Structure and Functions of Biopolymers

The properties of clots, formed out of desAA- and desAABB-fibrin with different surface structure

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It was shown that on the surface of fibrin clots, which are formed at the interphase on condition of excluding interpenetration of one phase into another one, there forms a structure (a surface layer), which differs from the clot structure. At the absence of the interphase, the formation of such layer in fibrin clot is not observed. The creation of this structure is assumed to be connected with the disturbance of the process of lateral association of fibrillar structures in the fibrin clot which takes place at the interphase. The surface layer is formed by protofibrillar structures which are spread flat on the interphase and form compact irregulated structure which can be seen in electron micrographs. The revealed peculiarities of the fibrin clot structure, caused by their formation conditions (presence or absence of the surface layer), determine the manner of clots interaction with the fibrinolytic system which can be the reason of discrepancies in the degree of fibrin clots resistance in blood circulation.

Key words: *fibrinogen, fibrin clot, electron microscopy*

Introduction. The plasma protein fibrinogen is known to transform under the influence of thrombin and ancistrone into monomeric fibrin which polymerizes further into two-stranded protofibrils [1-3]. With time protofibrils assemble laterally and form fibers of three-dimensional clot. The mechanism of protofibrils assembly is studied well enough [4, 5], but the processes which take place on the surface of the fibrin clot during the fibrin polymer formation are not understood completely.

Last years glues on the basis of fibrin or compositions of fibrinogen, thrombin and thrombin-like enzymes are widely used in clinic practice [6-9]. Moreover, the quantity of composite materials, made of plastic and metals, and used at different surgical operations (stents, plastic pros-

theses etc) has increased significantly. The surface of these substances can provoke the process of fibrinogen contact activation, and the structure of formed fibrin clots will be different from the structure of clots, formed in blood circulation. The research on the fibrin clots has shown for the first time that the structure of clots surface, called "surface layer", differs from the fibrin clot structure itself, Evidently, its presence or absence in the fibrin clot determines peculiarities of these clots interaction with fibrinollitic system components, which, in its turn, can result in thrombotic complications. The understanding of formation and interaction with fibrinollitic system components of the fibrin clot surface layer has both theoretical and practical meaning for biochemistry and practical medicine.

Materials and Methods. Fibrinogen was obtained from oxalate plasma of donor blood by precipitating with natrium sulphate [10]. It was cleaned from plasminogen admixtures by lysine solution treatment with subsequent ethanol re-precipitation of protein [11], or by affine chromatography on the lysine-sepharose. The content of fibrinogen, which clotted under the influence of thrombine, was 96-98%. A clot, formed of fibrinogen and purified from admixtures of plasminogen, is not destroyed in the presence of 20 international units of tissue plasminogen activator per 1 mg of protein for 120 minutes. Fibrinogen preparations were electrophoretically homogeneous according to the data of 10% DDs-Na-PAAG [12].

Plasmin was received by activating Glu-plasminogen on sepharose, conjugated with urokinase [13]. The activity of the obtained plasmin preparation was 10-15 catalytic units per 1 mg of protein.

Two model systems were used for clots formation: 1) blocks of agar from 1% bactoagar, and 2) plastic tubes. Electron microscopy showed that clots, formed in the first system, did not have a surface layer, while clots, obtained in system 2, had such a layer [14].

Initial concentration of fibrinogen, necessary for polymerization, was 20mg/ml, and trombin – 2 NIH/ml. Proper polymerization conditions were chosen for obtaining clots of different stability. Clots without fusion were formed during 30 minutes at room temperature, the ones, fused at -chain – 60 minutes at room temperature, and -, -chains – 60 minutes at the temperature of 37EC. The clot stabilization occurred due to the admixtures of factor XIII, activated by 2mM $CaCl_2$.

After polymerization the clots were carefully taken out of the reservoirs, in which they were polymerized, and put into the tubes with 0.05 M phosphate buffer, pH 7.4. Plasmin was added to the clots (10мg/ml), mixed and incubated at 37EC. The samples for electron microscopy were taken in defined time intervals during 60 minutes. After removing the sample from the tube it was put into phosphate buffer containing the solution of pNFGB (10^{-4} M) to prevent further clot hydrolysis by plasmin.

The formed fibrin gel was fixed in certain time intervals (depending on the task), using glutaric aldehyde as the first fixator (2.5% in 0.1 M cacodylatic buffer, pH 7.4). Then the gel was washed with 0.1 M cacodylatic buffer, pH 7.4, and fixed in the solutions of osmium oxide (from 2 to 50%). The clots were dehydrated on the surface of 4% solution of acetone, and when its concentration reached 50%, the clots were put completely in the solution. Afterwards they were polymerized in the resin Epon-Araldit (AGAR). The samples, prepared in such a way, were used for scanning and transmission microscopy.

Results and Discussion. The interphase is a necessary factor for the surface layer formation in the fibrin clot. This assumption is proved by an experiment in which the fibrin clot was formed inside agar-agar or gelatin block, the walls of which were soaked with water. In this case the gel inside is homogeneous phase for the protein solution and so the surface layer is not formed [14].

For further work with clots, different in surface structure, namely – presence or absence of the surface layer, the following models were chosen: clots polymerization in plastic tubes or in blocks of agar-agar.

It was shown by electron microscopy that the clots, formed in agar blocks, were deprived of the surface layer, while the clots, formed in the tubes, possessed this layer (Fig.1, A).

Electron microscopy of the clots, which have different surface structure, was performed on both model systems. The clots polymerization time was 30-40 minutes at room temperature, the volume - 0.2ml. The following samples were prepared: clots of desAABB-fibrin and desAA-fibrin with the surface layer and without it. The analysis of desAABB-fibrin clots with the surface layer and without it (Fig.1, A) showed that the thickness of the surface layer was 27.1 nm (15 measurements).

It was found that fibrin strands of the surface layer and those of the clot itself under the layer are connected structurally. The surface layer is formed of thinner fibrin strands (most probably protofibrillar structures) in comparison with the strands of the clot under it. The bundles of these thin surface layer strands, assembling laterally with each other, transform into thick gel fibers. Thin fibrils prevail in the clot with the surface layer (probably, there is a small amount of protofibrils which have no time to form fibrils); there are fibrils built into the surface layer or, as it was already mentioned, emerging from it. The clot with the surface layer contains less fibrils, the distance between them is bigger than in the clot, formed in agar block. The diameter

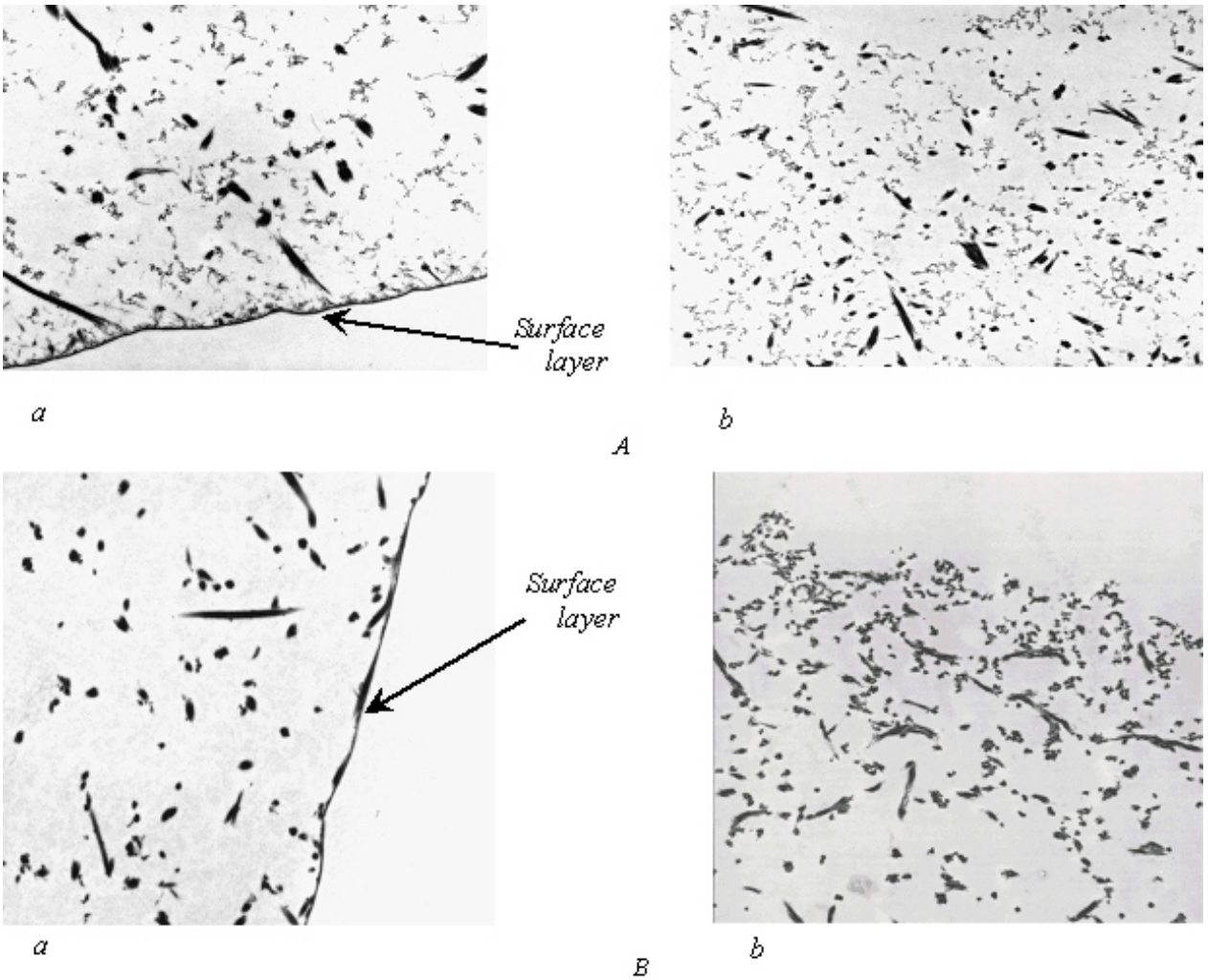

Fig.1. Electron micrographs of desAABB-fibrin (A) and desAA-fibrin (B) clots: a – polymerization in a tube; b - polymerization in an agar-agar block (x 35 000)

of fibrils in the clots with the surface layer is 30 - 170 nm, and in the clots without the surface layer is - 230 - 400 nm.

The analysis of desAA-fibrin clots with and without the surface layer (Fig.1, B) gave the same results which were obtained for the clots of desAABB-fibrin, having different surface structure.

The surface layer thickness was 26.8 nm (15 measurements), the diameter of fibrils in the clots with the surface layer and without it amounted to 85-114 and 120-200 nm correspondingly; the distance between fibrils was practically identical.

At first in the fibrin solution and interphase there appear protofibrils, which assemble into fibrils as a result of lateral association. The restricted mobility of protofibrils on the clot surface prevents the process of their lateral assembly. It may be due to the attachment of strands to the surface of tube walls, resin or (on the boundary with air) because of the surface tension. Fibrin strands, restricted in movements, form on the clots surface chaotically assembled thin fibers, which transform into fibrillar structures, a large degree of freedom for which inside the protein solution results in the fibrils formation. In the surface layer the process of fibrils formation is very slow due to the mobility restriction of protofibrillar structures. At the absence of interphase there is no surface layer in the fibrin clot, and the fibrils fill up a gel volume uniformly, therefore, the protofibrillar structures almost disappear from such clots after a certain time of polymerization. One of possible mechanisms of the surface layer formation may be spreading of protofibrils flat on interphase at their lateral growth

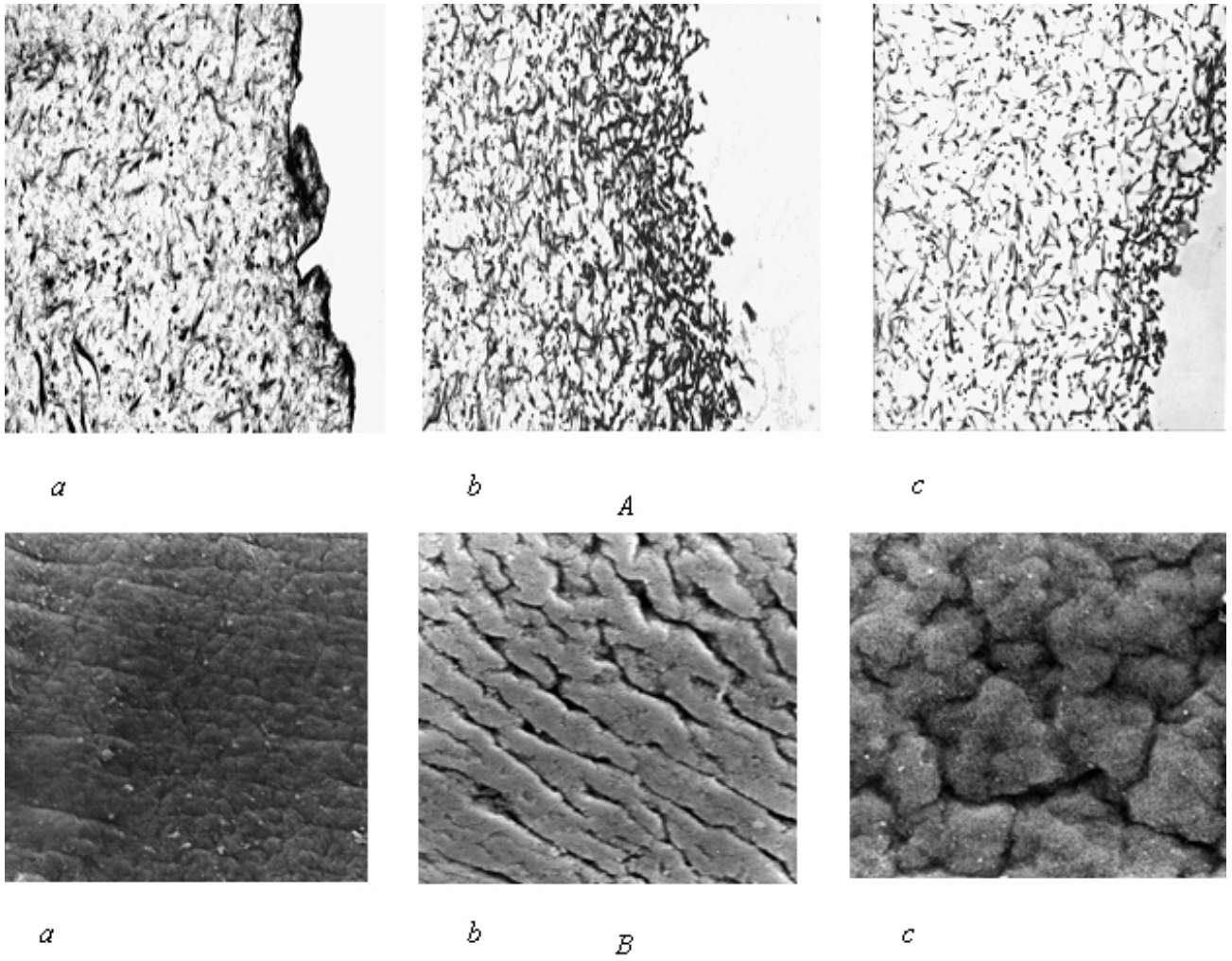

Fig.2. Electron micrographs of the hydrolysis with plasmin of the desAABB-fibrin clot with the surface layer at the enlargement in 35000 (A) and 7000 (B) times: a – 0 min of hydrolysis; b – 30 min of hydrolysis; c – 60 min of hydrolysis.

into the fibrin clot towards its surface. The evidence to it is electron micrographs on which there are fibrils, built into the surface layer, and it is evident that they are separated into thinner strands (protofibrils).

The difference in the thickness of the surface layer and fibrils inside clots, caused by the peptides removing (desAA-or desAABB-fibrin) from fibrinogen molecule, may be connected with the participation of the second pair of polymerization centres (D_2-E_2). The D_2-E_2 polymerization centres are known to stabilize early protofibrillar structures and provide lateral association to a great degree [15-17]. Consequently, this increases protofibrils affinity to each other which results in thickening of formed fibrils. The absence of such centres in desAA-fibrin results in thinning of fibrils, because of the decrease in protofibrils affinity to each other which is proved by the obtained data. The extension of fibrils is seen in the clot, which associate poorly laterally with each other due to the affinity decrease, this results in the appearance of a smaller quantity of protofibrillar structures, built into the surface layer. The consequence of this is the surface layer thinning in the desAA-clots in comparison with the desAABB-fibrin clots.

The obtained results suggest that such a peculiarity of the fibrin clot structure as presence or absence of the surface layer may change the interaction of the fibrin clot with the components of fibrinolytic system. To check this assumption, there was electron microscopy research of the process of destroying with plasmin those fibrin clots which have different surface structure. The analysis of the hydrolysis with plasmin of those desAABB-fibrin clots, which have the surface layer (Fig.2, A), showed that at first the clot structure undergoes changes which are analogous to the abovementioned ones. The surface layer disappears in 30 minutes of hydrolysis and the fibrin clot structure

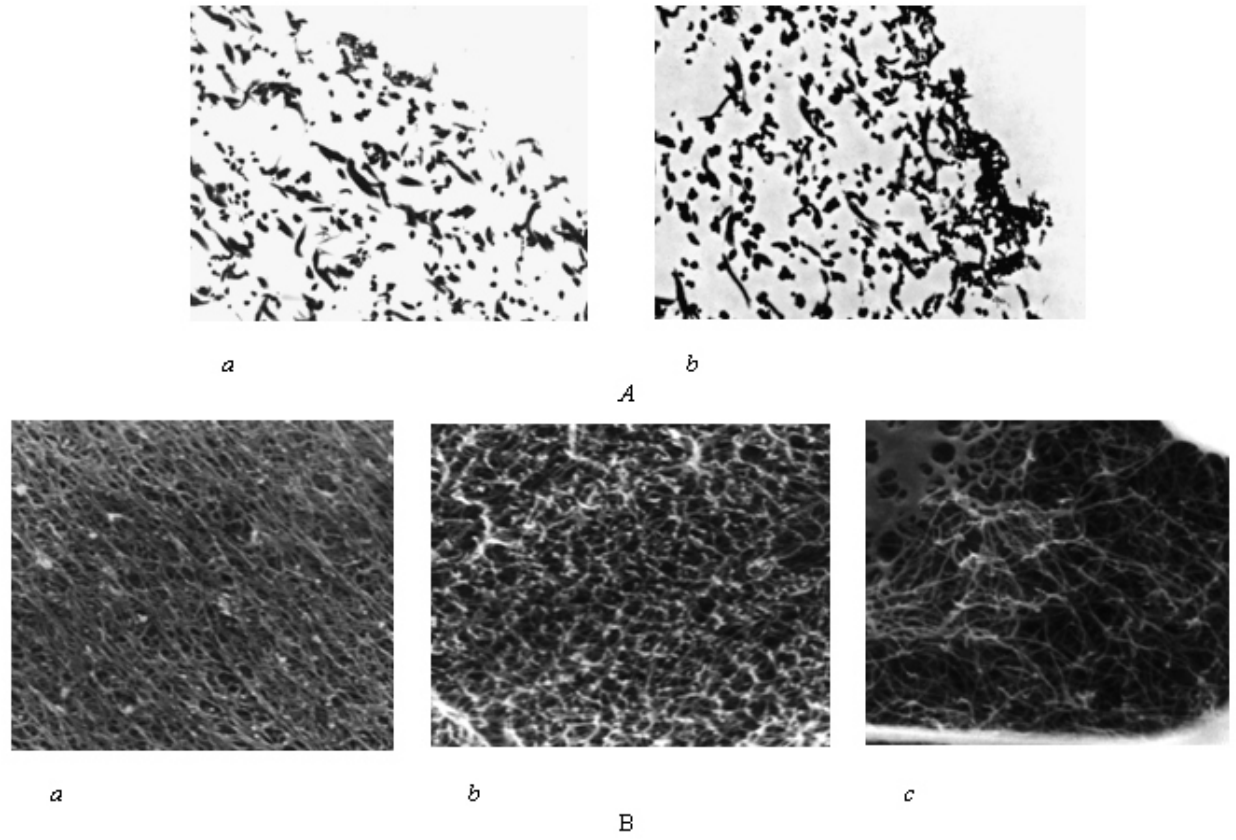

Fig.3. Electron micrographs of the hydrolysis with plasmin of the desAABB-fibrin clot without the surface layer at the enlargement in 35000 (A: a – 30 and b – 60 min of hydrolysis) and 7000 times (B: a – 30 and c – 60 min of hydrolysis)

changes significantly. There is also fibrils migration towards the clot surface.

The distance between fibrils is maximum in the clot centre, and on its surface fibrils almost stick together and form a complete belt. Three structural zones are seen in the clot: the first one is ~ 345 nm (the zone of maximum fibril density); the second one is ~ 400 nm (transitional one) and the third one is the rest of the clot.

The quantity of thin fibrils decreases, they become thicker, and the distance between them increases. After 60 minutes of hydrolysis (Fig.2, A, c) the zones remain, but their size changes. The zone of maximum fibrils density amounts to ~ 142 nm, and the transitional zone - ~ 310 nm. Thin fibrils almost disappear, the thicker ones remain. The distance between them increases considerably.

The appearance of zones inside the fibrin clot may be connected with peculiarities of the polymerization process, at which the surface layer forms on the fibrin clot surface. In the process of growth and subsequent lateral association protofibrils can endure significant pressure, caused by the clot volume restriction. Thus, they are in the conformation which resembles a spring that sets against the surface layer. After the layer destruction under the influence of plasmin, pressure disappears and fibrils start migrating towards the fibrin clot surface. This process may be an additional obstacle to complete the clot lysis, when plasmin molecules are not able to penetrate deeply into the fibrin clot. Then the hydrolysis occurs from the surface which serves as one more defending mechanism that allows keeping the integrity of blood vessels at their damage for a long time due to the clot, formed in the damaged place. In its turn it promotes preventing the penetration of foreign agents into blood circulation. This assumption agrees well with the literature data, where there are data concerning deviation in the degree of clots resistance to the action of the fibrinolytic system components in damaged and intact vessels [6-9].

The scanning electron photographs show that during hydrolysis some cleft appear on the fibrin clot surface

which become more vivid with the increase of hydrolysis time (Fig.3, B).

In 60 minutes of hydrolysis the clot surface is a chaotic, sharply lined surface.

The results of hydrolysis of the desAABB-fibrin clots with plasmin, which do not have the layer on their surface, are presented in Fig.1, A, b and Fig.3, A.

At zero hydrolysis time the clot structure (Fig.1, A, b) is analogous to the abovedescribed. The fibrin clot consists of only fibrillar structures. The changes begin with the hydrolysis starting. There become fewer fibrils, the distance between them increases. It is very difficult to find any zones inside the clot. Then there is slight migration of fibrils towards the clot surface (30-60 minutes of hydrolysis), however, this is most likely the fragments liberation which are removed by plasmin from the clots into the surrounding solution. There is no evident fibrils thickening during the hydrolysis as it takes place at clots hydrolysis which contain the surface layer. The absence of any zones in the fibrin clot is connected with the fact that in the process of growth and lateral association protofibrils do not have any restrictions of growth and formation of the fibrillar structure which is characteristic of the clot with the surface layer. Due to the absence of tension, caused by the surface layer, the fibrillar structures in the clot are distributed rather evenly, and during hydrolysis they do not migrate towards the void which appeared because of the destruction of the fibrin clot surface with plasmin.

At the same time the absence of steric obstacle (the surface layer) may result in quick liberation of the products of the fibrin clot hydrolysis with plasmin from outwards which, subsequently, will promote quick elimination of the fibrin clots with such a structure from blood circulation. On the one hand it is important for the prevention of vessels clotting, and, on the other hand, the fibrin clots without the surface layer may not take part in reparation processes connected with the damage of the inner vessel structure, as due to their structure peculiarities they are not able to prevent the penetration of foreign agents into blood circulation for a long time.

The fibrin clots without the surface layer (Fig. 3, B) are an accumulation of fibrillar structures, the quantity and branching of which do not decrease with the hydrolysis time. The picture, that can be seen, does not correspond to the changes which occur with the surface of the fibrin clots, which have the surface layer.

Conclusion. On the basis of the presented data the conclusion can be made that at any way of forming fibrin gel on its surface, which contacts with interphase, there forms a special structure which is different from the structure of the clot itself. This structure is presented by a thin surface layer. It is formed from fibrin strands which untwist into thinner fibres and protofibrils. It can be assumed that in blood circulation on the fibrin clot surface which was formed in the undamaged vessel, the surface layer is absent as there are no heterogeneous phases division surfaces. In the damaged vessels blood out of bloodstream coagulates. At blood clotting on the interphase with the air there forms a surface layer which prevents further hemorrhage and penetration of different microorganisms and viruses into the place of hemorrhage. The fibrin clot, formed only of thick fibrin fibres, can not provide such defense due to a significant distance between fibrils in the clot. The surface layer in the fibrin clot is the first mechanic hindrance on the way of possible infection of the organism, thus, together with the fibrin strands it serves as the first link in the chain of the organism defense mechanism at the damage of blood vessels.

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Исследование свойств сгустков, сформированных с дезАА- и дезААВВ-фибрина, с различной структурой поверхности

Резюме

Показано, что на поверхности фибриновых сгустков, формирующихся на границе раздела двух фаз при условии исключения взаимопроникновения одной фазы в другую, образуется структура (поверхностный слой), отличающаяся от таковой сгустка. При отсутствии границы раздела двух фаз формирования указанного слоя в фибриновом сгустке не наблюдается. Сделано предположение, что возникновение данной структуры связано с нарушением процесса латеральной ассоциации фибриллярных структур в фибриновом сгустке, происходящем на границе раздела фаз. Поверхностный слой образован протофибриллярными структурами, распластывающимися на границе раздела и формирующими плотную неупорядоченную структуру, которая и наблюдается на электронномикроскопических фотографиях. Обнаруженные особенности структуры фибриновых сгустков, связанные с условиями их формирования (наличие или отсутствие поверхностного слоя), определяют характер протекания процессов взаимодействия сгустков с компонентами фибринолитической системы, что может лежать в основе различий в степени устойчивости фибриновых сгустков в кровеносном русле.

Ключевые слова: фибриноген, фибриновый сгусток, электронная микроскопия.

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УДК 577.112.7+612.115

Надійшла до редакції 17.05.04