8. H. Mest, K. Blass, and W. Förster, Acta Biol. Med. Ger., 35, 63 (1976).
9. J. Neely and H. Morgan, Annu. Rev. Physiol., 36, 413 (1974).
10. D. Nugteren, E. Deckere, and F. Ten-Hur, J. Mol. Cell. Cardiol., 9, 35 (1977).
11. L. Opie, Am. Heart J., 76, 585 (1968).
12. J. Vusse, T. Roemen, F. Prinzen, et al., Circ. Res., 50, 538 (1982).
13. L. Wojtczak, FEBS Lett., 44, 25 (1974).

CHANGES IN PLATELET STRUCTURE AND FUNCTION IN EXPERIMENTAL ATHEROSCLEROSIS

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UDC 616.13-004.6-092.9-07:616.155.25-07

KEY WORDS: platelets; platelet membranes; viscosity of membranes; cholesterol; atherosclerosis.

The role of platelets in atherogenesis and the connection between platelet function and the pattern of lipid metabolism and its changes in atherosclerosis are widely familiar [7, 8, 12, 13]. What leads to changes in platelet function during atherogenesis? We know that the physiological activity of any cells, including platelets, largely depends on the physicochemical properties of their membranes. A dynamic regulatory role in biological membranes, which consists in particular of the control of fluidity, is played by cholesterol (Ch). A decrease in fluidity arising with an increase in the fraction of Ch prevents normal functioning of membrane-bound enzymes and disturbs some properties of the cells. It was shown previously [3] that incubation of platelets with low- and very-low-density lipoproteins, taken from patients with ischemic heart disease, enhances their aggregation, whereas incubation with high-density lipoproteins has the opposite effect. Considering the donor and acceptor character of these lipoproteins relative to Ch, it can be postulated that changes in platelet function are determined primarily by the quantity of this steroid in their membrane. We know that in hypercholesterolemia there is an increase in the Ch content in membranes of erythrocytes [1], affecting their function. It can be tentatively suggested that similar phenomena also take place in platelets, and that this is one cause of their hyperreactivity.

In the investigation described below changes in the physicochemical characteristics of platelet membranes and in the functional activity of the cells (ability to aggregate) were studied in rabbits with experimental atherosclerosis.

EXPERIMENTAL METHOD

Experiments were carried out on 35 Chinchilla rabbits. Experimental atherosclerosis was induced in 15 rabbits by feeding them daily for 3 months with 0.25 g Ch/kg body weight. The control group consisted of 20 rabbits. Blood was collected from the auricular vein into siliconized tubes containing 3.8% sodium citrate in the ratio of 9:1 by volume. Platelets were isolated by gel filtration from platelet-enriched plasma on Sepharose 2B. The content of Ch and phospholipids (PL) was determined after extraction of the lipids by Folch's method [6], by the methods of Abell and Vaskovsky [14]. The structure of the membranes was studied by the electron paramagnetic resonance (EPR) of spin probes method, using stearic acid derivatives with nitroxyl fragments in positions 5 (I) and 16 (II) relative to the carboxyl group. The appearance of the EPR spectra and the structural formulas of the probes used in rabbit platelets are shown in Fig. 1. The probes were introduced into the test samples in the form of ethanol solutions; their final concentration was 10^{-5} M per $6 \cdot 10^{8}$ cells/ml, and

Department of Biophysics, N. I. Pirogov Second Moscow Medical Institute. (Presented by Academician of the Academy of Medical Sciences of the USSR Yu. M. Lopukhin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 97, No. 2, pp. 149-152, February, 1984. Orignal article submitted April 7, 1983.

0007-4888/84/9702-0165$08.50

TABLE 1. Physicochemical and Functional Properties of Platelets of Rabbits with Experimental Atherosclerosis

Group of animals	Plasma Ch, mg%	Parameters of orderliness of probe I(S), relative units	Rotary correlation time of probe II, $\tau \cdot 10^{-9}$, sec	Molar ratio Ch/PL	Aggregation time, min
Control (n= 20)	123±28	0,619±0,018	1,85±0,30	0,55±0,12	6,81±1,18
Experiment (n= 15)	586±48	0,661±0,020	3,12±0,42	0,80±0,10	5,32±1,09
P	<0,001	<0,01	<0,02	<0,01	<0,05

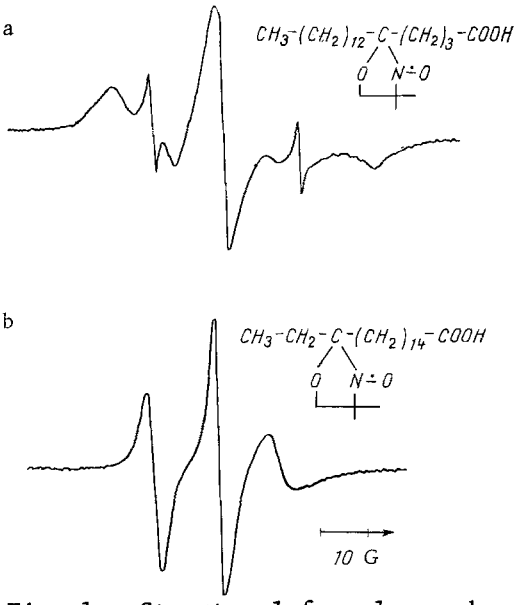


Fig. 1. Structural formulas and EPR spectra of spin probes I (a) and II (b) in rabbit platelets.

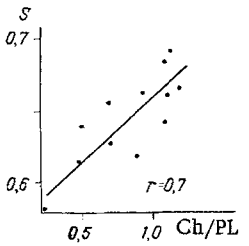


Fig. 2. Dependence of parameter of orderliness (S) of spin probe I on Ch content in rabbit platelets.

the ethanol content did not exceed 2% by volume. EPR spectra were recorded on an E-4 radiospectrometer (Varian, USA) in a thermostatically controlled flat cuvette at 37°C, with microwave power 10 mW, amplitude of high-frequency modulation 1-2 G, and rate of development of magnetic field 100 G in 8-16 min. To estimate the degree of randomness of movement of the acyl chains of platelet membrane PL in the region of probe I the parameter of orderliness S was used [2]. The microenvironment of probe II was characterized by calculating the rotary diffusion correlation time τ, which signifies the mean duration of rotation of the radical through an angle $\pi/2$ [2]. The rate of platelet aggregation was measured by the method [4] by recording the decrease in scattering of light by the cell suspension in buffer (134 mM NaCl, 15 mM Tris-HCl, 1 mM EDTA, 5 mM glucose, pH 7.35) after addition of 10^{-4} M ADP and

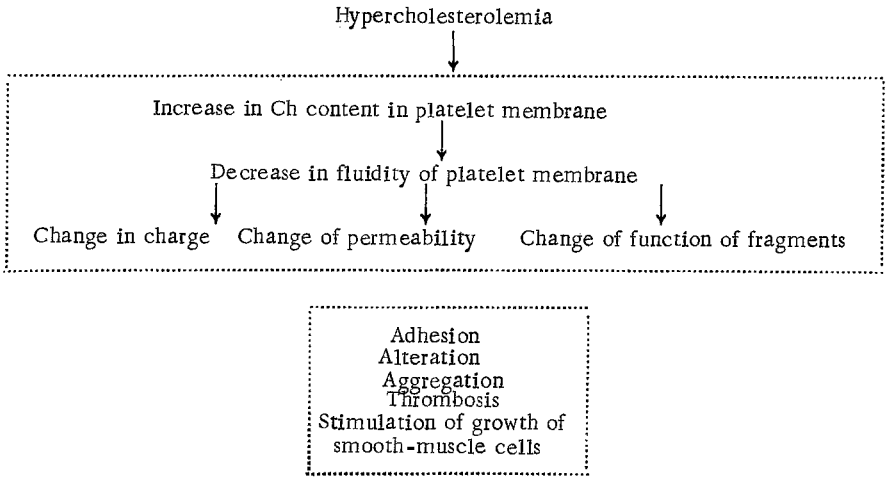
$3 \cdot 10^{-3}$ M $CaCl_2$ per 10^7 cells in 1 ml, and estimating the time taken to reach maximal translucency. Measurements were made at 37°C and at a wavelength of 620 nm.

EXPERIMENTAL RESULTS

During feeding of the animals with Ch, simultaneously with an increase in its plasma concentration, Ch also accumulated in platelets. This was shown by an increase in the molar ratio Ch/PL from 0.55 in platelets of control animals to 0.80 in rabbits kept on a high-cholesterol diet (Table 1). Since membranes of cell organelles contain little or no Ch, the significant differences in Ch concentration obtained can be attributed entirely to the outer cytoplasmic membranes of the platelets. Analysis of parameters of EPR spectra of spin probes I and II, depending on the microenvironment and characterizing the viscosity of the lipid phase of the membranes, showed that simultaneously with an increase in the Ch content there is also an increase in rigidity of the platelet membranes. This is manifested as an increase in the parameter of orderliness of probe I and the rotary correlation time of probe II (Table 1). It was shown by the use of fatty acid probes to study platelets [10] that the sites of these compounds are hydrophobic regions of the plasmalemma. Consequently, it can be concluded from the experimental results that the cytoplasmic membranes of platelets from rabbits with experimental atherosclerosis have a higher molecular packing density than normally, and this is evidently due to the excess of Ch. This hypothesis is confirmed by the direct dependence of the parameter of orderliness of probe I on the molar ratio of Ch to PL (Ch/PL) in rabbit platelets (Fig. 2). With an increase in the Ch/PL ratio the parameter of orderliness and, consequently, the microviscosity of the membranes rises. The coefficient of linear correlation is 0.7, a reliable result with a level of significance of 0.95. The significant increase in τ in the platelets of rabbits with hypercholesterolemia (Table 1) is evidence of the reduced mobility of the acyl chains of PL at a depth of about 22 Å (the depth at which the nitroxyl fragment of this probe lies in the bilayer) compared with the control. This may be due both to accumulation of Ch and also to other factors, such as changes in the fatty-acid composition of membrane PL. These results suggest that the increased activity of the platelets in plasma, discovered previously in a model of hypercholesterolemia, is due to modification of the lipid composition of the membranes by Ch, and this in turn leads to a disturbance of the structure of the bilayer and of functioning of the membranes, modulating cellular activity. A study of the functional properties of platelets isolated from plasma showed that in experimental atherosclerosis an increase in the Ch/PL ratio and in the microviscosity of the membranes is accompanied by shortening of the ATP-induced aggregation time (Table 1).

Some suggestions may be put forward regarding the mechanism of the increased aggregation properties of the platelets during accumulation of Ch. An increase in microviscosity of the platelet membranes leads to immobilization of membrane proteins, one result of which may be a decrease in the degree of sinking of the receptors (glycoproteins) into the bilayer, which must promote activation and facilitate intercellular contacts. Meanwhile an increase in rigidity of the membrane leads to inhibition of the membrane enzymes, such as Na^+, K^+-ATPase which, in turn, causes disturbance of the ionic balance, swelling, and finally, aggregation of the platelets [9]. One of the membrane enzymes is adenylate cyclase, which, through cAMP synthesis, controls the cell response to the action of the regulator. This enzyme is known [11] to control the content of intracellular Ca^{++}, which plays an important role in platelet activation. A fall in the cAMP level on account of reduced adenylate cyclase activity leads to changes in the Ca^{++}-binding properties of the membrane structures of the platelets, thus intensifying the flow of these cations into the cytoplasm. The excess of Ca^{++} causes disassembly of the cytoskeleton and activation of platelet phospholipase, an enzyme which limits the rate of synthesis of active metabolites of arachidonic acid and, in particular, of thromboxane A_2. This hypothesis is confirmed by recent investigations [5] which showed an increase in the blood concentration of thromboxane A_2 in animals with experimental atherosclerosis.

It can thus be concluded on the basis of these data and our own observations that one cause of acceleration of platelet aggregation in rabbits with experimental atherosclerosis is a change in the physicochemical state of their membranes on account of an excess of Ch. Some hitherto missing links can therefore now be introduced into the scheme for participation of platelets in the process of atherogenesis:



If our own results are compared with those obtained previously in studies of erythrocytes, fibroblasts, smooth-muscle cells, lymphocytes, and other cells it can be concluded that they all react equally to accumulation of Ch in the plasma membrane: by disturbance of permeability and of the function of membrane-bound enzymes, and by more intensive aggregation. These findings confirm yet again the primary nature of the membranotropic effect of Ch in atherosclerosis and they are evidence in support of the validity of the "membrane" hypothesis of atherogenesis.

LITERATURE CITED

1. L. G. Artemova, O. A. Azizova, T. S. Rudenko, et al., in: Magnetic Resonance in Biology and Medicine [in Russian], Chernogolovka (1981), p. 225.
2. L. Berliner (editor), The Spin Labeling Method. Theory and Applications, Academic Press, New York (1976).
3. A. V. Suvorov and V. A. Polesskii, in: Lesions of the Vessel Wall and Hemostasis. Abstracts of Proceedings of a Conference [in Russian], Poltava (1981), p. 194.
4. G. V. R. Born, Nature, 194, 927 (1962).
5. A. Dembinska-Kiec, W. Rücker, and P. Schönhöfer, Atherosclerosis, 33, 217 (1979).
6. J. Folch, M. Lees, and G. H. Sloane-Stanley, J. Biol. Chem., 226, 497 (1957).
7. J. S. Omeln, R. A. Hutton, and N. McIntyre, New Engl. J. Med., 302, 1424 (1980).
8. A. Nordoy, B. Svensson, D. Wiebe, et al., Circ. Res., 43, 527 (1978).
9. W. C. Sandler, G. C. LeBreton, and G. C. Feinberg, Biochim. Biophys. Acta, 600, 448 (1980).
10. R. D. Sauerheber, T. S. Zimmermann, Y. A. Esgate, et al., J. Membr. Biol., 52, 211 (1980).
11. A. K. Sinha, S. J. Shattil, and K. W. Colman, J. Biol. Chem., 252, 3310 (1977).
12. S. J. Shattil, J. Clin. Invest., 55, 636 (1980).
13. M. Stancioin, M. Schrobahici, I. Zatreanu, et al., Med. Interne, 16, 395 (1978).
14. V. E. Vaskovsky, E. J. Kosetsky, and I. Vasendin, J. Chromatogr., 114, 129 (1957).