Attachment of Cultured Human Endothelial Cells Is Promoted by Specific Association With S Protein (Vitronectin) as Well As With the Ternary S Protein–Thrombin–Antithrombin III Complex

By Klaus T. Preissner, Elisabeth Anders, Jürgen Grulich-Henn, and Gert Müller-Berghaus

The interaction of the multifunctional S protein (vitronectin) with cultured human endothelial cells of macrovascular and microvascular origin was investigated. Purified S protein, coated on polystyrene Petri dishes, induced dose-dependent and time-dependent attachment and spreading of human umbilical vein endothelial cells (HUVECs) as well as human omental tissue microvascular endothelial cells (HOTMECs) at 37°C. Not only isolated S protein, but also the ternary S protein–thrombin–antithrombin III (STAT) complex promoted attachment of ~90% of the cells within 2 hours at an S protein concentration of 0.13 μmol/L. Inhibition of attachment in these experiments was achieved by the addition of the cell-attachment pentapeptide Gly-Arg-Gly-Asp-Ser and by monoclonal antibodies against S protein, whereas nonrelated peptides or antibodies against fibronectin, fibrinogen, or von Willebrand factor (vWF) were ineffective. Direct binding of S protein to HUVECs and HOTMECs was studied with cells in suspension at a density of 1 x 10⁶ cells/mL and was maximal after 120 minutes. S protein bound to both cell types in a dose-dependent fashion with an estimated dissociation constant Kd = 0.2 μmol/L. At a 200-fold to 500-fold molar excess of unlabeled S protein, >80% of bound radiolabeled S protein was displaceable, whereas binding was reduced to 30% to 50% by addition of the pentapeptide, the STAT complex, or by physiologic concentrations of fibrinogen or vWF as well as Fab fragments of antihuman S protein IgG, but not by Fab rabbit IgG. These findings present evidence for the specific association of S protein with endothelial cells ultimately leading to attachment and spreading of cells. Moreover, a novel function for the ternary STAT complex, which induced endothelial cell attachment and spreading virtually identical to free S protein, is described. These data further suggest a possible role for S protein during coagulation as major vessel wall-related adhesive protein at sites of vascular injury.

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phoresis was used to quantify radiolabeled S protein. Dilutions of Gly-Asp-Ser was a product of Bachem, Bubendorf, Switzerland. Gen, antifibronectin and anti-vWF IgG fractions were purchased from Dakopatts, Hamburg, FRG. The synthetic peptide Gly-Arg-Gly-Asp-Ser was a product of a Zeiss Phase Microscope IM/35. Attachment was quantitated by removing the supernatants from the wells followed by gentle washes with SFM (combined solutions contained nonattached cells) and subsequently by detaching the adhered cells with trypsin/EDTA solution (attached cells). Cells were counted by the use of a hemocytometer, and results were expressed as percentage of attached cells. In addition, attachment of cells was quantitated after selected time intervals using precoating solutions containing 20 μg/mL S protein and the same methodology as described.

**Binding of S protein to endothelial cells.** The binding of S protein was measured on HUVECs as well as on HOTMECs in single-cell suspensions. Endothelial cell suspensions were prepared in SFM as described in the previous paragraph. The cells in suspension (final density 1 × 10^5 cell/mL) were incubated with various concentrations of 125I-S protein in a total volume of 120 μL PBS, containing 1% (wt/vol) HSA, 1 mmol/L CaCl₂, and 1 ATU/mL hirudin for various times at 37°C under gentle agitation. Different incubation mixtures also contained specified antibodies or other additives at desired concentrations. At selected time points, 50 μL cell suspension was layered on 300 μL 35% (wt/vol) sucrose dissolved in PBS–1% (wt/vol) HSA in conical propylene tubes and centrifuged for 2 minutes at room temperature in a microfuge (Heraeus Christ, Osterode, FRG). The tips of the tubes were cut off with a razor blade, and the amount of 125I-S protein associated with the cell pellet was determined. Part of this cell pellet was solubilized in sodium dodecyl sulfate (SDS) buffer and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions using the buffer system of Laemmli. After the gel had been fixed and dried, the distribution of radiolabel was detected by autoradiography. In addition, following binding of radiolabeled S protein and separation of unbound tracer, cells were exposed to 0.1 mg/mL trypsin for 3 minutes at 37°C and centrifuged again through sucrose solution to remove surface-associated label. Routinely, viability of cells after termination of binding experiments was determined by trypan blue exclusion.

**RESULTS**

**Promotion of attachment of endothelial cells by S protein and ternary STAT complex.** Polystyrene Petri dishes were precoated with different concentrations of S protein, with buffer alone, or 1% HSA solution. When HUVECs or HOTMECs were seeded at a density of 3 × 10^5 cells/mL on buffer or HSA-precoated dishes, only minimal cell attachment was observed within 2 hours at 37°C. In contrast, >50% of cells adhered under the same conditions when Petri dishes were precoated with S protein at concentrations between 0.3 and 3.0 μg/mL. Thus, >90% endothelial cells became attached within the same time period to dishes that had been precoated with S protein at concentrations >10 μg/mL (0.13 μmol/L) S protein. In a time-course experiment at a constant concentration of 20 μg/mL S protein, the induction of >90% cell attachment of HOTMECs within 1 hour of incubation at 37°C was observed (Fig 1, insert).

Inspection by light microscopy of the cell attachment process not only revealed cell adherence, but cells changed their morphological appearance and began to spread on the substratum shortly after being exposed to S protein (Fig 2). Eight different batches of S protein were tested for their cell...
attachment promotion activity and all were positive in exerting this activity at comparable concentrations. When compared with fibronectin at the same molar concentration, S protein exhibited similar attachment-promoting activity; the exposure of S protein to thrombin or trypsin prior to testing did not significantly reduce the attachment promotion activity of the protein (data not shown).

Because S protein associates with the thrombin–antithrombin III complex during blood coagulation to form a ternary complex, this macromolecular complex was also tested for its ability to promote endothelial cell attachment. Ternary STAT complex promoted attachment of endothelial cells to the same extent and virtually indistinguishably as compared with S protein (Fig 3). Because attachment studies with other cell types suggested the involvement of the cell attachment domain Arg-Gly-Asp of S protein in the adherence process, the synthetic pentapeptide Gly-Arg-Gly-Asp-Ser (GRGDS) was tested for its ability to inhibit the attachment-promoting activity of S protein for endothelial cells. The peptide GRGDS as well as monospecific antibodies against S protein significantly suppressed the attachment-promoting activity not only of free S protein but also of the ternary STAT complex (Fig 3), indicating that the attachment-promoting activity resides in the S protein component of this complex. Antibodies against thrombin or antithrombin III did not affect the activity of the ternary complex.

Furthermore, the pentapeptide GRGDS interfered with the attachment-promoting function of S protein in a concentration-dependent fashion and reduced endothelial cell attachment to 50% for both types of cells at 20 to 40 μg/mL (40 to 80 μmol/L) peptide (Fig 4). At concentrations >100 μg/mL (200 μmol/L) GRGDS, <10% of endothelial cells attached within 2 hours of incubation. Unrelated peptides such as the acidic fibrinopeptide A, the fibrin polymerization-inhibiting peptide Gly-Pro-Arg-Pro, or the basic tri-decapeptide of the heparin-binding domain in S protein (positions 347 through 359) did not interfere with the attachment-promoting activity of S protein when tested at a concentration of 200 μg/mL (Fig 4). Similar results were obtained for the ternary STAT complex (data not shown).

Monospecific IgG fractions of antisera against other adhesive proteins were used to evaluate further the specificity of the promoting effect of S protein on attachment of endothelial cells. The presence of antibodies against S protein significantly reduced attachment of HUVECs as well as of HOTMECs to S protein-coated dishes to <10% of control

attachment.
beled S protein were performed. Experiments with cells in suspension were carried out with HUVECs as well as with HOTMECs and gave similar results. In a time-course binding experiment, the direct interaction of 10 nmol/L $^{125}$I-S protein with HUVECs was assessed without or with a 200-fold molar excess of unlabeled S protein. In parallel, cells were treated after selected time periods of reaction with

Fig 4. Inhibition of S protein-promoted endothelial cell attachment by the pentapeptide GRGDS. Attachment of HUVECs ($\bigcirc$) as well as HOTMECs ($\blacklozenge$) was examined after 2 hours at 37°C on Petri dishes precoated with 20 μg/mL S protein. Shortly before the cells were added, various concentrations of GRGDS were added to the wells. In parallel, the effect of unrelated peptides fibronopeptide A ($\blacktriangle$), Gly-Pro-Arg-Pro ($\blacklozenge$), and the synthetic tridecapeptide ($\blacktriangledown$) at 200 μmol/L on attachment of HOTMECs was documented. Buffer controls ($\square$, $\blacksquare$) are shown (lower left corner). Values are means of duplicate determinations.

(Table 1). In contrast, irrelevant rabbit IgG as well as antibodies against fibrogen, fibronectin, or vWF showed only some reduction of S protein-promoted cell attachment such that ~80% of the cells still became attached. Antibody solutions alone did induce only minimal endothelial cell attachment and were comparable in their effect to the HSA control. Antibodies did, however, significantly block attachment of endothelial cells when the related antigen was used as substratum (Table 1).

Binding of S protein to endothelial cells. To elucidate whether S protein-induced attachment and spreading of endothelial cells was related to the specific association of the protein molecule with the cells, binding studies with radiola-

![Graph showing inhibition of S protein-promoted endothelial cell attachment by GRGDS](image)

**Fig 5.** (A) Binding of $^{125}$I-S protein to HUVECs. Radiolabeled S protein (10 nmol/L) was incubated with HUVECs in suspension at a cell density of $1 \times 10^5$ cells/mL in SFM containing 1% (wt/vol) HSA without ($\blacktriangle$) or with ($\blacklozenge$) a 150-fold molar excess of unlabeled S protein. At selected time intervals, subsamples were withdrawn from the reaction mixtures and cell-associated radioactivity was determined. In parallel, a third incubation mixture was treated at various time points with trypsin prior to separation of unbound radioactivity ($\blacktriangle$). Values represent means of four determinations. (B) Autoradiographs of starting material (lane 1) and radiolabeled S protein that remained associated with the cells (total cell pellet) at the end of the experiment (lane 2). The mol-wt markers along the margin are given in thousands.

**Table 1. Specificity of S Protein-Induced Attachment of Endothelial Cells**

<table>
<thead>
<tr>
<th>Protein Coating*</th>
<th>Competitor</th>
<th>Cells Attached (% Total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S Protein</td>
<td>None</td>
<td>92 ± 5</td>
</tr>
<tr>
<td>S Protein</td>
<td>Anti-S protein</td>
<td>8 ± 2</td>
</tr>
<tr>
<td>S Protein</td>
<td>Antifibronectin</td>
<td>76 ± 6</td>
</tr>
<tr>
<td>S Protein</td>
<td>Antifibronogen</td>
<td>75 ± 10</td>
</tr>
<tr>
<td>S Protein</td>
<td>Anti-vWF</td>
<td>81 ± 12</td>
</tr>
<tr>
<td>S Protein</td>
<td>Rabbit IgG</td>
<td>78 ± 15</td>
</tr>
<tr>
<td>Fibrinectin</td>
<td>None</td>
<td>94 ± 7</td>
</tr>
<tr>
<td>Fibrinectin</td>
<td>Antifibronectin</td>
<td>22 ± 9</td>
</tr>
<tr>
<td>vWF</td>
<td>None</td>
<td>83 ± 8</td>
</tr>
<tr>
<td>vWF</td>
<td>Anti-vWF</td>
<td>12 ± 6</td>
</tr>
<tr>
<td>HSA</td>
<td>None</td>
<td>6 ± 4</td>
</tr>
</tbody>
</table>

*Protein concentrations were 0.25 μmol/L.

**HOTMECs as well as HUVECs gave similar results. A typical experiment with HOTMECs is shown; values are mean ± SD, n = 4.
0.03% trypsin for 3 minutes at 37°C and cell-associated radioactivity was determined. Association of 125I-S protein with HUVECs reached saturation after ~100 to 120 minutes of incubation at 37°C (Fig 5A). Total binding after 120 minutes represented between 1% and 3% of the total radioactivity applied, and nonspecific binding corresponded to ~16% to 25% of this value. Trypsin treatment of cells prior to separation from unbound radioactivity further reduced the amount of cell-associated S protein to <10%. For further experiments, S protein binding was studied after a reaction time of 120 minutes, after which >90% of the cells were viable as judged by trypan blue exclusion.

The molecular integrity of bound radiolabeled S protein was tested by analyzing the complete cell pellet after the binding experiment by SDS-PAGE, followed by autoradiography of the gels. The reduced radiolabeled starting material gave the characteristic doublet pattern with mol wt 78,000 and 65,000 (Fig 5B, lane 1), and the extract from endothelial cells showed a likewise indistinguishable protein band pattern (Fig 5B, lane 2), indicating that the radioactive material that interacted with the cells was related to S protein.

The specificity of S protein–endothelial cell interaction was further established by competition studies: Whereas unspecific binding of S protein (±150-fold molar excess of unlabeled ligand) accounted for ~20% of total binding as demonstrated in the previous experiment (Fig 5A), unlabeled S protein at physiologic concentration (4 μmol/L, 50-fold molar excess) inhibited specific binding of 80 nmol/L 125I-S protein by ~80% (Table 2). Likewise, physiologic concentrations of fibrinogen or vWF reduced specific binding of ~60% to 70%, whereas fibronectin affected binding by only ~20%. Except for unlabeled S protein, higher concentrations of the other adhesive proteins did not lead to further reduction of S protein binding (data not shown). Both Fab fragments of anti(S protein) IgG and the ternary STAT complex were effective in reducing binding of 125I-S protein significantly to ~30% to 40% of control.

### Table 2. Competition of Various Components With Specific 125I-S Protein Binding to HOTMEC

<table>
<thead>
<tr>
<th>Competitor</th>
<th>125I-S Protein Bound (cpm, Mean ± SD, n = 3)</th>
<th>Binding (% Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>6,660 ± 860</td>
<td>100</td>
</tr>
<tr>
<td>Unlabeled S protein (4 μmol/L)</td>
<td>1,460 ± 190</td>
<td>21</td>
</tr>
<tr>
<td>Fibrinectin (1 μmol/L)</td>
<td>5,270 ± 330</td>
<td>79</td>
</tr>
<tr>
<td>Fibrinogen (9 μmol/L)</td>
<td>2,100 ± 190</td>
<td>31</td>
</tr>
<tr>
<td>vWF (0.5 μmol/L)</td>
<td>2,500 ± 170</td>
<td>37</td>
</tr>
<tr>
<td>STAT* (0.4 μmol/L)</td>
<td>2,160 ± 660</td>
<td>32</td>
</tr>
<tr>
<td>Gly-Arg-Gly-Asp-Ser (200 μmol/L)</td>
<td>3,500 ± 440</td>
<td>52</td>
</tr>
<tr>
<td>Fibrinopeptide A (200 μmol/L)</td>
<td>6,080 ± 600</td>
<td>91</td>
</tr>
<tr>
<td>Tridecapeptide (200 μmol/L)</td>
<td>14,400 ± 530</td>
<td>216</td>
</tr>
<tr>
<td>Anti(S protein) Fab (300 μg/mL)</td>
<td>2,590 ± 340</td>
<td>39</td>
</tr>
<tr>
<td>Rabbit Fab (300 μg/mL)</td>
<td>6,600 ± 1,300</td>
<td>99</td>
</tr>
</tbody>
</table>

*ternary STAT complex, assuming an apparent mol wt of 350,000.

Although the cell attachment pentapeptide GRGDS inhibited S protein binding by ~50%, neither the unrelated fibrinopeptide A nor the synthetic tridecapeptide (all at 200 μmol/L) interfered with binding. This latter heparin-binding peptide of S protein consistently increased specific binding of the protein by a factor of 2.

Binding of S protein to HUVECs was further characterized by using different concentrations of radiolabeled ligand. Saturation of binding was observed (Fig 6A), indicating the presence of a limited number of binding sites. When data were expressed in a Lineweaver-Burk plot, the analysis of the dissociation constant revealed an estimated value of Kd = 0.18 μmol/L (Fig 6A, insert), with a maximal number of binding sites of 2 to 3 x 10⁶. In an independent competition...
experiment, two different concentrations of $^{125}$I-S protein were allowed to bind to HUVECs in the presence of increasing concentrations of unlabeled S protein. Data were expressed in a Dixon plot and yielded an estimated dissociation constant $K_d = 0.2 \mu\text{mol/L}$, which is in good agreement with the former value (Fig 6B). Likewise, values of $K_d = 0.17$ to $0.2 \mu\text{mol/L}$ were determined from binding experiments with HOTMECs.

**DISCUSSION**

Adhesive proteins of the blood coagulation system, such as fibrinogen or vWF, the former by acting as cofactor for platelet aggregation, the latter by inducing the initial attachment and spreading of platelets to subendothelial matrices, are known as members of the substrate adhesion molecules family, all containing a unique cell attachment domain. Two other components of this family, S protein (vitronectin) and fibronectin, have been described as major attachment proteins for fibroblastic cells in plasma or serum. Among these four adhesive proteins, S protein (vitronectin) is unique since it exerts multiple regulatory functions in the terminal stage of coagulation by neutralizing heparin's anticoagulant function and by associating with the thrombin-antithrombin III complex. These diverse activities of S protein may particularly be expressed at the blood-endothelium interface, where important intrinsic mechanisms for regulation of thrombin activity occur. To approach the role of S protein in these vessel wall-related reactions, interactions of the adhesive protein with endothelial cells had to be defined.

Because S protein induced maximal attachment and spreading of HUVECs as well as HOTMECs in vitro within an incubation of 1 to 2 hours at 37°C at a concentration between 1 in 100 and 1 in 1,000 of its plasma concentration, we conclude that attachment and/or spreading of endothelial cells may also be significantly promoted by S protein in vivo. The rate of attachment and the type of spreading in response to S protein was virtually identical to that obtained on substrata coated with fibrinogen or fibronectin, with the organization of thick microfilament bundles of the stress fiber type and formation of focal contacts. With other cell types in culture, comparable low concentrations of vitronectin are sufficient to produce marked effects on spreading and differentiation. In our experiments, fibronectin and S protein showed comparable attachment-promoting activity in the same concentration range, suggesting that S protein is a major plasma component inducing endothelial cell adherence.

The relatively weak binding of the synthetic RGD-containing pentapeptide to other cells with a $K_d$ of $10^{-3}$ to $10^{-4}$ mol/L may account also for the low-affinity binding to endothelial cells and thus for the high concentration of the peptide required to obtain appreciable inhibition of attachment-promotion activity of S protein. Furthermore, the present findings also imply that S protein may be applicable for precoating Petri dishes for in vitro culture of endothelial cells similarly to the method used for fibronectin. Moreover, the coating of prosthetic vascular grafts with S protein prior to endothelial cell seeding may induce sufficient initial endothelial cell adherence and subsequent cell retention, as has been recently described for fibronectin.

Although S protein has only been attributed a scavenger molecule for the inactive thrombin-antithrombin III complex, our data rather suggest a prominent and novel functional role for the ternary STAT complex. This ternary complex induced endothelial cell attachment and spreading, virtually identical to free S protein. The adhesive properties of the ternary complex may become potentiated by the concomitant presence of both S protein and thrombin, since the latter has been described as mitogen. Moreover, since positions 413 through 415 of the B-chain of thrombin also contain the RGD cell-attachment sequence, an additional putative adhesive function for thrombin may even be proposed. Thus, the combined adhesive and mitogenic functions within the ternary complex may facilitate cell migration, cell attachment, and subsequent cell growth at the site of vessel-wall injury.

Binding experiments performed in the present study supported the concept of a cell attachment domain-dependent interaction of free S protein and of S protein within the ternary complex with endothelial cells in suspension. Although binding of free S protein appeared to be specific, as suggested from displacement with unlabeled ligand, antibodies, and the ternary complex, similar reduction of S protein binding to the cells was also observed with physiologic concentrations of fibrinogen or vWF as well as the RGD-containing peptide but not with fibronectin. These data are not only an indication for the limited recognition specificity of the binding site(s) for S protein on endothelial cells, but clearly demonstrate that this receptor site recognizes S protein at least in part in a RGD-dependent manner. Binding of the various RGD-containing adhesive proteins with overlapping specificity appears to be mediated by a family of receptor molecules, designated cytoadhesions, including surface receptors on osteosarcoma cells for fibronectin and vitronectin as well as the platelet glycoprotein IIb/IIIa complex. In particular, cultured endothelial cells do synthesize receptor molecules related to the glycoprotein IIb/IIIa complex and the recent observation that an RGD-dependent endothelial cell receptor shows specific interaction with fibrinogen, vWF, and S protein (vitronectin) but not with fibronectin confirms our direct binding data. Whether the RGD-dependent binding site on endothelial cells only recognizes a specific conformation of the according epitope in S protein or whether additional recognition sites are operative in S protein binding is unclear, however, since the same concentration of GRGDS that totally inhibited endothelial cell attachment to S protein produced only 50% inhibition of S protein binding.

From the present findings as well as from the fact that S protein may interact with vessel wall-derived heparan sulfate compounds, thereby neutralizing their anticoagulant activity, we may infer that, in addition, the heparin-binding domain of S protein is involved in its specific association with endothelial cells.

The reported rapid clearance rate of exogeneously administered thrombin-antithrombin III complex from the circulation in animals may be due to complex formation with free...
S protein. Alternatively, endothelial cell-associated S protein may be hypothesized to provide a recognition site for the thrombin-antithrombin III complex, such that the ternary complex may be formed on the endothelial cell surface. Subsequently, attachment, spreading, and migration of cells would be localized adjacent to the vascular site of injury.

In contrast to fibrinogen, S protein was not proteolytically modified during the binding process and only minor amounts may have become internalized within the cells. Although the affinity of S protein to endothelial cells in suspension appears to be similar to that of fibrinogen, differences may arise in the specificity when binding to single-cell suspensions and to endothelial cell monolayers is compared. Differences may be interpreted in terms of variations of receptor distribution because endothelial cell monolayers are polarized whereas cells in suspension apparently are not.

Endothelial cells themselves contained appreciable quantities of S protein as judged by indirect immunofluorescence on cell monolayers. By analogy with the production and storage of vWF as well as fibronectin in these cells, endothelial cell-derived S protein may also become secreted and may thereby induce attachment and spreading of cells at the site of injury. Because fibronectin and vWF are believed to be crucial in platelet and/or endothelial cell adherence to the subendothelial matrix, a similar role may thus be attributed to by S protein, in particular if the endothelial cells are a source for this adhesive protein. Because S protein has been identified in the extracellular matrix of certain cell types, it is also intriguing to extrapolate that S protein may be a constituent of the subendothelial matrix, which contains a variety of adhesive proteins such as fibronectin, vWF, thrombospondin, laminin, and collagens. The recently observed affinity of S protein for collagens may be an additional indication for this assumption and for the important role of this adhesive protein in endothelial cell-related phenomena.

The regulatory role of S protein (vitronectin) in the coagulation system and its here-described but yet unrecognized functions to bind to endothelial cells and promote their attachment and spreading point to its central position in vessel wall-related inflammatory processes.

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