Immunologic Identification of Tissue Factor (Thromboplastin) Synthesized by Cultured Fibroblasts

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Immunologic methods were employed in an attempt to identify a potent procoagulant present in homogenates of human skin fibroblasts cultured in vitro. The activity of this procoagulant was restricted to the early stages of coagulation and was heretofore considered to be due to tissue factor (tissue thromboplastin, factor III) either alone or in combination with one or more of the first-stage coagulation factors (VIII, IX, XI, XII). The present studies demonstrated that procoagulant activity was not diminished by incubation with anti-VIII or anti-IX antibodies of human origin or with anti-VIII antibody of rabbit origin. Furthermore, cell culture homogenates failed to bind anti-factor VIII antibody and did not contain an inhibitor of the reaction between factor VIII and its antibody. By contrast, procoagulant activity was obliterated by an antibody to tissue factor protein regardless of whether plasmas deficient in factor VIII, IX, XI, or XII were used in the assay system. The antitissue factor antibody failed to block the procoagulant effect after tissue factor had complexed factor VII. The procoagulant, therefore, consisted entirely of tissue factor.

A POTENT PROCOAGULANT is synthesized by cultured fibroblasts.[1,2] Activity of this procoagulant rises dramatically during the first 12–24 hr of incubation of trypsinized fibroblasts in fresh medium, as judged in assays on freeze-thaw cell lysates employing plasmas deficient in the four first-stage coagulation factors (VIII, IX, XI, XII). The activity of this procoagulant is restricted to the first stage of coagulation.[2]

These findings are subject to three possible interpretations: (1) tissue factor (thromboplastin, factor III), which causes rapid clotting in plasmas deficient in these factors, might account entirely for the results; (2) several first-stage factors might be present; or (3) tissue factor might exist in combination with one or more other specific factors within cultured cells.

We have previously shown that homogenates of cultured fibroblasts are capable of binding factor VII, thus demonstrating indirectly the presence of tissue factor.[2] Green et al.[3] suggested that this procoagulant was tissue factor, since factor VII was required for its expression. However, the presence of

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additional coagulation factors was not excluded. The dissimilarity of activity observed in assays using different factor-deficient plasmas (particularly factor VIII- and factor IX-deficient plasma) led us originally to conclude tentatively that one or more specific factors (such as factor VIII) might be present in the test material.[1]

Incubation of cell homogenates with sera from patients with acquired anti-VIII antibodies did not reduce procoagulant activity.[1,3] It is possible, however, that only a portion of factor VIII activity was susceptible to neutralization by antibody because of its spatial orientation within cell particles. Alternatively, antifactor VIII antibody might bind to factor VIII sites on cell particles, but effective neutralization of procoagulant activity could be negligbile because of coexisting tissue factor. Furthermore, the cell homogenate might contain an inhibitor of the reaction between factor VIII and its antibody.

To explore these alternatives, the ability of cell homogenate to bind anti-factor VIII antibody or to inhibit the reaction between factor VIII and its antibody was assessed. In addition, the effect of an antibody to tissue factor protein on procoagulant activity was determined. This antitissue factor anti body specifically inhibits factor X activation by tissue factor, provided complex formation with factor VII has not taken place (Yale Nemerson, personal communication). The antitissue factor antibody would permit definite identification of tissue factor and also possible associated coagulation factors. Residual activity after tissue factor inhibition in assays using plasmas deficient in factors VIII, IX, XI, or XII would signify that a procoagulant other than tissue factor was present in the homogenate.

MATERIALS AND METHODS

Citrated plasmas from patients with congenital deficiencies of factors VII, IX, XI, and XII were provided by Dr. Anthony F. H. Britten and were maintained frozen until used. Stock rabbit brain thromboplastin was obtained from Ortho Pharmaceutical Co. Celite-inosithin was prepared according to the method of Simon et al.[4] Details of preparation of imidazole-buffered saline, tissue culture technique, and the one-stage assays employing factor-deficient plasmas based on the activated partial thromboplastin time were described previously.[1]

Antibody Neutralization Experiments

Antibody to factor VIII was obtained from a patient who developed this anticoagulant after a penicillin reaction. The properties of this IgG antibody have been reported.[5] Antibody to factor IX (also and IgG) was obtained from a patient with severe Christmas disease who developed an anticoagulant. This antibody has been characterized by George et al.[6] Rabbit antihuman factor VIII was prepared by the method of Zimmerman et al.[7] The properties of this antibody have also been previously described.[8] Each of the antibodies was adsorbed with calcium phosphate (10 ml calcium triphosphate/ml of serum for 10 min at 25°C) and then was heated to 56°C for 30 min. The ability of antibody to reduce coagulation factor activity, as judged by one-stage assays with plasmas deficient in factors VIII or IX, was assessed by incubating equal volumes of fibroblast homogenate and dilutions of the antibody for 1 hr at 37°C.[9] Antibody binding by fibroblast homogenate was sought by incubating 0.5 ml of test material with 0.05 ml of a suitable dilution of antibody for 1 hr at 37°C and for 18 hr at 4°C. Since the procoagulant activity resides in the particulate portion of the fibroblast homogenate, the mixture was then centrifuged at 48,000 *g* for 30 min at 4°C, and the residual antibody in the supernatant was deter-

mined by incubating 0.2 ml with 0.2 ml of normal human plasma for 1 hr at 37°C. The antibody in U/ml is calculated from the factor VIII activity remaining after the incubation. One unit is defined as that amount of antibody that inactivates 75% of the factor VIII activity in an equal volume of normal human plasma (pooled standard) when the two are incubated together for 1 hr at 37°C. The human antifactor VIII antibody had 185 U/ml activity, the rabbit antifactor VIII antibody had 112 U/ml activity, and the human antifactor IX antibody had 18 U/ml activity.

The presence of a possible inhibitor of the reaction between factor VIII and its antibody was sought as follows: 1.0 ml of normal plasma was mixed with 0.5 ml of saline or fibroblast homogenate (frozen and thawed once in saline). In separate tubes, particulate material from 1 ml and 2 ml of homogenate was recovered by centrifugation at 4000 *g* for 30 min. This material was resuspended in 0.5 ml saline and was then mixed with 1.0 ml normal plasma. The resultant mixtures of normal plasma with either saline or particulate material from 0.5, 1.0, or 2.0 ml of homogenate were assayed with factor VIII-deficient plasma prior to, and 5 min and 1 hr following addition of 0.5 ml of antifactor VIII antibody. All incubations were carried out at room temperature in plastic tubes.

Preparation of Adsorbed Fetal Calf Serum

In certain of these experiments fetal calm serum was adsorbed three times with barium sulfate, 150 mg/ml, for ½ hr at room temperature prior to incorporation into the culture medium in which cells were cultivated. This was carried out to remove factor VII. The prothrombin time (0.1 ml plasma plus 0.2 ml stock thromboplastin that contains calcium chloride) of our current factor VII-deficient plasma was 34.3 sec. A mixture of equal volumes of factor VII-deficient plasma and unadsorbed (whole) fetal calf serum resulted in a prothrombin time of 26.0 sec. The prothrombin time of a mixture of equal volumes of factor VII-deficient plasma and adsorbed fetal calf serum was 34.9 sec. Thus the adsorbed fetal calf serum was considered to be devoid of factor VII.

Preparation of Antitissue Factor Antibody

Rabbit antitissue factor antibody was generously provided by Yale Nemerson. This serum and a sample of normal rabbit serum was adsorbed three times with 20 mg barium sulfate/ml for ½ at room temperature. The adsorbed sera were then heated to 56°C for 15 min. The antitissue factor antibody was assessed for its ability to inhibit the tissue factor activity of a saline suspension of acetone-extracted human brain (a fresh specimen obtained at autopsy). Equal volumes of human brain tissue factor were mixed with imidazole-buffered saline or various dilutions of control and antitissue factor serum in imidazole-buffered saline. Mixtures were incubated at room temperature for 1 hr. One-tenth milliliter of each mixture was added to 0.1 ml of normal plasma, then 0.1 ml of 0.02 *M* $CaCl_2$ was added, and the clotting time was determined. The clotting times were 25.6 and 25.8 sec in tests in which human brain tissue factor was incubated with saline or dilutions of normal rabbit serum as low as 1:4. By contrast, the clotting times observed in the mixture of human brain tissue factor with dilutions of antitissue factor rabbit serum of 1:4, 1:8, and 1:16 were 49.8, 49.0, and 49.5 sec, respectively. Thus, the rabbit antitissue factor antiserum (but not the normal rabbit serum) contained a potent inhibitor of human brain tissue factor. Both normal and antitissue factor rabbit sera were, therefore, tested in a dilution of 1:8 in imidazole-buffered saline in mixtures with cell culture homogenates.

Preparation of Cell Cultures

Trypsinized human foreskin fibroblasts were cultured in medium containing 20% whole (unadsorbed) or adsorbed fetal calf serum in Eagle's Minimum Essential Medium and fresh glutamine in a concentration of 292 mg/liter (Grant Island Biological Co). Suspensions of 200,000 cells/ml of medium were delivered in 5-ml aliquots to small, disposable tissue culture flasks (Falcon) and were incubated at 37°C. In an additional series of flasks in which cells were cultured in medium containing whole fetal calf serum, the monolayers

were washed three times with 5 ml of medium containing adsorbed fetal calf serum, and finally the monolayer was overlaid with 5 ml of medium containing adsorbed fetal calf serum. Duplicate flasks were opened at intervals of incubation. Monolayers were then mechanically scraped from the floor of the flask. Cell clumps were dispersed, and the cells were evenly distributed throughout the medium by repeated passage through a Pasteur pipet. Aliquots of cell suspensions were withdrawn from the flasks and placed into small, tightly capped glass vials for storage at −20°C prior to assay. We demonstrated in earlier experiments[10] that repeated freezing and thawing of cell homogenates resulted in reduction of activity, as judged in assays employing factor VIII- or XI-deficient plasmas. Therefore, in order to lyse the cells but preserve maximum procoagulant activity, samples were assayed after being frozen and thawed only once.

At the beginning of the experiment, and at various intervals of incubation, cell homogenates were prepared. These homogenates represented monolayers cultured and lysed in medium containing whole or adsorbed fetal calf serum or monolayers cultured in medium containing whole or adsorbed fetal calf serum but homogenized in medium containing adsorbed fetal calf serum. One-tenth milliliter of homogenate was mixed with 0.1 ml of either control or antitissue factor serum and was incubtaed 1 hr at room temperature. One-hundredth milliliter of each homogenate-serum mixture was added to 0.1 ml of plasma deficient in either factor VIII, IX, XI, or XII and 0.1 ml of celite-inosithin. After 5-min incubation at 37°C the samples were recalcified, and the clotting time was determined.

RESULTS

Experiments Employing Anti-VIII and Anti-IX Antibodies

Incubation of anti-VIII (rabbit or human origin) and anti-IX antibodies with fibroblast homogenates failed to reduce procoagulant activity as judged by assays with plasmas deficient in these factors despite the ability of these antibodies to inactivate factor activity of normal human plasma (Table 1). A more rigorous test for factor VIII antigen in the cell homogenates was also performed (Table 2). The diluted antiserum was adsorbed with fibroblast culture material, and the residual antibody titer determined. This experiment established that the cell culture material does not bind antifactor VIII antibody. However, anti-VIII antibody inhibited the factor VIII activity of normal plasma despite the presence of graded amounts of cell homogenate (Fig. 1). These data indicate that the fibroblast homogenate contained no substance immuno-

Table 1. Effect of Antibodies on Procoagulant Activity of Fibroblast Homogenates

Test Material	Antibody (Dilution)	Factor	Assay* Time (sec)	Inhibition %
Fibroblast homogenate	None	VIII	70	—
	Rabbit anti-VIII (1:10)	VIII	70	—
	Human anti-VIII (1:10)	VIII	72	—
Normal human plasma	None	VIII	84	0
	Rabbit anti-VIII (1:10)	VIII	108	97
	Human anti-VIII (1:10)	VIII	109	96
Fibroblast homogenate	None	IX	57	—
	Human anti-IX (1:25)	IX	57	—
Normal human plasma	None	IX	56	0
	Human anti-IX (1:25)	IX	68	79

*Test material, 0.2 ml, was incubated with 0.2 ml antibody dilution or with Veronal-buffered saline control for 1 hr at 37°C.

Table 2. Antibody Binding by Fibroblast Homogenate

Antibody	Fibroblast Homogenate	Residual Antifactor VIII* (U/ml) Cell-free Tissue Culture Medium	Buffer
Rabbit antifactor VIII (1:8 dilution of serum)	0.9, 1.2	1.2, 1.1	1.1, 1.0
Human antifactor VIII (1:20 dilution of serum)	1.2, 0.9	0.9, 0.8	0.9, 0.8

*Five-tenths milliliter of test material was incubated with 0.05 ml of antibody for 1 hr at 37°C and for 18 hr at 4°C. The mixture was then centrifuged at 48,000 *g* for 30 min, and antibody in the supernatant was determined by incubating 0.2 ml with 0.2 ml of normal plasma for 1 hr at 37°C.

logically identifiable as factor VIII or factor VIII or factor IX and no inhibitor of the reaction between factor VIII and its antibody.

Experiments Employing Antitissue Factor Antibody

Figure 2 illustrates results of clotting studies on homogenates of fibroblast monolayers cultured in medium containing either whole fetal calf serum, adsorbed fetal calf serum, or whole fetal calf serum that was replaced by adsorbed fetal calf serum as described above. The results illustrated are those obtained in assays with plasma congenitally deficient in factor VIII. It is apparent that somewhat less procoagulant was produced in cells cultured in medium containing adsorbed fetal calf serum. Homogenates incubated in the presence of normal rabbit serum demonstrated a rise in procoagulant activity, as previously described.[1] However, incubation of fibroblast homogenates with rabbit antitissue factor serum resulted in obliteration of the procoagulant activity of monolayers cultured in medium devoid of factor VII or monolayers in which the original medium was replaced by medium lacking factor

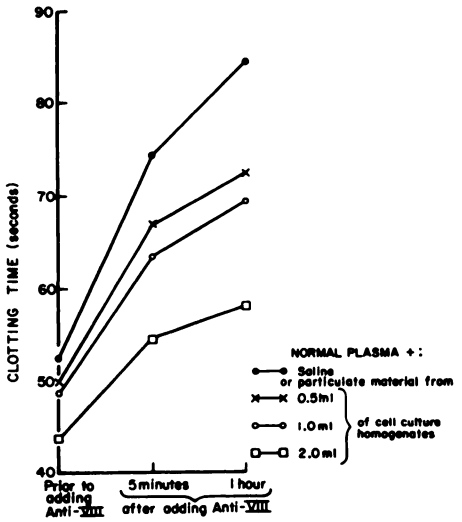

Fig. 1. Effect of various concentrations of fibroblast homogenate on interaction of factor VIII of normal plasma with antifactor VIII antibody.

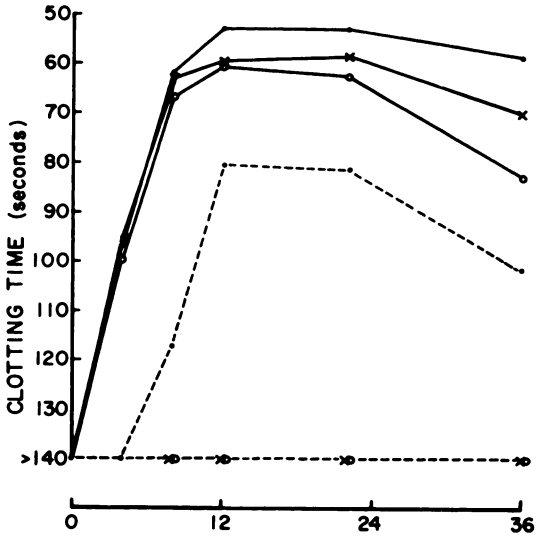

Fig. 2. Symbols connected by solid lines represent fibroblast homogenates harvested at various intervals and subsequently incubated in presence of normal rabbit serum. Symbols connected by broken lines represent homogenates similarly obtained but incubated in presence of rabbit antitissue factor serum. Black dots, cells cultured and homogenized in medium containing whole (unadsorbed) fetal calf serum; X, cells cultured in medium containing whole fetal calf serum but homogenized in medium containing adsorbed fetal calf serum; O, cells cultured and homogenized in medium containing adsorbed fetal calf serum. The clotting time is that observed in assays with factor VIII-deficient plasma (see text).

VII. Preservation of activity was observed when antitissue factor serum was incubated with cells cultured and homogenized in medium containing whole (unadsorbed) fetal calf serum, indicating that binding of factor VII had occurred prior to addition of the antiserum. Identical results were obtained when plasmas deficient in factors IX, XI, and XII were incorporated into the assay system in place of the factor VIII-deficient plasma. Thus, tissue factor appeared to account entirely for procoagulant activity observed in these assays.

DISCUSSION

Although previous studies by ourselves[2] and Green et al.[3] indicated that tissue factor is present in fibroblast homogenates, the presence of additional clotting factors was not ruled out. The relatively greater activity observed in assays with factor VIII-deficient plasma, in contrast to assays with factor IX-deficient plasma, added further credence to the hypothesis that specific factors (e.g., factor VIII) might be present.[2,3] In addition, variation in the stability of the procoagulant under the influence of heat, sonication, and freeze-thawing suggested that multiple procoagulants account for the activity observed in specific assays of first-stage coagulation factors.[10] However, the results reported here indicated that the procoagulant produced by cultured fibroblasts consists entirely of tissue factor. Activity is not neutralized by potent antibodies to circulating factors VIII or IX. The possibility that immunologically reactive factor VIII existed within these cells but that neutralizaion of activity is masked by tissue factors also present in the cells was excluded by the absence of binding of antifactor VIII antibody to the tissue culture homogenates. No inhibitor of the reaction between factor VIII and antifactor VIII

antibody was found. Furthermore, procoagulant activity observed in assays employing plasmas deficient in factors VIII, IX, XI, and XII was obliterated by antitissue factor antibody. Nemerson has shown that antitissue factor antibody specifically abolishes the procoagulant effect of tissue factor, i.e., its ability to activate factor X, provided complexing with factor VII has not occurred (personal communication). Thus, this antibody was of singular importance in identifying this procoagulant since residual activity after tissue factor inhibition might have signaled the presence of associated clotting factors. Residual activity was not found. Our data also indicated that tissue factor is capable of complexing factor VII in the serum component of the medium and therefore of exerting its procoagulant effect despite subsequent addition of antitissue factor antibody. The tissue factor of cells cultured in medium lacking factor VII or of monolayers washed free of factor VII was highly sensitive to the inhibitory effects of antitissue factor antibody.

The difference in activity noted in assays with various first-stage coagulation factor-deficient plasmas as outlined above is of great interest but is presently unexplained. Conceivably, the fibroblast tissue factor molecule might consist of subunits that interact differently with the various plasmas. Should this be the case, these results might indicate a previously unappreciated involvement of tissue factor in the intrinsic coagulation system.

The pathophysiologic significance of tissue factor observed in cultures of human skin fibroblasts is unknown. Ubiquitous connective tissue cells (fibroblasts) may perform a central role in arrest of hemorrhage since they synthesize collagen,[11] which may induce platelet aggregation[12] and factor XII activation,[13] and also provide tissue factor with which circulating factor VII may complex to initiate thrombin formation. Tissue culture methods would seem to provide a valuable tool in elucidating the role of tissue factor in hemostasis and thrombogenesis.

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