CONCISE REPORT

Human Endothelial Cells Synthesize Protein S

By Daryl S. Fair, Richard A. Marlar, and Eugene G. Levin

Human umbilical vein endothelial cells were analyzed for the presence of prothrombin, factor VII, protein C, and protein S in culture supernatants and cell extracts using specific radioimmunoassays. Only protein S was detected. Conditioned medium from 24-hour cultures and cell lysates contained 21.7 ng/mL and 88.8 ng/10⁷ cells of protein S, respectively. Intrinsic labeling and immunoprecipitation indicated that protein S was synthesized and secreted as a 75,000 molecular weight protein. Vitamin K, phorbol myristate acetate, and thrombin increased the production or specific activity (determined from activity/antigen ratios of 0.99 to 1.07, 0.93 to 1.04, and 0.90 to 1.04, respectively) of protein S. While untreated cells secreted a partially active protein S (activity/antigen = 0.40), warfarin greatly decreased the specific activity (<0.10) of this molecule, supporting the hypothesis that endothelial cells contain the enzymes required for the carboxylation of selected glutamic acid residues. The production of protein S by these cells supports the hypothesis that cofactor production and expression by the endothelial cells may play a significant regulatory role in the initiation, propagation, and suppression of hemostasis and thrombosis.

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MATERIALS AND METHODS

Endothelial cells were isolated from human umbilical cord veins as previously described and grown to confluence in T-75 flasks precoated with 2 mg/mL calf skin gelatin (Eastman Kodak Co., Rochester, NY). Cells were grown in RPMI 1640 supplemented with 20% fetal bovine serum (Reheis Co., Phoenix), 100 U/mL penicillin, and 100 µg/mL streptomycin (GIBCO, Grand Island, NY). Conditioned medium containing protein S was collected in RPMI 1640 in the absence of serum for 24 hours. In selected experiments, the cells were treated with phorbol myristate acetate (PMA, 400 ng/mL; Sigma Chemical Co., St Louis), alpha-thrombin (1 U/mL, a gift from Dr John Fenton, New York Department of Health, Albany), vitamin K (AquaMEPHYTON; Merck, Sharp & Dohme, West Point, PA; 10 µg/mL) or warfarin [3-(a-acetonyl benzyl)-4-hydroxycoumarin; Sigma; 1 µg/mL] for 16 to 24 hours in RPMI 1640 containing 0.1% bovine serum albumin.

Protein S was purified from plasma by the method of Dahlback. Measurement of protein S antigen was by either a double antibody competitive equilibrium radioimmunoassay on undiluted samples or a Laurell electroimmunoassay with Coomassie staining on culture supernatants concentrated 20-fold and dialyzed by ultrafiltration using an Amicon device (Amicon Corp, Danvers, Mass). Recovery of protein S was 80% to 85% as estimated, using iodinated protein S added before concentration. Activity was quantitated by a modification of the method of Comp et al., where Russell's viper venom (1:4,000 dilution; Sigma) was used in place of pure factor Xa. The sensitivity of the assay was to 3% of normal human plasma with a precision of 7% to 10% within and between assays. Dilutions of normal plasma and purified protein S were used to construct standard curves for activity and antigen measurements. The values were expressed as a percentage of normal plasma pool.

Cultures of confluent endothelial cells were washed with 4 mL of methionine-free minimal essential medium (Irvine Scientific, Santa Ana, Calif) and incubated in 4 mL of the same medium supplemented with 5 µg/mL vitamin K and 150 µCi/mL ³⁵S-methionine (Amersham Corp, Arlington Heights, Ill) for six hours at 37°C.
ENDOTHELIAL CELLS SYNTHESIZE PROTEIN S

After incubation, the supernatant was clarified by centrifugation at 100,000 g for 45 minutes at 4 °C, and the cell lysates were collected in 2 mL of 0.1% Triton X-100 (Eastman Kodak) containing 10 mmol/L benzamidine (Sigma), 1 mmol/L ethylenediamine tetraacetic acid (EDTA), and 100 U/mL Trasybol (Bayer, Leverkusen, West Germany). Bovine serum albumin (5 mg/mL) was added to both the supernatant and cell lysate and immunoprecipitated using rabbit anti-protein S and Staphylococcus aureus exactly as described previously. The immune complexes were solubilized into 2% sodium dodecyl sulfate (SDS) and electrophoresed on SDS-9% polyacrylamide slab gels containing 4C-protein standards (New England Nuclear Corp, Boston) in the presence of 1% 2-mercaptoethanol. Gels were fixed in 10% acetic acid, 25% methanol for one hour, washed extensively with water, incubated with 1 mol/L sodium salicylate for 30 minutes, dried, and subjected to autoradiography.

RESULTS

The production of protein S and other vitamin K-dependent proteins by human endothelial cells was analyzed by using specific radioimmunoassays for prothrombin, factor VII, protein C, and protein S. Only protein S was detected in significant quantities, with 88.8 ± 17.3 ng in the cell lysates from about 1 x 10⁷ cells. Conditioned medium from 24-hour cultures contained 21.7 ± 3.3 ng protein S per milliliter, but none of the other molecules. Thus, of the four vitamin K-dependent proteins tested, only the molecule with cofactor activity was observed within the limits of this analysis.

To demonstrate that the endothelial cell synthesized protein S, we cultured the cells for six hours in the presence of radioactive methionine and immunoprecipitated both the supernatants and cell extracts with antibodies to protein S. We analyzed these cells for the production of von Willebrand’s factor as control. Figure 1 is a representative example of five experiments in which the supernatant from endothelial cells was immunoprecipitated with rabbit anti-protein S in the absence and presence of 50 µg of exogenous purified protein S. A unique band of 75,300 ± 2,100 mol wt was precipitated with the antibody but was absent when unlabeled protein S was added to the supernatant before immunoprecipitation. The migration of this band was identical to iodinated native protein S (data not shown). Protein S represented about 0.1% of the total secreted protein relative to total trichloroacetic acid precipitable counts. Visualization of protein S in cell lysates was obscured by the coprecipitation of a nonspecific protein with a similar mol wt. When antibodies to von Willebrand’s factor were used, the characteristic polypeptide chains of >220,000 daltons were observed (data not shown). Thus human endothelial cells synthesize and secrete protein S.

The production of protein S from these cells implies that they may contain the carboxylase enzyme system required for the characteristic posttranslational modification of selected amino-terminal glutamic acid residues. To investigate this possibility, we tested the effect of warfarin, vitamin K, thrombin, and PMA on the ability of these cells to produce functional molecules. Confluent cells were cultured for 24 hours in the absence or presence of these agents, and the secreted protein S was assayed for activity and antigen using both normal plasma and purified protein S for standard curves (Table 1). Neither the medium or medium containing PMA in the absence of cells had protein S activity or antigen. Cells cultured in medium only released a partially active protein S as judged by the activity/antigen ratios of 0.35 to 0.40. Addition of thrombin did not increase the production of protein S significantly, but the functional capacity of the molecule was greater, as indicated by the increase in the activity/antigen ratio of 0.90 to 1.04. Both PMA and vitamin K enhanced the accumulation of protein S, which had specific activities of 0.93 to 1.04 and 0.99 to 1.07, respectively. Warfarin decreased the secretion of protein S slightly, but demonstration of a functional molecule was below the limits of detection in our bioassay. These trends were observed in three separate experiments. Within the sensitivity of our assay methods, the production of functionally active protein S can be increased by vitamin K, PMA, and thrombin and is significantly decreased by warfarin, a known inhibitor of the carboxylation reaction.
Table 1. Modulation of Protein S Activity and Antigen Secreted From Human Endothelial Cells

<table>
<thead>
<tr>
<th>Culture</th>
<th>Protein S</th>
<th>Activity NHP</th>
<th>Antigen NHP</th>
<th>Activity/Antigen NHP</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPMI</td>
<td>&lt;3</td>
<td>&lt;1</td>
<td>&lt;3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>RPMI + PMA</td>
<td>14</td>
<td>3.3</td>
<td>35</td>
<td>9.5</td>
</tr>
<tr>
<td>Control CM</td>
<td>51</td>
<td>22.2</td>
<td>49</td>
<td>23.8</td>
</tr>
<tr>
<td>PMA CM</td>
<td>35</td>
<td>15.0</td>
<td>39</td>
<td>14.4</td>
</tr>
<tr>
<td>Thrombin CM</td>
<td>48</td>
<td>20.0</td>
<td>45</td>
<td>20.2</td>
</tr>
<tr>
<td>Vitamin K CM</td>
<td>&lt;3</td>
<td>&lt;1</td>
<td>29</td>
<td>12.1</td>
</tr>
<tr>
<td>Warfarin CM</td>
<td>&lt;3</td>
<td>&lt;1</td>
<td>29</td>
<td>12.1</td>
</tr>
</tbody>
</table>

Confluent cultures of human umbilical vein endothelial cells were incubated in the presence of phorbol myristate acetate (PMA, 400 ng/mL), alpha-thrombin (1 U/mL), vitamin K (10 µg/mL), or warfarin (1 µg/mL) diluted in serum-free medium containing 0.1% bovine serum albumin. Condition medium (CM) from 24-hour cultures were collected and concentrated 20-fold before analysis for activity and antigen. Activity and antigen values are the means for two experiments and are expressed as percentages of pooled normal human plasma assuming protein S concentration in plasma was 29 µg/mL. Standard curves for activity and antigen measurements were based on dilutions of normal human plasma (NHP) or purified protein S (PS).

DISCUSSION

Synthesis of vitamin K-dependent blood coagulation proteins was once thought to be restricted to hepatocytes. Indeed, our lab has demonstrated that a human hepatoma cell line can produce most of these molecules, including protein S, which represent their plasma protein counterparts immunohistochemically, structurally, and functionally. Recently, the human monocyte and macrophage have been demonstrated to synthesize factor VII.

In this report, we have shown that the endothelial cell has the capacity to synthesize and secrete protein S, which has a similar mol wt as the plasma form, and that its synthesis and activity can be affected by known pharmacologic modulators of vitamin K-dependent proteins. The finding that vitamin K increases and warfarin decreases the synthesis and specific activity of protein S suggests that they contain the enzymes required for gamma carboxylation. Thus at least two additional cell types have the capacity to produce functionally active members of this protein family but appear to be limited or restricted to the synthesis of a particular component.

The increase of protein S levels after thrombin addition would be physiologically consistent with the role of thrombin in protein C activation. It can be envisioned that the increase in activated protein C mediated by thrombin-thrombomodulin would necessitate an increase in protein S levels to attain maximum protein C activity. The simultaneous occurrence of increased protein S-activated protein C levels could be coordinated by one endothelial cell. Studies determining the course of protein S release and the role thrombomodulin might play in protein S release will help to clarify the link between activation of protein C and protein S release.

The synthesis of protein S by endothelial cells may be biologically significant. Patients undergoing warfarin therapy show a decrease in vitamin K-dependent proteins, including protein S, of about 50%; however, individuals with liver disease show a discordant reduction in protein S (~25% decrease) relative to protein C (~50% reduction), suggesting that there is another site of synthesis for protein S. Our studies indicate the endothelium as a likely source of this molecule and would explain this apparent discrepancy. Recently, Stern et al have shown that the endothelium can serve as a functional surface for the assembly of the activated protein C–protein S complex. In addition, they have evidence for the internalization of protein S by these cells in the absence of activated protein C, but protein S remains surface associated in its presence. Our data suggest that the endothelial cell constitutively synthesizes and secretes protein S, and internalizes this molecule in a dynamic manner.

Once low levels of activated protein C are formed by thrombin–thrombomodulin on the endothelial surface, it may be in proximity to protein S (receptor), resulting in the formation of an active, stable inactivator complex for factors Va and V. The demonstration that this cofactor of activated protein C activity is produced by this cell supports our original hypothesis that cofactor production and expression by the endothelial cells may play a significant regulatory role in the initiation, propagation, and suppression of hemostasis and thrombosis.

ACKNOWLEDGMENT

We thank Marsha McDonald and David Revak for excellent technical assistance and Joy Lozano and Ellen Schmeding for preparation of the manuscript. We thank Cnr Scott and Peter P. Narwhal for providing preprints of their manuscripts.

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