Histidine-Rich Glycoprotein Does not Interfere With Interactions Between Antithrombin III and Heparin-Like Compounds on Vascular Endothelial Cells

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The role of histidine-rich glycoprotein in controlling heparin-like compounds on the endothelial cell surface is still unclear. The effects of this heparin-neutralizing protein on the interaction between antithrombin III and cultured porcine aortic endothelial cells were examined. Displacement of $^{125}$I-labeled antithrombin III specifically bound to endothelial cells by unlabelled histidine-rich glycoprotein was much less potent than that by unlabelled antithrombin III. One hundred-fold molar excess of histidine-rich glycoprotein displaced specific $^{125}$I-antithrombin III binding only by 20%. Furthermore, the endothelial cell-mediated acceleration of thrombin inactivation by antithrombin III was diminished by protamine sulfate, but was not affected by histidine-rich glycoprotein even at a histidine-rich glycoprotein/antithrombin III molar ratio of ~7:1. These data indicate that histidine-rich glycoprotein does not interfere with the interaction of endothelial cell heparin-like compounds with antithrombin III. Thus, it may not play an important role in the modulation of anticoagulant activity of endothelial cells in vivo, suggesting that the commonly accepted view of the probable function of this protein is erroneous.

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THE EXPRESSION OF the biologic effects of endogenous or exogenous heparin-like compounds in vivo may be restricted by the action of various binding and neutralizing proteins. Platelet factor 4, which is released from activated platelets, has been confirmed to inhibit the acceleration of thrombin-antithrombin complex formation by endogenous heparin-like material in vivo. Recently, a second major heparin-neutralizing protein has been identified called histidine-rich glycoprotein. It inhibits the anticoagulant effect of exogenous heparin in plasma in the presence of divalent cations. Histidine-rich glycoprotein is present in plasma in much higher concentrations (~100 μg/mL) than is platelet factor 4; thus, a role for this protein in modulating activities of endogenous heparin-related compounds within the circulation has been suggested. Heparan sulfates isolated from human aorta, however, recently have been shown to be relatively resistant to neutralization by histidine-rich glycoprotein compared with that by platelet factor 4.

We have performed the experiments to directly investigate whether histidine-rich glycoprotein influences the binding of antithrombin III with endothelial cell heparan sulfate, and inhibits heparin-like activity of the cell using purified ligands and endothelial cell cultures.

METHODS

Endothelial cell cultures. Aortic endothelial cells were isolated from the pig descending thoracic aorta and cultured in gelatin precoated 35-mm petri dishes containing RPMI-1640 medium supplemented with 30% fetal calf serum and antibiotics, as previously described. Cells in the second to third passage were used throughout the present study.

Purification and iodination of proteins. Porcine antithrombin III was purified by affinity chromatography using heparin-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden) according to the method of Koide et al. Purified antithrombin III had a specific activity of 6.6 U/mg protein, based on measurements of heparin cofactor activity of antithrombin III. Porcine histidine-rich glycoprotein was purified by two consecutive chromatographic procedures including heparin-Sepharose and DEAE-Sephacel according to the method of Koide et al. The purified proteins were homogenous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), corresponding to a molecular weight of 58,000 and 75,000, respectively.

Purified antithrombin III was labeled with $^{125}$I using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3,6-diphenyl glycoluril (IODogen, Pierce Chemical Co., Rockford, IL), as previously described. The specific radioactivity was ~1 x 10$^6$ cpn/mg. Neither heparin cofactor activity nor migration distance on gel electrophoresis in dodecyl sulfate was altered by the iodination procedure.

Binding experiments. Binding of antithrombin III to endothelial cell cultures was performed as previously described. Briefly, labeled antithrombin III with or without unlabelled proteins was placed upon the washed endothelial cell cultures in 0.2 mL of Hanks' balanced salt solution (HBSS) containing 1 mg/mL of bovine serum albumin for 15 minutes at 37°C. The medium was suctioned off and the cell layer was rinsed and then trypsinized. The cell suspension was centrifuged at 1,000 g for 10 minutes. The radioactivity in the supernatant was considered to represent antithrombin III bound to the cell surface; the radioactivity in the pellet represented radiolabeled protein internalized by the cells and accounted for <10% of total radioactivity associated with cells.

Heparin-like activity of endothelial cells. Inactivation of thrombin by antithrombin III in the presence of endothelial cells was performed by incubating bovine thrombin (3,900 NIH U/mg, provided by Dr Morita, Meiji Pharmaceutical University, Tokyo) in a petri dish together with antithrombin III. Washed endothelial cells were incubated with 50 μL of 0.15 mol/L NaCl, 2 mmol/L CaCl$_2$, and 0.01 mol/L Tris-HCl (pH 7.4) containing antithrombin III with or without heparin-neutralizing proteins for at least three minutes at 22°C, followed by the addition of 37.5 μL of the same buffer containing thrombin. The final concentrations of antithrombin III and thrombin were 296 nmol/L and 9.4 nmol/L, respectively. The reactions were performed at 22°C for five minutes. Residual thrombin activity was then determined by adding 25 μL of the chromo-
genic substrate (final concentration 0.48 mmol/L) to an incubation mixture and incubating it for ten minutes at 37°C. Amidolysis was stopped by the addition of 625 µL of citric acid (0.15%). The reaction mixture was removed and centrifuged at 12,000 rpm for five minutes. The absorbance of the supernate was read at 405 nm. Thrombin activity was linear with the amount of the enzyme in the assay system.

The influence of the addition of different divalent metal ions to the assay system was further investigated. Therefore, experiments were repeated with the addition of Zn²⁺, Cu²⁺, or Fe²⁺ (20 µmol/L final concentration) substituting Ca²⁺, with controls in the presence of ethylenediaminetetraacetic acid (EDTA, 10 µmol/L final concentration). The influence of divalent metal ions or EDTA on interference of histidine-rich glycoprotein with the interaction between antithrombin III and heparin was determined as follows. The reactions were performed at 37°C in 0.15 mol/L NaCl, 0.01 mol/L Tris-HCl (pH 7.4) that contained different divalent metal ions (20 µmol/L) or EDTA (10 µmol/L). Histidine-rich glycoprotein (222 nmol/L final concentration) was incubated together with antithrombin III (296 nmol/L), heparin (78 ng/mL, 183.3 IU/mg), and thrombin (9.4 nmol/L). After incubation for 30 seconds, residual enzyme concentration was determined.

RESULTS

Effects of histidine-rich glycoprotein on ¹²⁵I-antithrombin III binding to endothelial cells. Incubations of ¹²⁵I-antithrombin III with endothelial cells resulted in a time-dependent saturable binding that reached equilibrium by 15 minutes at 37°C. Endothelial cell heparan sulfate was previously shown to be responsible for this binding.¹⁰ Specific binding, which is defined as ¹²⁵I-antithrombin III binding displaceable by 100-fold excess amount of unlabeled protease inhibitor, accounted for ~70% of total binding. Competitive binding studies (Fig 1) showed progressive inhibition of ¹²⁵I-antithrombin III-endothelial cell binding in the presence of increasing concentrations of unlabeled antithrombin III. Histidine-rich glycoprotein had a much less effect on specific ¹²⁵I-antithrombin III binding to endothelial cells. One hundred-fold molar excess of histidine-rich glycoprotein displaced specific ¹²⁵I-antithrombin III binding only by 20%.

Effect of histidine-rich glycoprotein on endothelial cell heparin-like activity. Inactivation of thrombin by antithrombin III was significantly accelerated in the presence of endothelial cells (Fig 2). This antithrombin-cofactor (heparin-like) effect of the endothelial cells was greatly diminished by protamine sulfate (140 µg/mL), but was not affected by histidine-rich glycoprotein even at a histidine-rich glycoprotein/antithrombin III molar ratio of ~7:1, which was a 30-fold higher relative concentration than in physiologic conditions. Controls in which thrombin was incubated with endothelial cell cultures under identical conditions without antithrombin III demonstrated no loss of thrombin activity. In the absence of cells, neither histidine-rich glycoprotein nor protamine sulfate affected inactivation of thrombin by antithrombin III.

Even in the presence of 20 µmol/L of Zn²⁺, Cu²⁺, or Fe²⁺, histidine-rich glycoprotein did not affect the heparin-like antithrombin-cofactor activity of endothelial cells. It did inhibit thrombin inactivation by heparin-antithrombin III completely (Zn²⁺, Cu²⁺) or by 70% (Fe²⁺), as compared

![Graph 1](image1.png)

Fig 1. Displacement of ¹²⁵I-antithrombin III bound to endothelial cells by unlabeled antithrombin III and histidine-rich glycoprotein. Antithrombin III (8.8 nmol/L) was incubated with endothelial cells (108 µg per dish) in the absence or presence of various concentrations of unlabeled antithrombin III (++) or histidine-rich glycoprotein (C) for 15 minutes at 37°C. ¹²⁵I-antithrombin III specifically bound to cells at each concentration was obtained as described in Methods and expressed as a percent of the specific binding obtained in the absence of unlabeled proteins (114 femtomole/mg cell protein). Data are representative of two separate experiments with similar results.

![Graph 2](image2.png)

Fig 2. The effects of heparin-neutralizing proteins on endothelial cell heparin-like activity. Inactivation of thrombin (9.4 nmol/L) by antithrombin III (AT III, 296 nmol/L) on endothelial cell cultures (cell, +) or in a test tube (cell, −) was performed in the absence (−) or presence (+) of two different concentrations of histidine-rich glycoprotein (HRG, +; 222 nmol/L, + +: 2.22 µmol/L) or protamine sulfate (PS, 140 µg/mL) as described in Methods. Data represent the mean ± SEM of four determinations.
REFERENCES

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with no inhibition in the presence of EDTA (mean of duplicates). The displacement of specific 125I-antithrombin binding by histidine-rich glycoprotein in the presence of divalent metal ions was also not different from that in the presence of EDTA.

DISCUSSION

The various biochemical and functional properties of histidine-rich glycoprotein suggest apparently diverse physiologic roles of this protein in vivo.7 Its precise biologic function, however, has not been established yet. Our data have shown that histidine-rich glycoprotein has much less affinity for antithrombin III binding sites on endothelial cells than the protease inhibitor itself, and that it does not interfere with the endothelial cell-mediated acceleration of thrombin inactivation by antithrombin III even in the presence of different divalent metal ions. Thus, it seems unlikely that histidine-rich glycoprotein plays any physiologic role in controlling the endogenous vascular heparin-like compounds. This is in accordance with previous biochemical experiments showing a relatively low affinity of this protein for isolated heparan sulfates.4

These findings have implications for the expression of endogenous heparin-like activity on the vascular endothelium in vivo. The very low plasma concentration of platelet factor 4, which is released only at the site of vascular injury, suggests a highly localized procoagulant role for this heparin-neutralizing protein. On the other hand, another major heparin-neutralizing protein, histidine-rich glycoprotein, is always present in plasma in much higher concentrations, and cannot interfere with vascular heparin-like material at a concentration attained in clinical conditions. Therefore, it allows this endogenous anticoagulant mechanism to fully express its activity at the normal vascular surface in vivo.

REFERENCES


5. Shimada K, Ozawa T: Evidence that cell surface heparan sulfate is involved in the high affinity thrombin binding to cultured porcine aortic endothelial cells. J Clin Invest 75:1308, 1985


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