Membrane-mediated Synthesis of Tissue Factor (Thromboplastin) in Cultured Fibroblasts

By Leo R. Zacharski and O. Ross McIntyre

A potent procoagulant synthesized by cultured human skin fibroblasts has been identified as tissue factor (factor III, thromboplastin), since it binds factor VII and is blocked by a specific antitissue factor antibody. Fibroblast tissue factor is, at least in part, a labile superficial, membrane-associated substance the synthesis of which is mediated by cell adhesion but is inhibited by actinomycin-D and puromycin. Tissue factor production is related to the shape change that occurs as the cells spread on the floor of the culture vessel, but tissue factor is not synonymous with the configuration on the cell surface responsible for cell adhesion. These observations suggest that cell membranes may play a significant role in hemostasis and thrombosis by virtue of their tissue factor content.

WE HAVE DEMONSTRATED production of a potent procoagulant by a variety of cell types cultured in vitro.[1] The highest levels of procoagulant activity were observed in homogenates prepared from cultures of normal human skin fibroblasts. This procoagulant has been identified as tissue factor (thromboplastin, factor III), since fibroblast homogenates bind factor VII[2] and activity observed in assays using plasma congenitally deficient in factors VIII, IX, XI, and XII was obliterated by an antibody to tissue factor protein.[3]

Zeldis et al.[4] have recently demonstrated, by an immunohistochemical staining technique, tissue factor antigen on cell membranes in a variety of tissues. No tissue factor was detected in the cytoplasm. Our previous observations on cultured cells suggested that the clotting activity of tissue factor might also reside on the cell surface. Thus, activity was invariably low at the beginning of experiments on several cell types cultured in monolayer and rose to maximum after 12–24 hr of incubation. This rise in activity was independent of the time required for cells to double in number. Since trypsin used to separate the cells from each other and from the floor of the stock culture flasks, the possibility was entertained that initially low levels of activity reflected tryptic digestion of tissue factor on the cell surface. Activity might then be regenerated on incubation of cells in fresh medium. The present study was designed to elucidate the possible relationship between tissue factor and the surface membrane and protein synthetic capacity of cultured fibroblasts.

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MATERIALS AND HANDLING

Trypsin (0.25%) was obtained from Grand Island Biological Co.; phospholipase C *(Clostridium perfringens)*, neuraminidase *(C. perfringens)*, collagenase *(Clostridium histolyticum)*, and puromycin was obtained from Nutritional Biochemical Co.; soybean trypsin inhibitor was purchased from Sigma, and actinomycin D from Merck. Reagents were dissolved in sterile distilled water prior to incorporation into these experiments.

Details concerning procurement and preparation of the tissue culture medium and methods of cell culture and specimen sampling were described previously.[1,2] Although fibroblast tissue factor accelerates the coagulation of normal as well as factor VIII-, IX-, XI-, or XII-deficient plasma, incorporation of fibroblast homogenates in an assay based on the activated partial thromboplastin time employing factor VIII-deficient plasma (obtained from a patient with a severe classic hemophilia) proved to be a convenient means of assessing tissue factor activity and was employed in these studies as described previously.[1,2]

RESULTS

Separation of cells in potent 24-hr monolayers from each other and from the culture flask to which they were adherent by trypsinization resulted in reduction of tissue factor activity. This was demonstrated in the following experiment. The medium overlying intact 24-hr fibroblast monolayers in small, disposable plastic flasks (Falcon) was removed; the monolayers were washed with 0.5 ml of either 0.25% trypsin in saline, saline alone, Eagle's Minimum Essential Medium, or fresh tissue culture medium (Minimum Essential Medium plus 20% fetal calf serum, fresh glutamine, penicillin, and streptomycin[1,2]). This wash was immediately removed and replaced with a second 0.5 ml aliquot of the same solution. Monolayers were harvested after incubation at 37°C for 5, 30, and 60 min in the presence of the various solutions; 4.5 ml of fresh medium (the fetal calf serum of which contains excess antitrypsin activity) was then added. The tops of the plastic tissue culture flasks were then cut away with a heating element and all adherent cells were dislodged from the flask floor with a plastic spatula. Cells were evenly distributed throughout the medium by repeated passage through a Pasteur pipette, and finally an aliquot was withdrawn and frozen and thawed three times in preparation for assay. The results of a typical experiment are presented in Fig. 1. A progressive prolongation of the clotting time of factor VIII-deficient plasma was observed when cells were incubated in the presence

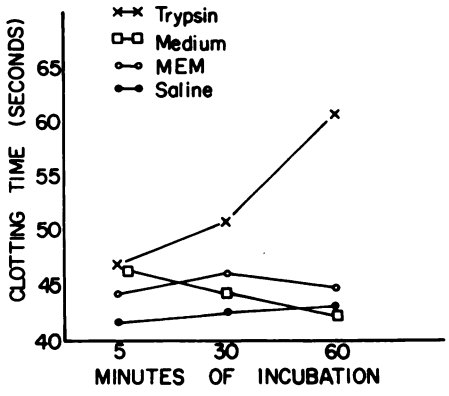

Fig. 1. Tissue factor activity of cultured fibroblasts incubated in presence of trypsin, tissue culture medium, Eagle's Minimum Essential Medium, or saline.

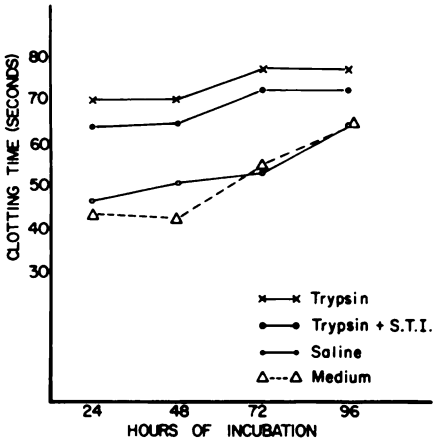

Fig. 2. Effect of trypsin, trypsin + soybean trypsin inhibitor, saline, and tissue culture medium on the tissue factor activity of fibroblast monolayers harvested at various intervals of incubation.

of trypsin in contrast to the other solutions. In an additional experiment, monolayers were harvested for assay (after 24, 48, 72, and 96 hr of incubation) in either their overlying medium or, after 20 min of incubation at 37°C, with either saline, trypsin in saline, or trypsin plus soybean trypsin inhibitor in saline as described in the previous experiment. In the trypsin-soybean trypsin inhibitor experiment, the final concentration of trypsin was 0.19% and soybean trypsin inhibitor was 0.25%. Results, presented in Fig. 2, suggest that reduction in tissue factor activity by trypsin was the result of proteolysis, since the trypsin effect was counteracted by soybean trypsin inhibitor. This conclusion must be regarded as tentative because inhibition was incomplete, and higher concentrations of soybean trypsin inhibitor markedly prolonged clotting times, presumably by direct inhibition of tissue factor.[5] It is of interest, however, that microscopic observation of monolayers revealed eventual complete separation of cells incubated in the presence of trypsin, while monolayers incubated in the presence of saline or trypsin-soybean trypsin inhibitor remained intact.

Neuraminidase also caused cell release from the flask suface. The influence of neuraminidase on tissue factor levels in cultured fibroblasts was therefore investigated. Addition of 0.5 ml of this enzyme (in a concentration of 200 μg/ml) to monolayers from which the overlying medium had been decanted produced complete separation of cells. Cells were harvested for assay after incubation with enzyme for 45 min. In contrast to the trypsin effect, the effect of neuraminidase was an 8-sec acceleration of the clotting time in comparison to that of mechanically dislodged cells. The clotting time of nonenzymatically treated, mechanically dislodged cells was 65.6 sec and the clotting time of neuraminidase-treated cells was 57.7 sec (mean of six determinations). Lower concentrations of neuraminidase (e.g., 2 μg/ml) had little effect on cell adhesion or tissue factor activity. Neuraminidase alone, when incorporated in the assay system, was without procoagulant effect. Incubation of monolayers with phospholipase C in concentrations up to 1000 μg/ml and collagenase in concentrations up to 10 mg/ml for 1 hr had little effect on procoagulant activity of cultured fibroblasts.

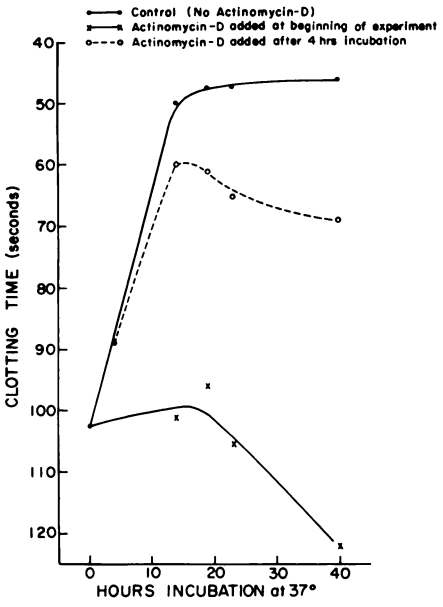

Fig. 3. Effect of actinomycin D on tissue factor synthesis by cultured fibroblasts.

The ability of repeated washing of intact monolayers with saline to reduce tissue factor activity was also demonstrated. Whereas the average clotting time of unwashed monolayers was 41.4 sec, the clotting time after washing three times with saline was 63.1 sec, a prolongation of 21.7 sec.

The above results might simply reflect removal of an activated clotting intermediate (e.g., activated factor X) from the medium surrounding the cells. However, assay of cell-free medium by our standard technique[1,2] (the activated partial thromboplastin time employing factor VIII-deficient plasma) revealed no change in the clotting time after the medium had been in contact with cells. In a simple assay system, in which 0.01 ml of medium was mixed with 0.1 ml of factor VIII-deficient plasma plus 0.1 ml saline and the clotting time was determined on addition of 0.1 ml of 0.02 *M* calcium chloride, clotting times of 210 and 182 sec, respectively, were obtained on cell-free medium before and after contact with cells. That this difference could bear meaningfully on the previously described experiments is highly unlikely, since these clotting times were 150 sec or more longer than those observed in the presence of cells.

The possibility was entertained that tissue factor and the cell surface configuration responsible for adhesion were one and the same. Several observations suggested that this was not the case: (1) Hemocytometer counts on the contents of flasks inoculated with cell suspensions revealed that cell adhesion was complete after 5–6-hr incubation, whereas tissue factor activity did not reach maximum until 12–24 hr incubation. (2) Cells incubated in medium lacking fetal calf serum became adherent but did not synthesize tissue factor. This was demonstrated in experiments in which trypsinized fibroblasts were washed three times in Minimal Essential Medium to remove the trypsin and then cultured in Minimum Essential Medium in the absence

of fetal calf serum at 4°, 25°, and 37° centrigrade. In cultures incubated at 25°C and 37°C, adhesion was complete after 6-hr incubation. Agitation of the culture flasks failed to dislodge the cells. In cultures incubated at 4°C, adherent cells numbered less than 50% of those originally cultured. A notable difference existed, however, between these cultures and cultures in which fetal calf serum was incorporated into the medium. Whereas cells cultured in the absence of fetal calf serum were capable of adhering, they failed to spread on the floor of the culture flask and to acquire the spindled, fusiform configuration characteristic of cells cultured in the presence of fetal calf serum. Thus, generation of tissue factor activity was related to the ability of the cells to spread on the floor of the culture vessel, rather than simply to the ability of the cells to adhere.

To determine whether the RNA and protein synthesis was required for tissue factor production, experiments were carried out in which actinomycin D and puromycin were incorporated in various concentrations into the culture medium. Figure 3 illustrates that the rise in tissue factor activity was obliterated in fibroblast cultures incubated with actinomycin D in concentrations of 2 μg/ml or greater. However, when addition of actinomycin D was delayed until 4 hr after incubation was begun, significant tissue factor-producing capability was preserved. Similar inhibition of tissue factor synthesis was observed in cultures incubated with 2 μg/ml or more of puromycin.

DISCUSSION

The procoagulant synthesized by cultured human skin fibroblasts has been identified as tissue factor.[1,3] Thus, the effect of this procoagulant is restricted to the early stages of coagulation.[2] It is capable of binding factor VII,[2] and its activity, detected in assays employing plasmas congenitally deficient in factors VIII, IX, XI, and XII, is obliterated by a specific antitissue factor antibody[3] and by soybean trypsin inhibitor. Fibroblast tissue factor, however, manifests a peculiar and unexplained dissimilarity of reactivity with various first-stage factor-deficient plasmas that suggest involvement of tissue factor in the intrinsic coagulation system.[2,6]

We have previously shown that cells incubated in suspension culture (leukocytes) generated far less procoagulant activity than several cell types incubated in monolayer culture.[1] The rise in activity observed in several cell types cultured in monolayer was independent of the time required for the cells to double in number and paralleled the period during which the cells were becoming adherent to the floor of the flask.[1] Cells cultured in high concentration, such that the area of cell contact with the floor of the flask and subsequent cell spreading were limited, generated less activity than cells cultured in lower concentrations.[7] Furthermore, prevention of cell adhesion (and, therefore, subsequent spreading) by mechanical agitation of culture flasks completely inhibited tissue factor synthesis. Cultures removed from the shaking device at intervals and placed at rest began to generate tissue factor reflecting gravitation and subsequent adhesion and spreading of cells on the floor of the flask.[7] These results suggested that cell adhesion preceded and

permitted the shape change that was associated with tissue factor synthesis.

Zeldis et al.[4] have demonstrated tissue factor antigen on cell membranes in several tissues. Data reported in the present study corroborate their findings and suggest that the biologic activity of tissue factor resides at the cell surface. The difficulty with which fibroblast cell membranes are isolated and the lability of tissue factor have hampered more direct identification and isolation of this substance.

Niemitz[8] has shown that inhibitors of protein synthesis inhibit tissue factor production by cultured leukocytes. Our data on cultured fibroblasts confirm those observations, and we postulate the following series of events: (1) adhesion and spreading of cells on the floor of the culture flask; (2) initiation of new RNA and protein synthesis in response to this signal from the cell surface; and (3) production of tissue factor (which may be newly synthesized or assembled from inactive precursors) that resides in part if not exclusively at the cell surface.

The relation of these findings to the pathophysiology of tissue factor in vivo is unknown. However, it is tempting to speculate that local endothelial damage might lead to several surface-related events that enhance blood coagulation locally. For example, the making and breaking of cell surface contacts is necessarily involved in cell infiltration, migration, and proliferation.

It has been shown that endotoxin induces tissue factor synthesis in leukocytes.[9] Such leukocytes might then be eminently suited for adhesion to endothelium and marginiation into tissues. Zeldis et al.[4] found tissue factor antigen to be particularly abundant in the vicinity of atherosclerotic plaques where it virtually encompassed cholesterol crystals. They speculated that tissue factor-initiated coagulation might commence in the face of endothelial damage or in the event of plaque disruption. Location of tissue factor on the cell membrane renders it readily available for complexing with plasma coagulation factor VII.

Although these studies were originally designed to elucidate the physiology of coagulation, the detection of tissue factor production of cultured cells would also seem to provide a simple means for studying membrane-initiated protein synthesis.

ACKNOWLEDGMENT

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