CELLULAR RECEPTORS FOR ACTIVE BLOOD CLOTTING FACTORS

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The writers' recent investigations have shown that thrombin and factor Xa, Stuart-Prower factor, are not only enzymes for hemostatic reactions. They also belong to the class of biological catalysts and can influence the functional state of cells in the blood stream (erythrocytes, lymphocytes, monocytes), the stroma (fibroblasts) and the structural elements of parenchymatous organs [3].

The object of this investigation was to examine the surface of fibroblasts for receptors for active blood clotting factors and to study them.

EXPERIMENTAL METHOD

The following materials were used.

1. Human thrombin (Central Blood Transfusion Institute), additionally purified by gel-filtration on Sephadex G-100 [1].

2. Factor Xa, obtained from human blood serum by ion-exchange chromatography on DEAE-Sephadex A-50, using a stepwise phosphate buffer elution gradient [5]. The resulting preparation of factor Xa was degraded by BAMe (Reanal, Hungary), and prothrombin — factor II was converted into thrombin — factor IIa in a system of factor II + factor Xa + $CaCl_2$. The factors IIa and Xa used were homogeneous in polyacrylamide gel.

3. A primary culture of fibroblasts, obtained from human embryos [11]. The cells were cultured for 3 days in medium 199 with antibiotics (penicillin 100 U/ml, streptomycin 200 U/ml) and 10% serum. The growth medium was then replaced by a serum-free medium. After 4 days the incubation medium was poured off, sedimenting cells were removed by centrifugation, and the medium was used for affinity chromatography in order to detect compounds with affinity for factors IIa and Xa.

To discover sites on the cells binding thrombin and factor Xa the following methods were used.

1. The fluorescent antibodies method, based on Coons' [8] method, with indirect immunofluorescence staining [2]. The principle of the experiments was that fibroblasts, incubated beforehand with factor Xa, were treated with antiserum against factor Xa obtained by immunization of rabbits. The location of the bound factor Xa was determined by the immunologic reaction between antibody against factor Xa and fluorescent serum against rabbit globulin (N. F. Gamaleya Research Institute of Epidemiology and Microbiology, Academy of Medical Sciences of the USSR). Parallel controls of immunologic specificity and reactions of labeled and normal sera were set up [2]. The preparations obtained were tested in UV light in a luminescence microscope.

2. Adsorption of cells on enzymes immobilized in the solid phase. The method of lymphocyte selection for immobilized antigens [10] was used as the basis. For this purpose factor IIa (3 mg/ml and factor Xa (1 mg/ml), dissolved in physiological saline, were applied to the polystyrene surface of Petri dishes. After immobilization of the enzymes, 8 ml of Hanks' solution with fibroblasts was poured into each dish. After 2 h the unfixed cells were washed off and counted. The degree of adsorption of fibroblasts on the immobilized enzymes was judged from the difference between the number of cells before and after incubation.

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TABLE 1. Results of Adsorption of Fibroblasts on Polystyrene Surface with Immobilized Thrombin

Experimental conditions	Number of unfixed cells in 1 ml	Number of adsorbed cells, %
Experiment	75 000	47
Control 1	105 000	32
Control 2	117 000	24

Legend. Experiment – polystyrene surface covered with active thrombin. Control 1 – surface covered with inactivated thrombin. Control 2 – surface without enzyme.

The polystyrene surface without enzymes, with immobilized enzymes but inactivated by heating for 30 min to 56°C, and with fibroblasts treated beforehand with the enzymes, were used as the control.

3. The method of affinity chromatography in the modified version [9]. For this purpose, serum-free medium after growth of a primary culture of fibroblasts (20 ml) for 4 days was passed through chromatography columns (10 × 100 mm) filled with factor IIa–Sepharose (one column) and factor Xa–Sepharose (second column). Enzymes were immobilized with Sepharose 4B, activated by cyanogen bromide, by the method in [6]. Compounds with affinity for thrombin and factor Xa were desorbed with HCl-glycine buffer, pH 2.4. The eluates were at once neutralized with 1M K_2HPO_4, dialyzed in the cold against physiological saline, and tested for possible antienzymic activity in plasma and in a pure system of proteins.

The original growth medium taken before contact with cells, passed through immobilized enzymes in the same way as the experimental samples, was used as a control; the antienzymic activity of the desorbed compounds was determined.

EXPERIMENTAL RESULTS

The use of the indirect fluorescence staining method revealed binding of factor Xa on the surface of the fibroblasts (Fig. 1). The specificity of the observed reaction was confirmed by experiments with control preparations, performed in several versions. Other workers also have shown that adsorption of thrombin on the surface of fibroblasts is possible [7].

In our view, the leading role in contact between cells and enzymes is played by active sites of the hemostasis factors. To prove this, we inactivated the catalytic centers of the enzymes by heating them for 30 min to 56°C, and then determined the degree of their adsorption on the cells. For this purpose we used the method of selection of fibroblasts on polystyrene dishes with immobilized thrombin and factor Xa (Tables 1 and 2).

According to the results, selection of fibroblasts on a surface covered with inactivated enzymes was at a much lower level than on the active enzymes. Consequently, the catalytic centers of thrombin and factor Xa play a leading role in the response of the cells to these blood clotting factors. Cells incubated beforehand with factor Xa were adsorbed to a lesser degree on the immobilized enzyme. This general pattern, in our view, can be explained on the grounds that some of the receptor sites were blocked by factor Xa.

In the last series of experiments an attempt was made to distinguish the structures of the cell surface with which factors IIa and Xa interact. The basis for identification of the receptors was a paper [7] which showed that cell surface receptors for thrombin are mobile and they can be found in fibroblast growth medium. As they diffuse into the pericellular space, the thrombin-binding structures preserve their activity and can inhibit thrombin through a mechanism of action of antithrombin III. Considering the feature of the cell receptors mentioned above, it was decided to identify compounds with affinity for factor IIa and factor Xa. To do this, fibroblast growth medium, into which fragments of membranes with

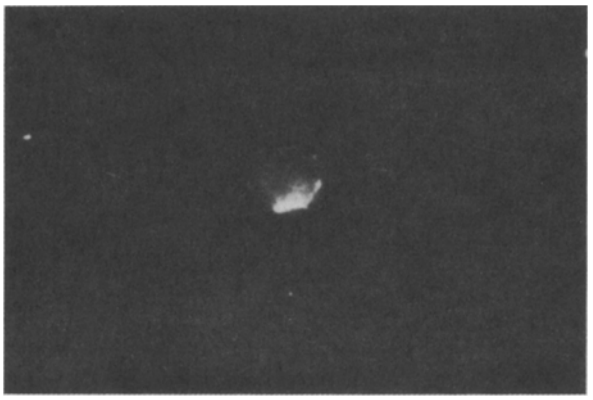

Fig. 1. Fluorescent fibroblast after incubation for 30 min with factor Xa followed by treatment with labeled antiserum against factor Xa.

TABLE 2. Results of Adsorption of Fibroblasts on Polystyrene Surface with Immobilized Factor Xa

Experimental conditions	Number of cells in 1 ml of added solution	Number of cells in 1 ml of washings	Number of adsorbed cells, %
Experiment	335 000	95 000	71
Control 1	245 000	119 000	52
Control 2	6 670	6 350	5

Legend. Experiment – adsorption of cells on polystyrene surface covered with active factor Xa. Control 1 – fibroblasts treated beforehand with factor Xa. Control 2 – adsorption of cells on surface with immbolized inactivated enzyme.

the hypothetical receptors for thrombin and factor Xa pass in the course of the metabolic activity of the cells, was passed through enzymes immobilized to Sepharose 4B: thrombin in one column and factor Xa in the other.

Compounds desorbed from factor IIa-Sepharose lengthened the plasma thrombin time fourfold [4] in a system of 0.1 ml plasma + 0.2 ml mixture (0.1 ml thrombin + 0.1 ml eluate), after preliminary incubation of the thrombin with the column eluate for 40 min. Proteins desorbed from factor Xa-Sepharose lengthened the plasma clotting time by 59% in a system of 0.1 ml plasma + 0.1 ml factor Xa + 0.1 ml eluate + 0.1 ml $CaCl_2$. The effect of inactivation of factor Xa also was detected in a pure system of protein: 0.1 ml factor Xa + 0.1 ml eluate + 0.2 ml factor II + 0.1 ml hemolysate + 0.1 ml 1% $CaCl_2$ (incubation for 5 min) + fibrinogen. In this version of the experiment the fibrin formation time was 180% longer than in the control. Column eluates of growth medium, taken before incubation of the cells, gave no such effect.

The results of affinity chromatography indicate that fibroblast growth medium contains cell fragments with affinity for factor IIa and factor Xa. They are able to inhibit the activity of these enzymes. It can be tentatively suggested that proteins eluted from factor IIa-Sepharose and factor Xa-Sepharose are one and the same compound, for the immobilized enzymes have a uniform catalytic center.

The results given above thus confirm that enzyme-binding sites for thrombin and factor Xa are present on the cell surface. These structures react selectively with the catalytic centers of the enzymes studied. Cellular receptors for factors IIa and Xa possess anticoagulant properties and, since they are mobile membrane structures, they can be secreted into the pericellular space.

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