878) with flame-ionization detector. Concentrations of conjugated dienes (CD) were determined in accordance with recommendations in [5], conjugated trienes (CT) as in [2]. Synthesis of malonic dialdehyde (MDA) by platelets was estimated by the method in [17]. The content of Schiff bases (SB) was determined as in [11]. The concentration of total lipids (TL) was determined by the sulfophosphovanillin method, and protein in platelet digests was estimated by Lowry's method.

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TABLE 1. Characteristics of Structural and Functional State of Platelet Membranes (M ± m)

Parameter tested	Control (n = 38)	Activated platelets (n = 55)
Parameter of orderliness (S)	0,550±0,003	0,591±0,002*
Correlation time (τ), sec	$(1,520\pm0,007)\cdot10^{-9}$	$(1,200\pm0,005)\cdot10^{-9}$*

Legend. Here and in Tables 2 and 3, asterisk indicates values for which $p < 0.05$.

TABLE 2. Content of LPO Products and NEC in Activates Platelets (M ± m)

Parameter tested	Control (n = 59)	Activated platelets (n = 55)
CD, μmoles/mg TL	11,42±0,86	29,35±1,30*
CT, optical density units/mg TL	1,08±0,10	2,38±0,13*
MDA, nmoles/mg TL	6,50±0,49	11,61±0,61*
SB, fluorescence units/mg TL	35,81±2,21	97,83±5,67*
NEC, μg/10^9 cells	47,98±3,65	70,56±4,84*

TABLE 3. Correlation between Membrane Parameters and LPO Products in Platelets

LPO	Membrane parameters			
	parameter (n = 55)		correlation time (τ) (n = 32)	
	control	activated platelets	control	activated platelets
CD	+0,74*	+0,88*	−0,63*	−0,61*
CT	+50*	+0,71*	−0,80*	−0,60*
MDA	+0,55*	+0,74*	−0,65*	−0,70*
SB	+0,53*	+0,64*	−0,83*	−0,59*
S—τ	−0,97*			−0,76*

To assess the physical state of the membranes electron paramagnetic resonance (EPR) was used. As probes derivatives of stearic acid with a nitroxyl radical in positions 5 and 16 (from "Syva") were used. The final concentration of the probes in the sample was 10^{-6} M to $(4\text{-}6)\cdot10^9$ cells. EPR spectra were recorded on a type SEPR-02 radiospectrometer, and from them the parameter of orderliness (S) and the correlation time (τ) were calculated by standard formulas [1].

The experimental results were analyzed by single-factor and multiple-factor parametric statistical tests [6]. Mean values were analyzed by Student's test. The strength of correlation between individual variables was characterized by Pearson's coefficient of correlation. Multiple-factor linear regression analysis of the results was carried out by the standard procedure: construction of a model, identification, investigation. Construction of a model consisted of the choice of dependent variable and a list of independent variables, with whose values the level of the dependent variable may be connected. As an independent variable we used the factor of the patient's belonging to a group of patients with UA (B), which in the control group assumed the value of zero, whereas for subjects with increased

functional activity of their platelets, it assumed the value 1. The accuracy of the model was characterized by a multiple correlation coefficient (ρ). The model was identified on interactive mode with the aid of a program of multiple-factor dialog regression on an SM-4 computer.

EXPERIMENTAL RESULTS

By using the spin probes method it was possible to assess changes in the membranes of platelets with increased functional activity. The use of two lipid-soluble spin labels (5- and 16-doxylstearate) with radical centers at different depths from the membrane surface, enabled the mobility of the acyl chains of the phospholipids in different layers of the platelet membrane to be characterized.

Data on mean values of the parameters S and τ of the platelet membranes are given in Table 1. A significant increase in the mean values of the parameter S for the spin label 5-doxylstearate in activated platelets indicates an increase in orderliness and orientation of the hydrocarbon chains of the lipids at a depth of 0.6-0.8 nm from the membrane surface. The significant decrease in mean values of τ during platelet activation for spin-labeled stearic acid with a nitroxyl radical in position 16 characterizes increased mobility of the fatty acid residues of the phospholipids in the 2.0-2.2 nm region. Thus an increase in mobility of the acyl chains of the phospholipids was observed in activated platelets with increasing depth in the membrane. This is evidence of the existence of a "flexibility profile" [7] of the hydrocarbon chains of the phospholipids in platelets. On the basis of this finding, two regions of the platelet membrane with different orientations of the fatty-acid chains of their phospholipids can be distinguished: close to the membrane surface – a region of "dense" packing with an orderly arrangement of the phospholipid chains and a region of "loose" packing of polyunsaturated fatty-acid residues of lipids in the deep layers of the membrane.

In our experiments, during platelet activation, besides considerable changes in the membrane parameters S and τ, an increase in the concentration of LPO metabolites and of NEC also was observed (Table 2).

It follows from the positive correlation found between values of the parameter S and concentrations of LPO metabolites (Table 3) that lipid peroxidation products have a direct modifying action on the structural state of platelet membranes. Meanwhile negative correlation was found between the correlation time (τ) and accumulation of LPO products. Parameters of the physicochemical state of the platelet membranes (S and τ) also show negative correlation with one another (Table 3).

The changes discovered suggest that the mechanism of regulation of the structural state of platelet membranes by LPO products is as follows. In the case of the derivative 16-doxylstearate the nitroxyl radical is located at a depth of 2.0-2.2 nm in the region of long-chain acyl residues of phospholipids – the principal substrates of LPO. In the course of activation of LPO, the formation of diene and triene conjugation is coupled with an increase in the degree of unsaturation of membrane phospholipids in the process of intramolecular structural changes. A change in microviscosity of the membranes is accompanied by generation of free-radical intermediates and by an increase in concentration of secondary LPO products. Oligomerization of membrane proteins by dialdehydes (in particular, by MDA) and, possibly, by active forms of oxygen [10], facilitates an increase in the "rigidity" of the surface layers (at a depth of 0.6-0.8 nm) of the platelet membranes. Consequently, orderliness and orientation of juxtamembranous structures of platelets depend on the degree of unsaturation and the degree of oxidization of lipids in the depth of the bilayer, a result of activation of LPO reactions.

By regression analysis it was possible to assess the contribution of individual factors to the structural state of platelet membranes and to describe them by a system of equations:

$$S=0{,}544+0{,}001\cdot CD+0{,}0001\cdot NEC \qquad (\varrho=0{,}89;\ p<0{,}001), \tag{1}$$

$$S=0{,}540+0{,}00\quad CD+0{,}001\cdot MDA+0{,}001\cdot SB+0{,}0033\cdot CT \qquad (\varrho=0{,}98;\ p<0{,}001). \tag{2}$$

It follows from equations (1) and (2) that increased concentrations of LPO products (CD, CT, MDA, SB) and of NEC have a direct effect on the "rigidity" of platelet membranes. The absence of the B factor in these equations is evidence that in normal and activated platelets the orderliness of the platelet membranes depends on the concentration of LPO metabolites and of NEC. Thus LPO products and NEC have a direct influence on the structural state of the platelets. However, as follows from equation (2), LPO metabolites (CD, CT, MDA, SB), in the absence of increased NEC concentrations, can themselves change the "rigidity" of platelet membranes.

The concentration of CD introduces a component into the above-mentioned multiple-factor equations. Analysis of the CD level in the platelets led to the following regression equation ($\rho = 0.93$; $p < 0.001$):

$$CD = 419{,}5 \cdot S + 10{,}5 \cdot B - 226{,}0, \quad (3)$$

into which the parameter S was introduced. The presence of the B factor in equation (3) means that whatever the orderliness and orientation of the membrane phospholipids, activated platelets are characterized by a higher CD concentration.

Analysis of all these equations suggests the existence of the following relationships. Activation of LPO with accumulation of its primary products, namely CD, on the one hand leads to increased orderliness of the platet membranes (Eq. 1). On the other hand, an increase in "rigidity" of the platelet membrane promotes, through indirect mechanisms, initiation of the first stage of the chain of lipid peroxidation reactions (Eq. 3), and also, evidently, the further development of the process as a whole (Eq. 2). The level of CD and "rigidity" of the platelet membranes act as mutually regulatory factors in platelets (Eqs. 2 and 3).

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