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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EDTA (10 μmol/L) or by the stopped genic substrate (final concentration 0.48 mmol/L) to an incubation of histidine-rich glycoprotein with the interaction between antithrombin III and heparin was determined as follows. The absorbance of the supernate was read at 405 nm. Amidolysis 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 Fe³⁺ (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. Specific binding, which is defined as ¹²⁵I-antithrombin III binding displaceable by 100-fold excess amount of unlabeled protein 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 Fe³⁺, 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% (Fe³⁺), as compared

**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 (○) 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.

**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 sulfates (PS, 140 μg/mL) as described in Methods. Data represent the mean ± SEM of four determinations.
with no inhibition in the presence of EDTA (mean of duplicates). The displacement of specific $^{125}$I-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. 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.

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