Tissue-Factor Coagulant Activity of Cultured Human Endothelial and Smooth Muscle Cells and Fibroblasts
By James R. Maynard, Barbara E. Dreyer, Michael B. Stemerman, and Frances Ann Pitlick

The tissue-factor (thromboplastic) activity of cultured human endothelial cells and fibroblasts is low at time of transfer into fresh medium but increases 3-10-fold. Endothelial cells reach peak activity (400 U/10^5 cells) 5-8 hr after subculture. Activity in fibroblast cultures peaks (3000-12,000 U/b5 cells) 7-12 hr after subculture. After attaining maximum activity, endothelial and fibroblast tissue-factor content decreases in a time course similar to other cells studied in this laboratory, approaching basal levels by 24-50 hr after subculture. If medium over fibroblasts is changed every 12 hr, activity can be sustained at the peak level for an additional day but cannot be maintained at a high level indefinitely. The kinetics of expression of smooth muscle cell tissue factor are markedly different from other cell types. There is always a pronounced lag (30 hr or more) before the activity increases, and then, in most cases, there is no subsequent decline in activity even though the cells are not refed or restimulated. The activity of each of these cell types is cryptic but becomes available after freeze-thaw disruption of cells.

Although vascular activators of coagulation have been postulated, their localization and effectiveness remain to be demonstrated. Purified collagen, one component of the vessel wall matrix, activates Hageman factor, thus initiating coagulation through the intrinsic pathway. In situ, however, basement membrane is protected by endothelium, and fibrillar collagen lies relatively remote from plasma in the media and adventitia. Therefore, damage to one or more layers of overlying cells (endothelial and smooth muscle) may be required before effective contact between components of the blood plasma and collagen can occur. While other substances which can initiate the intrinsic pathway may be present in the intima and media, their existence has not yet been demonstrated. The vessel wall has also been examined for activators of the extrinsic system. From studies using homogenates of dissected strata of the wall, the existence of thromboplastin or tissue factor has been inferred. However, there is disagreement in the literature about the relative potency of the intima, media, and adventitia. Interpretation of these studies is ambiguous since the assay employed, the shortening of plasma clotting times, fails to distinguish between intrinsic and extrinsic coagulation. Furthermore, clean separation of the layers of the vessel wall is technically difficult.

From the Department of Molecular Biophysics and Biochemistry and the Department of Internal Medicine, Yale University Medical School, New Haven, Conn., and the Department of Medicine, Montefiore Hospital and Medical Center, Albert Einstein College of Medicine, Bronx, N. Y.


Supported in part by Biomaterials Program Contract NO1-HB-42968 from the National Heart, Lung, and Blood Institute, a John Hartford Foundation grant, and Program Projects Grants 16126 and 054/5 from the National Heart, Lung, and Blood Institute.

Address for reprint requests: Dr. Frances Ann Pitlick, Department of Internal Medicine, Yale University Medical School, 333 Cedar Street, New Haven, Conn. 06510.

© 1977 by Grune & Stratton, Inc. ISSN 0006-4971.
We have therefore utilized cell culture techniques and specific assays to evaluate tissue-factor activity of the three cell types that occur in blood vessels. In these experiments we show that endothelial cells, smooth muscle cells, and fibroblasts all have measurable tissue-factor activity.

MATERIALS AND METHODS

Endothelial-type and smooth muscle-type cells were cultured from collagenase digests of human umbilical veins using the techniques of Jaffe et al.4 as described by Gimbrone et al.5 Fibroblasts were cultured from human foreskin after collagenase digestion (Worthington, Type CLS): 125–200 U/mg, 0.2%, in Puck’s saline A6 buffered at pH 7.4 with 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; Sigma) for 3 hr. The cells were grown in a humid atmosphere of 95% air–5% CO₂ in medium 199 (Gibco) containing 50 U/ml penicillin (Gibco), 50 μg/ml streptomycin (Gibco), and 2 mM glutamine (Gibco). All sera were heated for 30 min at 60°C and coagulation factors IX, VII, X, and II were removed by adsorption with calcium phosphate as previously described.7

Fibroblasts were cultured in medium supplemented with 10% fetal calf serum (Gibco); medium for smooth muscle cell cultures contained 15%, fetal calf serum; medium for endothelial-type cell cultures contained 25%, fetal calf serum and 5%, human serum, type AB (Gibco). Endothelial and fibroblast cultures were lifted for transfer by brief trypsin (Sigma; Type III) digestion (0.2%, trypsin in buffered Puck’s saline A for 2–5 min). Smooth muscle cell cultures became detached from the plastic growth support only after 10–30 min of incubation with trypsin. After lifting, cell viability was always greater than 90% by the trypan blue dye exclusion test.

Human antibodies to human blood group antigens were obtained from Ortho Diagnostics. A crude globulin fraction was prepared by precipitation with an equal volume of saturated ammonium sulfate. The precipitate was dialyzed against 0.05 M sodium phosphate 0.1 M sodium chloride, pH 7.5. The globulin was stored as a 20-mg/ml solution and diluted 1:10 for the rosette formation technique. To detect blood group antigens, a marker for endothelial cells described by Jaffe et al.,4 endothelial cells were lifted with 0.1% collagenase-0.01% EDTA in buffered Puck’s saline A and washed three times before incubation with type A or type B antibodies for 1 hr. The cells were then washed five times and incubated with type A or type B red cells for 5 min.

The coagulant activity of the washed monolayer was determined by adding purified coagulation factors to the Petri dish as previously described.7 Coagulation factors VII, X, and prothrombin were purified from bovine plasma by previously described techniques.9 Thrombin generated in this mixture was assayed with purified fibrinogen (Kabi). A companion set of washed monolayers was disrupted by freezing in dry ice–ethanol three times before incubation with the plasma coagulation factors. Cells were counted after detachment from yet another washed monolayer by incubation for 30 min with 0.0025%, trypsin.

For assessment of tissue-factor activity using a two-stage assay,7 cells were lifted from their support by incubation with 0.0025% trypsin in buffered Puck’s saline A for 30 min at 37°C. A portion was counted and another portion was frozen and thawed three times for development of maximum activity and was then assayed directly. Activity was expressed as units of tissue factor relative to the EDTA bovine brain standard in use in this laboratory.10 Undiluted Simplastin (General Diagnostics) was equivalent to about 2500 EDTA brain units in this assay.

RESULTS

Characterization of Cell Types

Endothelial cell cultures derived from gentle collagenase digestion of untraumatized umbilical veins contain primarily polygonal cells which grow in isolated patches until confluent in a sheet. These cells grow more readily on plastic than glass. Brief trypsin digestion is used routinely during subculture to leave smooth muscle cells attached to the growth support (see below). In our experience human serum promotes the growth and maintenance of endothelial cells in culture.
When endothelial cells derived from a cord with type A erythrocytes were examined for blood group antigens using the rosette formation technique, 6% of the cells formed complete rosettes with type A antibody erythrocytes. No rosettes were observed with type B antibody or type B erythrocytes. These results are identical to those previously reported. The rod-shaped bodies characteristic of endothelial cells have also been observed in these cultures (results not shown). Thus, these cells had the endothelial characteristics described by other observers.

Occasional elongated smooth muscle-type cells are found in primary cultures of endothelial cells. After several passages, these elongated cells begin to represent a significant proportion of the population; with continued passage, the mixed population of these two cell types persists. In order to have enough cells for the experiments described here, endothelial cells were cultured through two passages before use. Only those cultures with minimal smooth muscle cell contamination were employed. From the results described below, it is evident that significant smooth muscle cell contamination of such endothelial cultures would have been reflected in the tissue-factor assay.

Cells cultured from traumatized umbilical veins appear to be the smooth muscle cells described by Jaffé et al. and Gimbrone and Cotran. These cells are elongated and appear larger than endothelial cells on the growth support. When confluent, these cultures demonstrate morphologic characteristics, including "hills and valleys," similar to those described by Ross for cells derived from guinea pig aortic medial explants and Gimbrone and Cotran for umbilical vein smooth muscle cells. These cells attach poorly to glass, but grow well on plastic. Unlike endothelial cells and fibroblasts, prolonged digestion with trypsin is usually required to lift these cells from their growth support.

The foreskin fibroblasts are only slightly smaller than the smooth muscle-type cells, but they grow readily on glass and are easily lifted from glass or plastic by brief digestion with trypsin. When confluent, these cells form typical whorl patterns ascribed to fibroblasts.

**Kinetics of Tissue-Factor Expression**

The three types of cells were transferred into plastic Petri dishes and at several intervals the monolayers were washed, lifted with trypsin, frozen and thawed, and then assayed for tissue-factor activity (Fig. 1). Within 5–8 hr after transfer, activity reached a maximum in endothelial cells, then began to decline (Fig. 1). The peak specific activity of these cells was similar to that of WISH amnion cells studied in this laboratory. Although activity was low at later time points, clotting times were still faster than buffer controls and the cells remained viable throughout the experiment. Fibroblast activity peaked at 7–12 hr, and was about 10–50-fold greater than that of endothelial cells. This activity also quickly returned to a basal level.

A number of different endothelial and fibroblast strains behaved in the manner illustrated with little deviation around the time when peak activity was reached. Fibroblasts showed approximately a fourfold variation in the peak specific activity, while endothelial cells had even less variation.

The decline of tissue-factor activity might be due to depletion of a stimulus or
Fig. 1. Tissue-factor activity of lifted cells. Confluent cultures of the three cell types were transferred to 35-mm Petri dishes (0.5–1.5 x 10^4 cells/dish) in their respective media. At the indicated intervals the cells were lifted by incubation with 0.0025% trypsin for 60 min. A portion of the cells was counted and another was frozen and thawed three times and assayed for tissue-factor activity.

Fig. 2. Effect of medium changes on tissue-factor activity. Confluent fibroblasts were diluted and transferred into fresh medium in 35-mm plastic Petri dishes. One set of cultures served as a control and was not refed (•); the medium on a second set was changed once at 12 hr (□); the medium on another set was changed at 12 and 24 hr (○); the final set was fed at 12, 24, and 36 hr (○). At the times indicated washed cells were lifted from their growth support by incubation with trypsin. Tissue-factor activity was assayed on this suspension after freezing and thawing three times.
a constituent necessary for the production of activity or the appearance of an inhibitor. We tested this hypothesis by changing the medium in fibroblast cultures every 12 hr (Fig. 2). Medium replacement at 12 hr produced about a 20% increment in activity over control cells. Cells fed again at 24 hr sustained activity at a peak level for 12 hr longer than control cells or those fed once. An additional feeding at 36 hr, however, could not prolong the peak. During the first 36 hr, the cell count was relatively constant; after this period, the cells were in logarithmic phase growth with a doubling time of 20 hr.

Smooth muscle cells showed a much more pronounced variability in both specific activity and in the profile of activity changes following subculture into fresh growth medium. Table 1 summarizes some of the parameters of cell growth and tissue-factor activity changes for 12 smooth muscle cell strains. Two of the strains (131 and 132) were at their final subculture and did not show any increase in cell number. In most cases the activity increased and then remained constant, sometimes at a very high specific activity. In all cases, smooth muscle cells had a characteristic lag before the peak level of activity was reached, the fastest generation of maximal activity occurring 34 hr after subculture. More than half of the strains reached peak activity by 48 hr; the remainder required at least 140 hr, and one culture was still increasing in activity when the experiment was terminated at 192 hr (8 days). Most of the cultures (7 of 11) stayed at or near the peak activity for the remainder of the experiment, even though none of the cultures was refed. Three strains had a peak in their activity profile and a subsequent decline not unlike the general behavior of other cultures. Even with these strains there was a pronounced lag before the peak activity occurred and the decline in activity was more gradual than that seen for fibroblast cultures.

To ensure that the observed differences in activity among the three cell types were not due to differences in serum concentration in the culture media, fibro-

Table 1. Comparison of the Tissue-Factor Activity of Several Smooth Muscle Cell Strains

<table>
<thead>
<tr>
<th>Cell Strain No.</th>
<th>Peak Specific Activity</th>
<th>Time to Reach Maximum Specific Activity (hours after subculture)</th>
<th>Specific Activity Increase Over the Initial Value</th>
<th>Behavior After Reaching Peak Activity</th>
<th>Cell Number Doubling Time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>14,000</td>
<td>40</td>
<td>3.7</td>
<td>Not determined</td>
<td>34</td>
</tr>
<tr>
<td>62</td>
<td>4,000</td>
<td>34</td>
<td>3.7</td>
<td>Remains elevated</td>
<td>32</td>
</tr>
<tr>
<td>65</td>
<td>12,000</td>
<td>48</td>
<td>5.0</td>
<td>Remains elevated</td>
<td>96</td>
</tr>
<tr>
<td>86</td>
<td>15,000</td>
<td>48</td>
<td>2.5</td>
<td>Declines</td>
<td>34</td>
</tr>
<tr>
<td>87</td>
<td>19,000</td>
<td>48</td>
<td>3.4</td>
<td>Declines</td>
<td>28</td>
</tr>
<tr>
<td>89</td>
<td>7,300</td>
<td>140</td>
<td>6.5</td>
<td>Remains elevated</td>
<td>70</td>
</tr>
<tr>
<td>96</td>
<td>2,200</td>
<td>140</td>
<td>3.5</td>
<td>Remains elevated</td>
<td>37</td>
</tr>
<tr>
<td>131</td>
<td>28,000</td>
<td>46</td>
<td>1.5</td>
<td>Remains elevated</td>
<td>No change in cell number</td>
</tr>
<tr>
<td>132</td>
<td>31,000</td>
<td>46</td>
<td>2.0</td>
<td>Remains elevated</td>
<td>No change in cell number</td>
</tr>
<tr>
<td>134</td>
<td>11,000</td>
<td>192</td>
<td>5.0</td>
<td>Still increasing</td>
<td>40</td>
</tr>
<tr>
<td>149</td>
<td>6,700</td>
<td>168</td>
<td>2.1</td>
<td>Remains elevated</td>
<td>34</td>
</tr>
<tr>
<td>152</td>
<td>5,300</td>
<td>48</td>
<td>3.8</td>
<td>Declines</td>
<td>58</td>
</tr>
</tbody>
</table>

Smooth muscle cells isolated from different umbilical cords (22-152) were assayed for tissue-factor activity at different times following subculture into fresh medium. The experiments were continued for a total of 123 hr (cells from cord 62 and 65), 166 hr (86,87,89,96), or 192 hr (131,132,134,149,152). None of the cultures was refed. In each of these experiments the tissue-factor activity increase lag was similar to that for smooth muscle cells isolated from cord 22, which is shown in Fig. 1 and is also included in this table.
blasts and smooth muscle cells were cultured in all three media and the tissue-factor activity was assayed. Fibroblasts were assayed at several time points during the 24 hr following subculture. At each time point, the deviation from median activity was less than 20%; furthermore, the cells grown in one type of medium were randomly high or low at the several time points (data not shown). Similar results were obtained with smooth muscle cell cultures. Thus, the observed differences in activity for the three cell types in their respective media were not due simply to media composition differences.

Protected Nature of Coagulant Activity

In order to study the availability of coagulant activity in undisturbed cells, washed monolayer cultures were incubated with purified coagulation factors and thrombin generation was measured. This assay has been previously described. Another set of monolayers was frozen three times before addition of coagulation factors, and a third set was lifted with trypsin and counted. These results are summarized in Table 2. In fibroblast cultures, twice as much thrombin was generated in freeze-thaw disrupted cultures as those which had been simply washed (at 12 hr, 21 versus 10 NIH U/ml/5 min); similar increases after freezing were noted with smooth muscle cells and endothelial cells. Endothelial cells had much lower activity than the other two cell types. At 56 hr after subculture, the amount of activity in the fibroblast cultures had decreased even though the number of cells had increased; in the endothelial culture, even after freeze disruption, only a trace of thrombin was generated. On the other hand, although intact smooth muscle cells had almost no activity at this time, disrupted smooth muscle cells retained their ability to initiate coagulation.

The assay described in Table 2 avoided the use of trypsin to detach the cells

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Hours After Transfer</th>
<th>Cells per Dish ($\times 10^5$)</th>
<th>Thrombin Generated (NIH U/ml/5 min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Intact Frozen-Thawed</td>
</tr>
<tr>
<td>Fibroblast</td>
<td>12</td>
<td>1.32</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>3.52</td>
<td>3</td>
</tr>
<tr>
<td>Smooth</td>
<td>12</td>
<td>0.45</td>
<td>10</td>
</tr>
<tr>
<td>Muscle</td>
<td>56</td>
<td>0.69</td>
<td>&lt;1*</td>
</tr>
<tr>
<td>Endothelial</td>
<td>11</td>
<td>1.22</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>56</td>
<td>ND†</td>
<td>ND†</td>
</tr>
</tbody>
</table>

Confluent cultures of the three cell types were transferred to 35-mm Petri dishes. At 12 and 56 hr, one Petri dish of each culture was frozen and thawed three times. Two ml of saline A was then added to the intact or disrupted cultures and the following reagents were added for the final concentrations indicated: factor VII (50 U/ml), factor X (0.4 mg/ml), prothrombin (3 mg/ml), cephalin (100 µg/ml), and CaCl$_2$ (5 mM). At 1–2-min intervals 100-µl portions were withdrawn and added to 200 µl fibrinogen (6 mg/ml). A standard curve was prepared by converting prothrombin to thrombin with Taipan venom (Sigma). Companion cultures were washed and incubated with 0.0025% trypsin for 30 min and the lifted cells were counted. Cell counts were confirmed by cell counts of photomicrographs of untreated monolayers and by examination of the Petri dishes for total cell removal in the trypsin-lifting process.

* Lower limit of detection.

† ND, not determined. Sufficient Petri dish cultures of endothelial cells were available for only one assay at this point; prior to addition of coagulation factors, the culture appeared normal and healthy with no loss of cells.
TISSUE-FACTOR COAGULANT ACTIVITY

prior to assay. We still saw that coagulant activity was more available after cellular disruption and that a ranking order for coagulant activity for the different cell types existed: fibroblast > smooth muscle cells > endothelial cells. Furthermore, the decline in total activity in fibroblast and endothelial cultures was again noted but activity was retained in smooth muscle cell cultures. Thus trypsin treatment alone did not account for the activity differences observed (Fig. 1). This experiment has been duplicated with other strains of each cell type with the same results.

DISCUSSION

We have compared the kinetics of tissue-factor expression in cultured fibroblasts with the kinetics of expression and absolute amounts of activity of cultures of two other cell types which normally comprise the blood vessel wall, endothelial cells and smooth muscle cells. Cultures of these cell types may represent an in vitro model for the tissue-factor hemostatic potential.

Tissue factor, the procoagulant activity found in cultured cells, is associated with the cell surface. While some cellular disruption is necessary for its expression, this activity is a component of the cell surface of fibroblasts and a transformed cell line, WISH amnion cells, and can be released into the cell-free supernatant under conditions which maintain cell integrity.

When each of these cell types growing in monolayers was exposed to a mixture of the purified components of the extrinsic pathway the rate of thrombin formation was low (Table 2). If the cells were frozen and thawed prior to addition of the coagulation factors the activity was markedly increased. Thus, as we have previously demonstrated for WISH amnion cells, we conclude that tissue-factor activity also exists in a protected state on the three cell types and some cellular trauma is required for the full expression of activity.

The amount of activity in cultured cells is not constant, but increases severalfold after subculture into fresh medium. This change reflects total activity in the cells rather than just a shift from an intracellular to surface compartment since the same amount of trauma is required for expression of activity, and trypsin releases the same proportion into the supernatant throughout the growth cycle.

Endothelial cells respond to transfer to fresh medium in a manner similar to normal fibroblasts and WISH amnion cells. In this study the activity quickly increased (Fig. 1) then subsequently returned to the basal level before 24 hr after subculture. A consistency in both specific activity and kinetics of expression has been observed for four different endothelial strains. The kinetics are similar to other cell types we have studied, but endothelial cells have a lower specific activity than either fibroblasts or smooth muscle cells. The endothelial cell kinetics are remarkably different from smooth muscle cells (see below), indicating that these observations are due to the activity of the endothelial cells and not to trace smooth muscle cell contamination in the endothelial cultures.

The results relating cell type to coagulant activity reported here do not agree with our histochemical localization of tissue factor using antibodies to rabbit or human tissue factor. We are currently attempting to resolve this conflict, which may arise for any of several reasons, including a failure of culture systems to mimic the situation in situ, and the possibility that the original antigens
are heterogeneous and precipitate with a highly antigenic marker on endothelial cells, which is unrelated to coagulant activity.

In contrast with the behavior typical of all the other cell types we have studied, umbilical vein smooth muscle cells showed greater variability in both their total tissue-factor activity and the kinetics of the activity expression which occurred in response to subculture. While there was considerable variation between different strains (Table 1), the activity was always much greater than in endothelial cells. In all cases there was a pronounced lag before the activity increased, and most of the cultures maintained a high level of activity for an extended period of time. Both of these features of the activity appear to be unique to smooth muscle cells. Even the culture which did decline in activity after reaching a maximum did so gradually over a period of several days (data not shown). The smooth muscle cells were never refed or restimulated after the initial subculture. Since the same isolation and culture techniques were employed for all the strains reported in Table 1, we cannot account for the variability observed and must at this time ascribe it to unknown variations in donor tissue.

The refeeding experiments were designed as a closer approximation of the situation of a cell in a tissue, in which continual nutrient replenishment and metabolite removal occurs. Even under these conditions, activity, although prolonged, was not sustained. We thus infer from this in vitro model that the activity of these cells in the vascular wall approximates that in established cultures rather than in those cultures which have not reached confluence.

Although the coagulant activity of endothelial cells is much less than that of smooth muscle cells and fibroblasts, there is as yet no way to determine the absolute amount of initiator required to produce an intravascular coagulum. Endothelial cells in vitro do have some activity, so the endothelium may act as the first line of defense against hemorrhage initiated by the tissue-factor pathway. On the other hand, their activity may be low enough such that they are an effective barrier between plasma and the underlying vessel wall. Injury exposing the subendothelial cell layers will result in a greatly increased hemostatic potential due both to the greater coagulant activity of smooth muscle cells and fibroblasts and to their greater numbers. This behavior is particularly true of smooth muscle cells since it appears from these studies in vitro that the normal basal level of tissue factor can be much higher than that of other cell types.

From these experiments it is clear that all three cell types in the vessel wall could contain tissue-factor activity and that two of these cell types, fibroblasts and endothelial cells, may have a greater coagulant potential in an area of active cell proliferation. Since the cells must be disrupted for full expression of coagulant activity, we assume from this model that tissue factor in vivo is also in a protected state and normally unavailable to react with plasma coagulation factors.

The data presented above serve simply to define which cells in the vessel wall could have tissue-factor activity. If the situation holds in vivo, a coagulant potential will be found across the wall, not only in terms of specific activity of each cell type, but also involving the numbers of each cell type across the wall. The possible contribution of collagen to activate the intrinsic system would add to the potential.
On the other hand, individual cell types may interact with each other and their intercellular matrices to modulate tissue-factor activity such that the endothelial cells have more activity than the underlying smooth muscle cells and fibroblasts. Presently, however, we have no data to describe tissue-factor activity in situ.

While we propose these experiments as a model of vascular hemostatic potential, two reservations are operative: (1) the cells are cultured in vitro and their similarity to cells in vivo is unknown, and (2) the cell types are not obtained from adult human vascular tissue. However, the cell culture model is currently one of the few techniques available to quantify activity of given cell types in which trauma and extracellular components may be externally controlled. As culture technology improves, these observations will be extended to cells of adult origin so that a more reliable approximation can be made.

ACKNOWLEDGMENT

We thank Dr. Robert Radcliffe and Dr. Sidonie Silverberg for the purified coagulation factors, and Dr. Yale Nemerson for helpful criticism of the manuscript. We acknowledge special thanks to the staff of the Obstetrics Unit of Yale-New Haven Hospital for providing the umbilical cords used in this work.

REFERENCES

18. Zacharski LR, Bowie EJW, Titus JL, Owen CA Jr: Cell-culture synthesis of a factor


Tissue-factor coagulant activity of cultured human endothelial and smooth muscle cells and fibroblasts

JR Maynard, BE Dreyer, MB Stemerman and FA Pitlick