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ANTI-CLOTTING ACTIVITY OF ENDOTHELIAL CELL CULTURES AND HEPARAN SULFATE PROTEOGLYCANS

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SUMMARY: Endothelial cell culture medium inhibits the thromboplastin-stimulated conversion of prothrombin to thrombin and induces a lengthening of the clotting time which is concentration dependent. Antibodies to the heparan sulfate proteoglycans synthesized by this endothelial cell line abolish the inhibitory effect on clotting. Our results suggest that heparan sulfate proteoglycans may be the cell products that impart the property of blood-compatibility to the endothelial cell surface.

The electrophoretic spectrum of the sulfated mucopolysaccharides synthesized by endothelial cell cultures is quite different from that of a subendothelial cell line with respect to both the type of chains synthesized and their relative proportions (1). Although both cell types release heparan sulfate proteoglycans into the culture medium, the endothelial cells secrete a large number of species that differ widely with respect to their electrophoretic mobility on a polyacrylamide gel whereas only one species is released by the other cell line (2). In addition, the heparan sulfate proteoglycans of these two cell types can be distinguished on the basis of their immunological properties (2). The purpose of these studies was to determine whether a correlation could be established between the type of proteoglycans synthesized and a specialized function of the cells of the vessel wall. We report here that antibodies to heparan sulfate proteoglycans isolated from the medium of an endothelial cell line neutralize the inhibitory effect on clotting which is characteristic of these cultures. Thus, endothelial cell proteoglycans appear to be functionally different from those synthesized by other cells of the vessel wall that do not inhibit clot formation.

METHODS: The two cell lines used for these studies, a subendothelial cell line and an endothelial cell line, were grown in F-12 medium supplemented with 10%

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fetal bovine serum as previously described (1). Conditioned medium was obtained from confluent cultures (60 x 15 mm dish, 4 ml of supernatant growth medium) after 24 hours of incubation.

To study the interaction between the goat antiserum and the heparan sulfate proteoglycans present in the endothelial cell conditioned medium, cultures were labeled with carrier-free [^{35}S]sulfuric acid (50 μCi/ml) for 24 hr. Aliquots of 1 ml (5 x 10^5 cpm as acid-insoluble radioactivity) were mixed with the antiserum under conditions of antibody excess. After the addition of the second antibody, the precipitate was collected by centrifugation, redissolved in sample buffer (10 mM Tris-HCl, pH 8.8; 5% sodium dodecyl sulfate (SDS); and 5% β-mercaptoethanol) and boiled for 2 minutes. Aliquots of 10 μl/well were applied to a 2 mm thick, 2.9-20% gradient polyacrylamide gel. A discontinuous buffer system (3) with no stacking gel was used and the electrode buffer was made 0.1% with respect to thioglycolic acid. The samples were electrophoresed for 4 hours at a constant voltage of 20 volts/cm. After electrophoresis, the gel was dried and exposed to XR-5 X-ray film (Kodak).

The anti-clotting activity of endothelial cell cultures was assayed by adding 0.2 ml of fresh citrated rabbit plasma to post-confluent cultures containing 3 ml of conditioned medium; pre-immune or immune goat serum (0.2 ml) was added to the conditioned medium 5 minutes prior to the addition of rabbit plasma. When only culture medium (conditioned or non-conditioned) was used, the incubation mixture was (in order of addition): medium, 0.45 ml; goat serum, 5 μl (pre-immune or immune); fibrinogen, 50 μl (1% solution); $CaCl_2$, 50 μl (25 mM); and thromboplastin, 5 μl (Sigma). In these and in the other experiments outlined below, the addition of thromboplastin was sufficient to initiate clotting; apparently, the other coagulation factors were supplied by the fetal bovine serum used to supplement the culture medium. To determine the effect of conditioned medium on the conversion of radioiodinated (4) prothrombin to thrombin 5 μl of thromboplastin were added to an incubation mixture containing 0.45 ml of conditioned medium, 50 μl of $CaCl_2$ (25 mM) and 5 μl of [^{125}I]prothrombin (1 unit/ml; 10 Ci/unit). The assay for the neutralization of activated factor X by conditioned medium was carried out essentially as described for the determination of plasma heparin in human patients (5) except that conditioned medium was used instead of test plasma and non-conditioned medium was used as diluent for both the experimental and standard heparin curves. The components for the assay, including heparin (169 USP K units/mg), were obtained from Sigma. All experiments were performed at room temperature.

RESULTS AND DISCUSSION: The two cell lines used for these studies differ greatly in their ability to inhibit clotting of the culture medium in the presence of added plasma. The addition of rabbit plasma to subendothelial cell cultures causes the prompt formation of large platelet aggregates (Fig. 1a) followed by the deposition of fibrin strands (Fig. 1b). This phenomenon is not observed in endothelial cell cultures 1 hour after the addition of plasma (Fig. 1c). We hypothesized that structural differences of the heparan sulfate proteoglycans may be the reason for this striking difference between the two cell lines. With the development of a goat antiserum to the heparan sulfate proteoglycans which are released into the supernatant medium by endothelial cell cultures (Fig. 2) it became possible to determine whether these compounds play a role as anti-

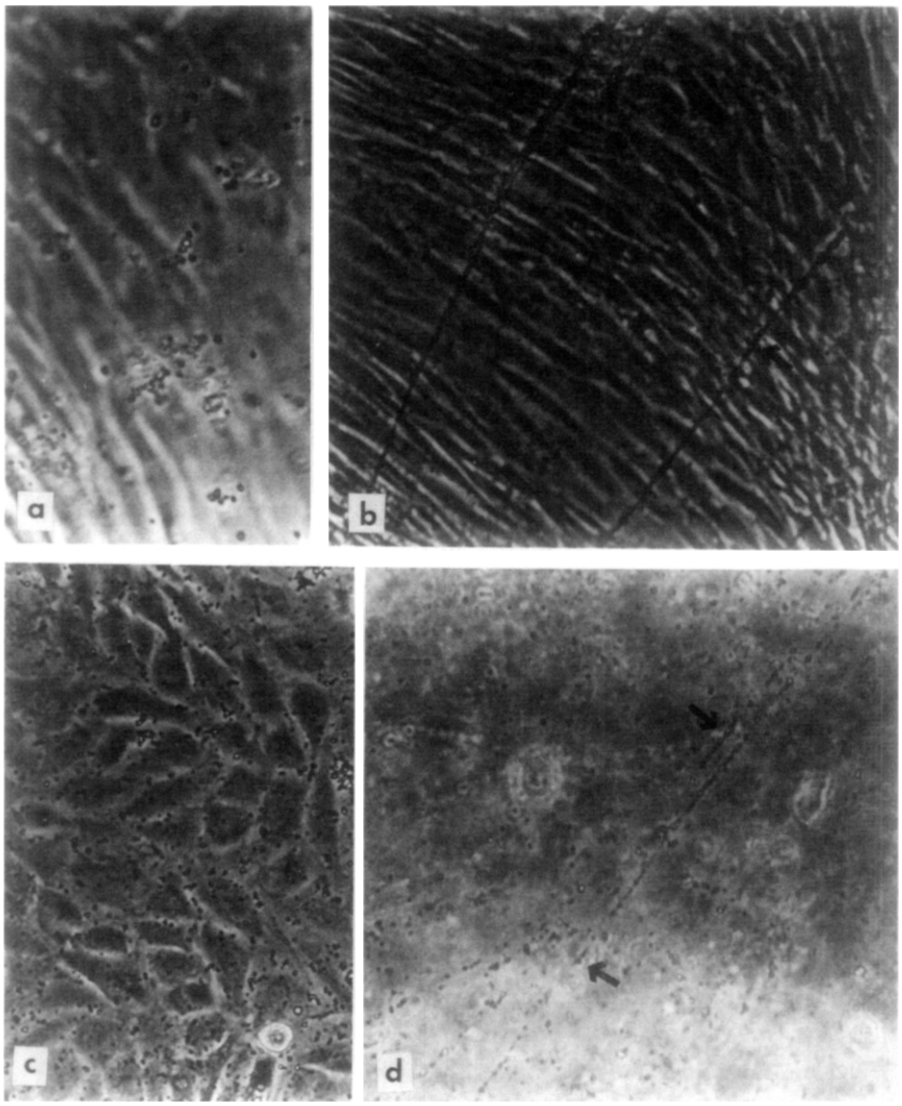

Fig. 1 Anti-clotting activity of endothelial cell cultures and its reversal by antibodies to heparan sulfate proteoglycans. a) Subendothelial cells 1 min after the addition of plasma; b) same as (a) after 15 min; c) endothelial cells 1 hr after the addition of plasma; and d) endothelial cells 15 min after the addition of immune serum and rabbit plasma. The appearance of the endothelial cell culture 1 hr after the addition of pre-immune serum and rabbit plasma was as in (c). The plane of focus is the fibrin strands (indicated by arrows) or the platelets. The visualization of the fibrin strands (which may be detected in subendothelial cell cultures as early as 5 min after the addition of plasma) is accompanied by the formation of a solid clot. Phase contrast inverted microscope, 100x.

clotting agents. We found that the addition of the antiserum to the endothelial cell culture dish allows the formation of fibrin strands (Fig. 1d) thereby abolishing the difference between the two cell lines.

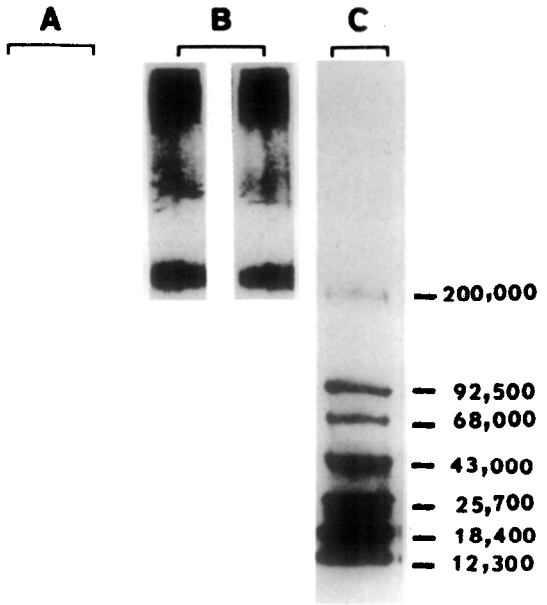

Fig. 2 Interaction of ^{35}S-labeled heparan sulfate proteoglycans with the goat antiserum. The conditioned medium from endothelial cell cultures that had been labeled with [^{35}S]sulfuric acid was mixed with the goat antiserum and the immunoprecipitate analyzed by polyacrylamide gel electrophoresis (see Methods). The autoradiography is shown. The ^{35}S-labeled mucopolysaccharide chains of the proteoglycans precipitated by the antiserum are nitrous acid degradable (2), a reaction characteristic for heparin-related mucopolysaccharides. A, pre-immune serum; B, immune serum; C, ^{14}C-labeled protein molecular weight markers.

We carried out a series of studies to determine at which point in the clotting process the inhibitor present in endothelial cell cultures may be active. We measured the level of thrombin activity present under various conditions by determining the clotting time. Thus, when the experiment shown in Fig. 1 was repeated using only medium in the presence of fibrinogen and thromboplastin, clotting times were as follows: non-conditioned medium, 5 min; subendothelial cell conditioned medium, 5.5 min; endothelial cell conditioned medium, 25 min. In the presence of 5 µl of antiserum, the clotting time was reduced to 10 min. The addition of 5 µl of pre-immune serum to conditioned endothelial cell culture medium reduced the clotting time by only 5%. No decrease in anti-clotting activity was observed when the conditioned medium was tested after it had been pre-incubated for 30 min at 37°C. The following evidence confirms that in our system, as in the accepted coagulation scheme,

the formation of a clot requires the catalytic conversion of prothrombin to thrombin and suggests that thrombin is not the enzyme which is directly inhibited by the endothelial cell conditioned medium: i) in the presence of non-conditioned medium supplemented with fibrinogen, thromboplastin (a tissue factor that participates in the process that results in the conversion of the proenzyme to the active enzyme) induces prompt formation of a clot; in the absence of thromboplastin, no clotting takes place, ii) no clotting is observed in the presence of hirudin, an inhibitor of thrombin, and iii) clotting is not inhibited when prothrombin is activated to thrombin before the addition of endothelial cell conditioned medium.

In other studies we found that the thromboplastin-dependent conversion of prothrombin to thrombin is inhibited in the presence of endothelial cell conditioned medium. As shown in Fig. 3, the electrophoretic mobility of [^{125}I]prothrombin appears to be unchanged after 5 minutes of incubation in this medium; in contrast, the presence of subendothelial cell conditioned medium in the incubation mixture does not prevent the rapid disappearance of the electrophoretic band attributable to the proenzyme. The experiment summarized in Fig. 4 suggests that the endothelial cell inhibitor affects activated factor X (a serine protease which converts prothrombin to thrombin) since the addition of increasing concentrations of conditioned medium causes the neutralization of increasing amounts of this enzyme as measured by the lengthening of the clotting time. Comparison with a standard heparin curve (5) showed that one ml of conditioned medium contained an amount of neutralizing activity equal to 0.21 unit of heparin. Our experiments do not exclude the possibility that the endothelial cell proteoglycans may interact with prothrombin but suggest that an important mechanism through which these endothelial cell products inhibit clotting is by neutralizing activated factor X. According to the classical coagulation scheme, the neutralization of this enzyme is accomplished by its inhibitor, antithrombin III (6). In the presence of heparin the rate of neutralization of activated factor X is greatly enhanced (7). If this model also applies to our system, the role of heparan sulfate proteoglycans could be

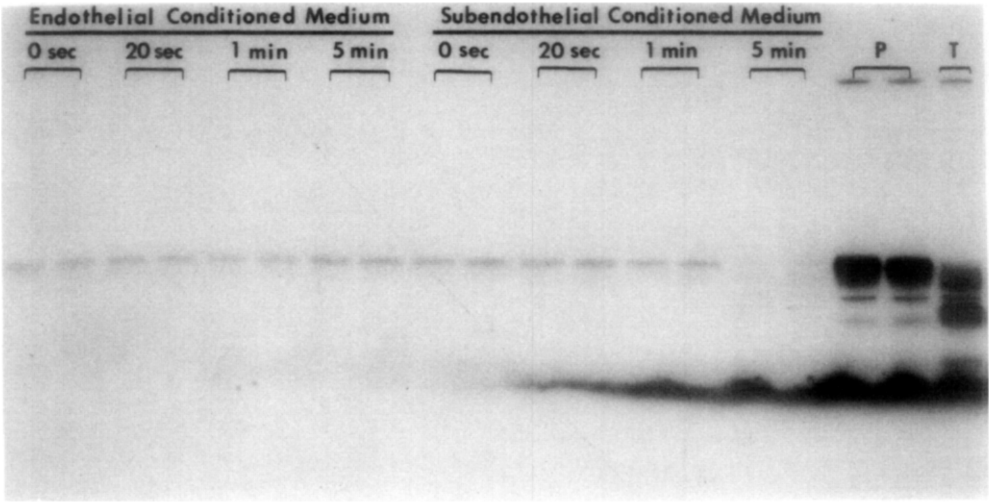

Fig. 3 Inhibition of conversion of [^{125}I]prothrombin to thrombin by endothelial cell conditioned medium. Conditioned medium from either endothelial or subendothelial cell cultures was added to reaction mixtures containing $CaCl_2$ and [^{125}I]prothrombin as specified in Methods. The reaction was initiated by the addition of thromboplastin. Aliquots of 50 µl of incubation mixture were removed at the times indicated in the figure and analyzed by polyacrylamide gel electrophoresis. The autoradiograph is shown. Endothelial Conditioned Medium: samples obtained from a tube containing conditioned endothelial cell medium in the reaction mixture. Subendothelial Conditioned Medium: samples obtained from a tube containing conditioned subendothelial cell medium in the reaction mixture. P: [^{125}I]prothrombin alone; T: [^{125}I]prothrombin incubated with activated factor X.

that of accelerating the rate of inactivation of activated factor X by its inhibitor. Since more than one proteoglycan species is found in the conditioned medium, one could expect that these endothelial cell products may also inhibit (perhaps even more efficiently) some other step of the thromboplastin-dependent pathway prior to the activation of factor X.

Sulfated mucopolysaccharides covalently bound to a protein core (sulfated proteoglycans) have been shown to be present in various tissues and at the surface of a number of cell types. The biological significance of these compounds is still unclear. As a result of our work it appears that definite functional differences exist between the heparan sulfate proteoglycans synthesized by the two cell types used for these studies.

The observation that rabbit endothelial cells under culture conditions secrete sulfated proteoglycans into the supernatant growth medium prompted the

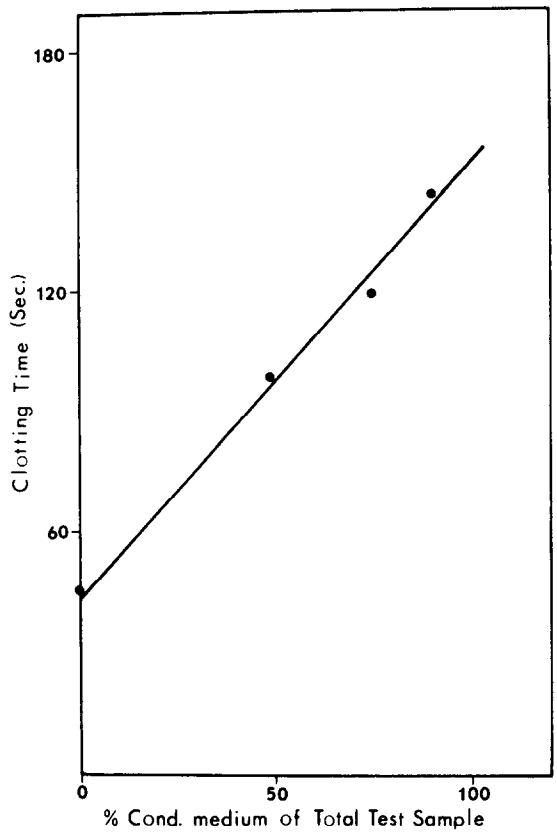

Fig. 4 Neutralization of activated factor X by endothelial cell culture conditioned medium. A two-stage assay (5) was used with minor modifications as outlined in Methods. The amount of active activated factor X remaining in the reaction mixture was determined by measuring the clotting time.

suggestion that by releasing these compounds into the blood stream the endothelium may contribute to the regulation of certain blood homeostatic mechanisms (8). In the light of our results, it is reasonable to postulate that, if the endothelium releases into the blood stream sulfated proteoglycans, these compounds may participate in the control of some enzymatic systems either locally or at distance. The availability of the antiserum will allow us to ascertain whether heparan sulfate proteoglycans of endothelial origin are actually present in the circulation. Also, suggestions have been made that the heparan sulfate proteoglycans present at the surface of the endothelial cells may represent a binding site for certain proteins or enzymes that regulate exchanges between blood and tissues (9,10,11). If the surface proteoglycans function in a manner similar to that of the proteoglycans released into the

culture medium, namely, if they also bind certain enzymes which participate in the coagulation process, with these studies we may have obtained some information on the biochemical mechanism underlying the "blood-compatibility" of the endothelial cell surface.

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