STRUCTURAL AND FUNCTIONAL ORGANIZATION OF THE HEMOSTATIC CLOT

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UDC 612.115.1:612.111.72

Experimental data confirming the authors' hypothesis relating to structural and functional organization of the hemostatic clot are described. The basis of the clot is a fibrin–platelet structure in the meshes of which erythrocytes and leukocytes are distributed. Spontaneous compaction of the clot (retraction) takes place on account of the contractile properties of the surface structure of the activated platelets – extruded platelet cytogel. It is demonstrated that disturbance of the structure of the clot during aggregation of the platelets leads to inhibition of retraction. The sequence of processes taking place during formation and contraction of the clot is discussed.

KEY WORDS: structure of the blood clot; platelets; retraction; aggregation.

Recent experimental data have led to the suggestion that in the blood clot there is a strictly regular structure which is essential for performance of the hemostatic function. The most important of these data are: 1) the connection between the mechanical properties of the clot and the number and functional activity of the platelets [5, 9]; 2) the presence of an actomyosin-like protein (thrombosthenin) and Ca-ADP in the platelets [6]; 3) the existence of a specific surface structure of the activated platelet, or extruded cytogel, capable of contraction and containing thrombosthenin [3, 7, 11]; 4) the catalytic activity of the platelet surface [10].

The object of this investigation was to verify experimentally the structural and functional concept of the organization of the hemostatic clot.

EXPERIMENTAL METHOD

Blood was obtained by cardiac puncture from adult rabbits and mixed with 3.8% Na citrate in the ratio of 9:1. Platelet-enriched plasma (PEP) was obtained by centrifugation. The number of cells was counted in a Goryaev's chamber. Photomicrography was carried out with the ML-3 luminescence microscope [3]. Coagulation of specimens of recalcified PEP was recorded on the N333 coagulograph, and the mechanical properties of the clot were recorded on the Tromb-2 thromboelastograph. Aggregation of the platelets was recorded by Born's nephelometric method [8].

A fluorometric method [2] was used for the objective recording of the kinetics of transformations of the platelets during clotting. The method was based on the use of the ability of lysosome-like α granules of the platelets to accumulate actively the fluorescent label 3,6-bis-dimethylaminoacridine (acridine orange, AO). The recalcification test was carried out with 1.29% isotonic $CaCl_2$.

EXPERIMENTAL RESULTS

To study the order of the processes taking place in recalcified PEP, the coagulogram (OG), thromboelastogram (TEG), and fluorogram (FG) were recorded simultaneously in the same sample of plasma at different concentrations of $CaCl_2$. To 2 ml PEP at 37°C 0.05 ml of AO solution was added, after incubation for 10 min the $CaCl_2$ was added, and samples of 0.36, 0.28, and 1.5 ml respectively were taken for recording the TEG, CG, and FG. The curves obtained were combined by using a single time scale (Fig. 1).

With three of the concentrations of Ca^{2+} ions tested the TEG was located on the time scale to the right of the CG and FG, which practically coincided, with only a slight shift relative to one another. Similar information is given in the literature; for instance, the reaction time (r) for whole healthy human blood is 8 min and the

Laboratory of Thromboresistant Materials, Institute of Transplantation of Organs and Tissues, Ministry of Health of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR V. S. Savel'ev.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 85, No. 2, pp. 132-136, February, 1978. Original article submitted June 24, 1977.

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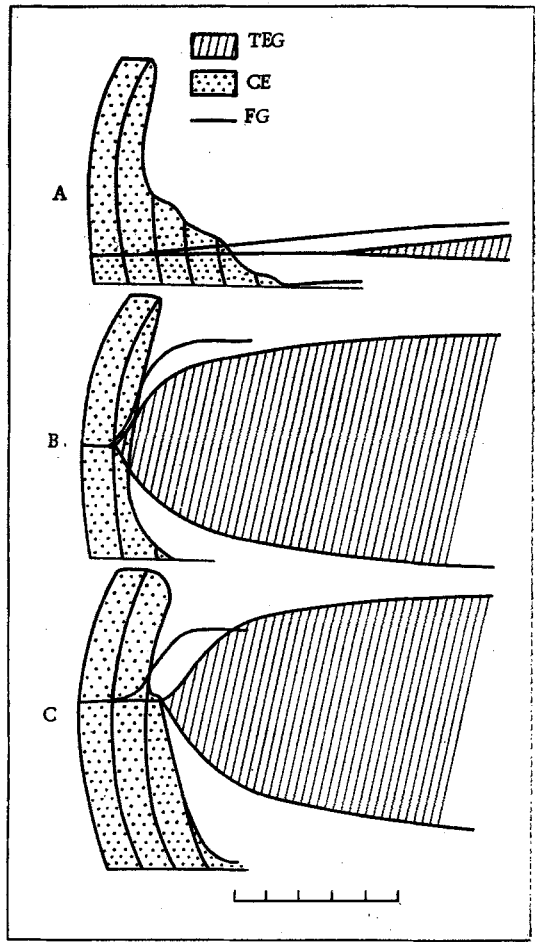

Fig. 1

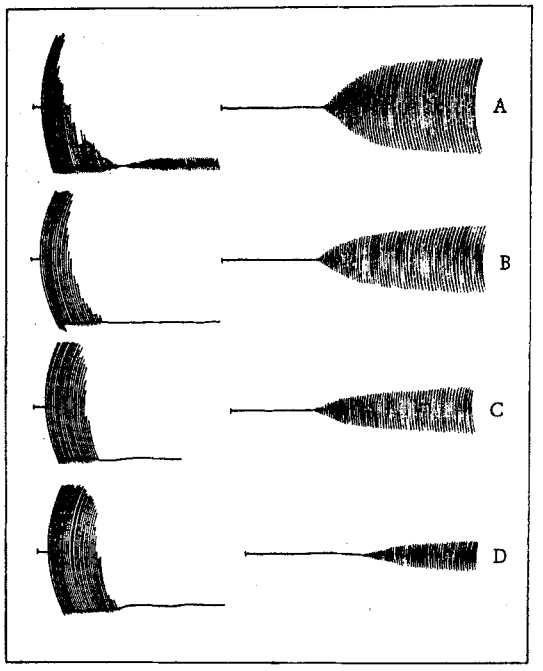

Fig. 2

Fig. 1. Dynamics of processes taking place in recalcified plasma. A) 0.3 ml, B) 0.6 ml, C) 1.4 ml $CaCl_2$. Number of platelets in PEP 707,000/mm^3. Time scale shown below (1 division = 1 min).

Fig. 2. Effect of change in number of platelets on parameters of TEG and CG. A) 917,500, B) 370,000, C) 160,000, D) 20,000 cells/mm^3.

CG index – the time for the end of clotting and beginning of retraction and fibrinolysis – is 6-11 min [1]. This fact can be explained if it is assumed that the clot forms first and subsequently contracts. The first process is well reflected in the CG, for the structure formed deprives the sample in the cuvette of the coagulograph of its mobility, whereas the second is reflected in the TEG, for the contractile forces arising in the clot lead to twisting of the detector of the instrument.

Since the platelets play an important role in clot formation, it was necessary to study the effect of a change in their number on these processes. For this purpose the TEG and CG were recorded simultaneously in samples of PEP containing different numbers of platelets.

A decrease in the number of platelets in the PEP led to a very small increase in the time of beginning of clotting T_1, as recorded on the CG, whereas a considerable increase was found in the maximal amplitude (a_m) and lengthening of the reaction time (r) during recording of the TEG (Fig. 2). Just as in the previous series of experiments the beginning of divergence of the branches of the TEG was observed to be at the end of the CG. The amplitude of the TEG was thus directly dependent on the number of platelets. These general principles can be explained by the fact that the CG reflects the precipitation of fibrin filaments whereas the TEG reflects contraction of the clot. The time of precipitation of fibrin filaments depends to a lesser degree on the number of platelets than the degree of retraction.

To verify the hypothesis that contraction of the clot is due to the contractile activity of the platelets, it was necessary to show how the distribution of platelets in the sample of plasma (from diffuse to a high degree of aggregation) influences the parameters of the TEG.

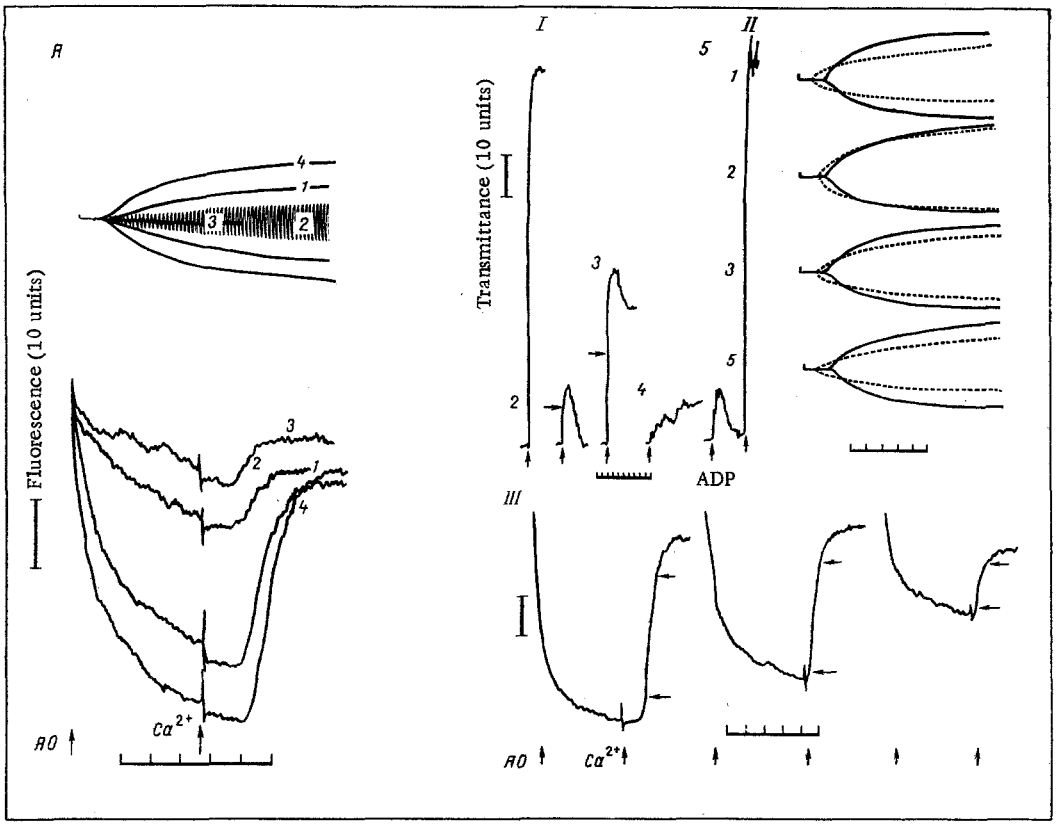

Fig. 3. Effect of state of aggregation of platelets on mechanical properties of clot and ability of platelet to accumulate and liberate AO. A) ADP-induced platelet aggregation. 1) 10^{-4} M ADP without mixing; 2) mixing for 2 min; 3) mixing for 6 min; 4) control. In FG, here and later: time scale below (1 division = 1 min), fluorescence on left (10 conventional units). Arrows indicate addition of AO and recalcification. Aggregation recorded at 22°C, TEG and FG at 37°C. Number of cells in TEG 625,000/mm^3; B) "spontaneous" aggregation of platelets (with mixing). I) "Spontaneous" aggregation (by Born's method) with different times of mixing and incubation of PEP. 1) Initial "spontaneous" aggregation; 2-3) mixing for different times; 4) after plasma had stood for 30 min at 22°C; 5) same plasma after mixing and addition of ADP. Vertical line marks time of starting mixer; horizontal line marks time of stopping mixer. Time scale below (1 division = 1 min). II) TEG after aggregation in same samples. Continuous line shows initial TEG; broken line experimental TEG. Remainder of legend as in I. III) FG and CG after "spontaneous" aggregation of platelets. From left to right: control, sample 1, sample 5. Horizontal lines mark beginning and end of clotting on CG.

Aggregation of platelets is known to take place as a result of the action of an inducing agent (ADP, for example) and contact of the cells (mixing) [8]. To 0.9 ml PEP 0.1 ml of a solution of ADP in physiological saline was added, the mixture was diluted 1:1 with physiological saline, and the TEG, CG, and FG were recorded after the addition of 0.3 ml $CaCl_2$. The complete FG (accumulation and liberation of AO) was recorded, for which purpose 0.05 ml of AO solution was added to the suspension, and the absorption, followed by the liberation, after addition of $CaCl_2$ were recorded.

The experiments showed that addition of ADP without mixing (final concentration 10^{-4} M) caused the formation of small aggregates of platelets (Fig. 3A : 1, 4; B) and a decrease in the value of a_m. Mixing (1000 rpm) after addition of ADP led to a decrease in a_m and caused the formation of very large aggregates of platelets (Fig. 3B, 5). The effect was proportional to the mixing time (Fig. 3A: 2, 3). Considerable changes in FG were observed: a progressive decrease in the quantity of fluorescent label absorbed and liberated.

During the recording of platelet aggregation the following feature was noted. In some samples of PEP strong "spontaneous" aggregation of platelets arising immediately after mixing without addition of ADP was observed; in the course of incubation of the plasma the degree of aggregation changed (Fig. 3B, I, 1-4), and ultimately the plasma became insensitive to mixing. The ability of the platelets to aggregate after addition of ADP and mixing still remained (Fig. 3B, I, 5).

Although the cause of this phenomenon is unknown, it was considered interesting to study how mixing this plasma with a high degree of "spontaneous" aggregation affects the indices of the TEG and FG. By switching on the mixer for a short time it was possible to obtain different degrees of aggregation – from completely reversible to irreversible. Under these circumstances changes characteristic of ADP-induced aggregation, as described above, were observed. Similar changes also were found in the character of the FG. The CG and FG recorded simultaneously coincided (Fig. 3B: II, III).

Very recently attempts to estimate the energy of contraction of the fibrin–platelet structure of the clot quantitatively have been published. This can be done both thromboelastographically [5] and by direct measurement of retraction of the clot by means of a mechano-electrical transducer [4]. In this last paper proof of the complete absence of retraction in platelet-free plasma and also that the maximal strength of retraction is a linear function of the number of platelets was obtained.

Direct luminescence-microscopic observations led to the conclusion that rapid self-contraction (retraction) of the clot takes place on account of contraction of a specialized surface structure of the platelet, which has been called extruded platelet cytogel. These results, together with the facts mentioned above concerning the presence of contractile protein (thrombosthenin) in platelets and its liberation during viscous metamorphosis, present a new picture of the process of clot formation. The hemostatic clot is a strictly orderly structure, consisting of platelets connected by filaments of fibrin. In the meshes of this network lie erythrocytes and leukocytes. Such a structure is capable of self-compaction on account of the contractility of the platelets. Under these circumstances the thrombosthenin which is a component of the specialized surface structure of the activated platelets – the platelet cytogel – contracts. Disturbance of the structure leads to a disturbance of its hemostatic function, for the fibrin–platelet network becomes unable to contract and cannot effectively hold up the blood flow. In the course of the local hemostatic process the following chain of principal events thus takes place: 1) activation of the blood cells (mainly platelets) and procoagulant enzymes; 2) adhesion of some of the platelets at the edges of the region of injury to the blood vessel and liberation of thrombosthenin (cytogel) on the surface of the free platelets; 3) polymerization of fibrinogen of the surface of the platelets with the formation of fibrin bridges between the cells and the formation of the fibrin–platelet structure; 4) contraction of the thrombosthenin, leading to tightening of the fibrin–platelet structure, retraction of the clot, and the arrest of bleeding.

The problem of the mechanisms responsible for the formation of the orderly fibrin–platelet structure is still unsolved. An important role evidently belongs to the catalytic activity of the surface of the activated platelets [10], which is the site of self-assembly of the fibrin filaments.

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