Protein C Inhibits Endocytosis of Thrombin–Thrombomodulin Complexes in A549 Lung Cancer Cells and Human Umbilical Vein Endothelial Cells

By Ikuo Maruyama and Philip W. Majerus

We investigated the effect of protein C on the endocytosis of thrombin–thrombomodulin complexes. We previously showed that exposure of umbilical vein endothelial cells to thrombin stimulated the internalization and degradation of thrombin. A similar internalization was stimulated by a monoclonal antithrombomodulin antibody. We have repeated these studies in the presence of protein C and found that endocytosis of 125I-thrombin–thrombomodulin complexes, but not 125I-antithrombomodulin–thrombomodulin complexes, is inhibited. Activated protein C did not inhibit endocytosis of thrombin–thrombomodulin complexes.

Rapid Protein C activation by thrombin depends on the endothelial cell surface protein, thrombomodulin.1,2 Thrombin binds to thrombomodulin in a 1:1 complex that catalyzes protein C activation.3,4 Previously, we found that exposure of cultured human umbilical vein endothelial cells or A549 human lung cancer cells to thrombin decreased thrombomodulin on the cell surface, suggesting that internalization of thrombin–thrombomodulin had occurred. The internalized 125I-thrombin was degraded in the cells, and thrombomodulin reappeared on the cell surface after 30 minutes, implying the recycling of thrombomodulin.3 From these observations, we proposed that the uptake and degradation of thrombin bound to thrombomodulin on the endothelial cell surface may stop protein C activation and provide a mechanism for clearance of thrombin from the circulation. Our previous results indicated that within 30 minutes of exposure to thrombin, all active thrombin–thrombomodulin complexes were cleared from the surface of A549 cells. This result was in apparent conflict with the original study of Esmo and Owen1 who showed a linear rate of protein C activation for at least 1 hour when thrombin and protein C were incubated with human umbilical vein endothelial cells. We studied endocytosis of thrombin–thrombomodulin complexes in the presence of protein C to resolve this apparent conflict, and found that protein C, but not activated protein C, inhibits the endocytosis of thrombin–thrombomodulin complexes both in human umbilical vein endothelial cell cultures and in A549 cell cultures.

Materials and Methods

Materials were obtained from the following sources: Medium 199 (M199) and fetal calf serum (FCS) from KC Biological (Lenexa, KS); penicillin, streptomycin, and L-glutamine from Gibco Laboratories (Grand Island, NY); carrier-free 125I-sodium iodide from Amersham (Arlington Heights, IL); tissue culture flasks and centrifuge tubes from Corning Glass Works (Science Products, Corning, NY); and Falcon Labware (Becton Dickinson, Oxnard, CA); Clusters 24-, 48-dish wells from Costar (Cambridge, MA); and protein A-Sepharose from Pharmacia Fine Chemicals (Piscataway, NJ). All other chemicals were reagent grade products of Sigma Chemical (St Louis) or Fisher Scientific (Pittsburgh). Protein C6 and thrombin7 were of human origin and isolated as indicated. Protein S from human plasma was provided by Dr Koji Suzuki (Mie University School of Medicine, Tsu, Japan). The proteins were homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) with bovine serum albumin (BSA) as standard. Monoclonal antithrombomodulin antibody was produced as described previously.5 Antithrombomodulin monoclonal IgG (designated IgG–TM) was isolated from ascites using protein A-Sepharose.7 Protein C was fully activated by thrombin, and the activated protein was subsequently isolated.5 Protein iodination. 125I–TM (7,000 to 20,000 cpm/ng) and thrombin (1,000 to 2,000 cpm/ng) were labeled with 125I–iodine by a modified chloramine T procedure.9 Protein was separated from free iodide on a Sephadex G-25 column. The specific activity in a coagulation assay of the α-thrombin used was 2,200 NIH U/mg of protein. Iodinated thrombin was treated with diisopropyl fluorophosphate (DFP) as described previously.5 Unreacted DFP was removed by gel filtration on a column of Sephadex G-25.

Cell cultures. Human umbilical vein endothelial cells were prepared by the method of Jaffe and co-workers.9 A human lung cancer (A549) cell line was obtained from the American Type Culture Collection. Cells were grown in 6- to 16-mm multidiwell plates using M199 containing 20% FCS, 2 mM/g/L of glucose, 100 U/ml of penicillin, and 100 μg/mL of streptomycin in a 5% CO2 atmosphere at 37°C. Cell number was determined using a Coulter Counter model F (Coulter Electronics, Hialeah, FL).9

Binding studies. Confluent monolayers of human umbilical vein endothelial cells in 10-mm culture wells were washed three times with 0.14 mol/L of NaCl, 0.004 mol/L of KCl, 0.001 mol/L of phosphate buffer, pH 7.4, 0.011 mol/L of glucose containing 0.2% BSA (henceforth referred to as wash buffer). Binding studies were carried out in reaction mixtures containing M199 supplemented...
with 2.5 mg/mL of ovalbumin and 10 mmol/L of Heps, pH 7.4 (thrombin-binding medium) for \( ^{125}\text{I}-\text{thrombin} \) binding, or M199 containing 5 mg/mL of BSA and 50 \( \mu \text{g/mL} \) normal human IgG (IgG-TM binding buffer) for \( ^{125}\text{I}-\text{IgG-TM} \) binding in a total volume of 0.3 mL. After incubation of cells with \( ^{125}\text{I}-\text{labeled protein} \), the cells were washed rapidly five times with cold (0°C) wash buffer.

The washed monolayers were solubilized in 0.5 mL of buffer containing 15% glycerol, 2% sodium dodecyl sulfate (SDS), 75 mmol/L of Tris-HCl (pH 6.8), and 2 mmol/L of EDTA (cell-solubilizing buffer). The solubilized cell solution was assayed for radioactivity in a Biogamma II scintillation counter (Beckman Instruments, Fullerton, CA). Specific binding was determined by measuring the difference in cell-bound radioactivity with and without 100-fold excess unlabeled ligand. The cell-bound radioactivity with an excess amount of unlabeled ligand was considered nonspecific and was subtracted from the total binding. Nonspecific binding was 20% to 40% of total binding using \( ^{125}\text{I}-\text{thrombin} \) and 10% to 25% of total binding using \( ^{125}\text{I}-\text{IgG-TM} \). In experiments in which endocytosis and degradation of thrombin were measured, bound thrombin was estimated as that displaced by unlabeled thrombin, degraded thrombin was estimated as TCA soluble radioactivity in the medium, and internalized thrombin was that not displaced by unlabeled thrombin after 60 minutes at 4°C.

**RESULTS**

*Inhibition by protein C of the endocytosis of thrombin- or diisopropylphosphoryl (DIP)-thrombin-thrombomodulin complexes in human umbilical vein endothelial cells.* We previously showed that thrombomodulin disappears from the surface of human umbilical vein endothelial cells after 5-minute exposure to thrombin. Cell surface thrombomodulin was estimated by measuring the binding of \( ^{125}\text{I}-\text{IgG-TM} \) at various times after exposure of cells to thrombin. We repeated this experiment with and without protein C, as shown in Fig 1. Exposure of human umbilical vein endothelial cells to thrombin or DIP-thrombin resulted in the reduction of thrombomodulin molecules on the cell surface. The decrease in cell surface thrombomodulin was greater than that induced by thrombin alone. The effect of protein C on IgG-TM-induced decrease of thrombomodulin molecules on human umbilical vein endothelial cells. Monoclonal anti-thrombomodulin IgG, designated IgG-TM, also induces a decrease in thrombomodulin molecules on endothelial cells. As shown in Fig 2, exposure of human umbilical vein endothelial cells to IgG-TM resulted in the reduction of thrombomodulin molecules on the cell surface. The decrease in surface thrombomodulin was greater than that induced by thrombin or DIP-thrombin. Reappearance of thrombomodulin molecules on the cell surface before exposure to thrombin was 44,000 molecules per cell.

**Table 1. Effect of Protein C or Activated Protein C on Thrombin-Induced Endocytosis of Thrombomodulin in Human Umbilical Vein Endothelial Cells**

<table>
<thead>
<tr>
<th>Agent</th>
<th>Thrombomodulin (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein C (( \mu \text{mol/L} ))</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>0.03</td>
<td>70</td>
</tr>
<tr>
<td>0.06</td>
<td>77</td>
</tr>
<tr>
<td>0.12</td>
<td>99</td>
</tr>
<tr>
<td>Activated protein C</td>
<td></td>
</tr>
<tr>
<td>0.06</td>
<td>61</td>
</tr>
<tr>
<td>0.12</td>
<td>63</td>
</tr>
</tbody>
</table>

Confluent monolayers of human umbilical vein endothelial cells \((1 \times 10^5)\) were washed three times with wash buffer and then exposed to 8 nmol/L of unlabeled thrombin for 5 minutes at 37°C. The monolayers were washed once with wash buffer at 0°C, and \( ^{125}\text{I}-\text{IgG-TM} \) binding medium was added with \( [\bullet=\bullet] \) or without \( [\bullet=[\bullet] \) 0.12 \( \mu \text{mol/L} \) of protein C. At the times indicated, \( ^{125}\text{I}-\text{IgG-TM} \) (17 nmol/L) was added and after a 5-minute incubation at 37°C bound \( ^{125}\text{I}-\text{IgG-TM} \) (15,000 cpm/ng) was determined as described in the Materials and Methods section. Nonspecific binding was 6,000 cpm/well; total binding before exposure to thrombin was 37,000 and 36,000 cpm/well.

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*The 100% control value for cell surface thrombomodulin (thrombomodulin molecules on the cell surface before exposure to thrombin) was 44,000 molecules per cell.*
PROTEIN C INHIBITS ENDOCYTOSIS

Fig 2. Thrombomodulin endocytosis on human umbilical vein endothelial cells after exposure to IgG-TM. Monolayers of human umbilical vein endothelial cells were washed three times with wash buffer, then exposed to 25 nmol/L of unlabeled IgG-TM for 5 minutes at 37°C. The monolayers were washed once with wash buffer at 0°C, and 125I-IgG-TM binding medium was added with (O) or without (△) 0.1 μmol/L of protein C or activated protein C (A). At times indicated, 125I-IgG-TM (25 nmol/L) was added, and after a 5-minute incubation at 37°C, bound 125I-IgG-TM (7,500 cpm/ng) was determined. Nonspecific binding in this experiment was 6,500 cpm. Total bound before exposure to unlabeled IgG-TM was 23,000 cpm/well.

Effect of protein C on the time course of binding, internalization, and degradation of 125I-DIP-thrombin in A549 cells. We investigated the effect of protein C on the time course of binding, internalization, and degradation of 125I-DIP-thrombin in A549 Cells at 37°C, as shown in Fig 3. At each incubation period, monolayers were washed with cold wash buffer, and fresh thrombin-binding medium (300 μL) containing 100 nmol/L of unlabeled DIP-thrombin was added and incubated at 4°C for an additional 60 minutes. TCA soluble radioactivity from the first incubation medium reflects degraded 125I-DIP-thrombin, and displaced radioactivity by unlabeled excess DIP-thrombin in the second incubation reflects cell surface bound 125I-DIP-thrombin. As shown in the top panel of Fig 3, there was no significant difference in surface-bound 125I-DIP-thrombin in experiments with or without protein C. 125I-DIP-thrombin degradation (middle panel) and 125I-DIP-thrombin internalization (bottom panel), however, were significantly inhibited by the addition of protein C. Next, we investigated the inhibition of endocytosis and degradation of 125I-DIP-thrombin at varying protein concentrations, as shown in Fig 4. In this experiment, A549 cell monolayers were incubated with or without various amounts of protein C and 2.5 nmol/L of 125I-DIP-thrombin for 4 hours. Protein C inhibited endocytosis and degradation of 125I-DIP-thrombin but not the binding to the cell surface. There was 40% to 70% inhibition of endocytosis and degradation at the highest concentration of protein C used.

DISCUSSION

In this study, we investigated the effect of protein C or activated protein C on thrombin-, DIP-thrombin-, or IgG-TM-induced endocytosis of thrombomodulin in cultured human umbilical vein endothelial cells and in A549 human lung cancer cells. Protein C, but not activated protein C, at physiological concentrations inhibited thrombin or DIP-thrombin-induced endocytosis of thrombomodulin and subsequent degradation of thrombin or DIP-thrombin on human umbilical vein endothelial and A549 cells. IgG-TM also induced endocytosis of cell-surface thrombomodulin in
human umbilical vein endothelial cells and A549 cells, but protein C did not inhibit this reaction. Protein S, a potentiation of the activity of activated protein C in the inactivation of coagulation factors Va and VIIIa had no effect on the protein C inhibition of endocytosis of thrombomodulin. The physiological significance of the finding that protein C inhibits thrombin-induced endocytosis of thrombomodulin in vitro remains uncertain. On the basis of our findings, we propose that protein C in situ may serve to regulate thrombin-thrombomodulin complex on endothelial cells, thereby inhibiting procoagulant reactions of thrombin and lead to protein C activation. The activated protein C formed may then combine with protein S, bind to another site on endothelium where factors Va and VIIIa are inactivated, and thereby inhibit fibrin formation and act as a local anticoagulant. Once protein C is depleted locally, endocytosis of thrombin–thrombomodulin complex and subsequent degradation of thrombin may occur. Thus, endocytosis of thrombin–thrombomodulin and regulation of this process by protein C may provide a control mechanism for the thrombin–thrombomodulin system on the endothelial cell surface.

**REFERENCES**

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