Histidine-rich glycoprotein (HRG) circulates in plasma at a concentration of 2μM and binds plasminogen, fibrinogen, and thrombospondin. Despite these interactions, the physiologic role of HRG is unknown. Previous studies have shown that mice and humans deficient in HRG have shortened plasma clotting times. To better understand this phenomenon, we examined the effect of HRG on clotting tests. HRG prolongs the activated partial thromboplastin time in a concentration-dependent fashion but has no effect on tissue factor–induced clotting, localizing its effect to the contact pathway. Plasma immunodepleted of HRG exhibits a shortened activated partial thromboplastin time that is restored to baseline with HRG replenishment. To explore how HRG affects the contact pathway, we examined its binding to factors XII, XIIa, XI, and Xla. HRG binds factor XIIa with high affinity, an interaction that is enhanced in the presence of Zn2+, but does not bind factors XII, XI, or Xla. In addition, HRG inhibits autoactivation of factor XII and factor XIIa–mediated activation of factor XI. These results suggest that, by binding to factor XIIa, HRG modulates the intrinsic pathway of coagulation, particularly in the vicinity of a thrombus where platelet release of HRG and Zn2+ will promote this interaction. (Blood. 2011;117(15):4134-4141)

Introduction

Despite the capacity of the intrinsic pathway to enhance thrombin generation, patients deficient in factor XII (FXII) do not bleed.1 Even patients with FXI deficiency rarely have hemorrhagic complications, except with surgery or major trauma.2 Because of these observations, it is well accepted that the contact system plays little part in hemostasis; and, by extension, it was also thought to be unimportant for thrombosis. However, a number of recent studies challenge this thinking. First, mice with deficiencies of high-molecular-weight kininogen (HK), bradykinin B2 receptor, FXII, or FXI are protected against injury-induced thrombosis, and an antibody against FXI inhibits thrombus formation in a baboon arteriovenous shunt model.3-7 These observations raise the possibility that the contact pathway contributes to thrombogenesis.8 Second, potential physiologic activators of FXII have been identified, which could initiate the contact system at sites of vascular injury. In addition to glycosaminoglycans and collagen, novel activators include polyphosphates and RNA.9,10 Polyphosphates, which are released from the dense granules of platelets activated at sites of injury, trigger coagulation in a FXII-dependent fashion. Likewise, RNA released from the damaged vessel wall also can activate FXII, and RNAase administration to animals attenuates thrombosis at sites of injury, observations that have sparked a renewed interest in the contact pathway.10 Consequently, it is important to better understand this pathway and how it is regulated.

Histidine-rich glycoprotein (HRG) is an abundant plasma protein whose role is largely unknown.11 HRG circulates at a concentration of approximately 2μM. Its concentration may increase locally because HRG also is found in α-granules of platelets where it can be released on activation.12 HRG is a modular protein composed of 2 NH2-terminal cystatin-like domains, a central histidine-rich region, and a COOH-terminal domain. These domains contribute to binding of Zn2+ and numerous ligands, including plasminogen, fibrinogen, thrombospondin, and heparan sulfate. Because of its multiple interactions, it has been hypothesized that HRG contributes to hemostasis as an accessory or adapter protein.11,13 In support of this concept, HRG-deficient mice exhibit shortened clotting times.14 Furthermore, HRG has been shown to inhibit contact activation of plasma in vitro, presumably because the histidine-rich region of HRG binds to negatively charged surfaces and prevents autoactivation of FXII.15 Modulation of the contact system may impact thrombosis because studies have linked deficiency of HRG in humans with thrombophilia.16 The current study was undertaken to more precisely define the role of HRG in the modulation of coagulation.

Methods

Reagents

Deaminated heparin (molecular weight 6700), biotinaminidohexanoic acid hydrazide (biotin-hydrazide), Protease Inhibitor Cocktail for His-Tagged Proteins, and silica (0.5-10 μm particle size) were from Sigma-Aldrich. Dextran sulfate (500 kDa) was from GE Healthcare. Sulfosuccinimidyl-6-biotinamido hexanoate was from Thermo Fisher Scientific. All coagulation


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Histidine-rich glycoprotein binds factor XIIa with high affinity and inhibits contact-initiated coagulation

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factors and corn trypsin inhibitor (CTI) were from Enzyme Research Laboratories. A sheep polyclonal antibody against human HRG, FXII-deficient plasma, and the FXII enzyme immunoassay kit were from Affinity Biologicals. Chromogenic substrates S-2302, S-2366, and S-2251 were from DiaPharma Group, Biopen CS11–22 was from Aniara, and Pefachrome XIA was supplied by Pentapharm. Human C1 inhibitor was from EMD Chemicals. Recombinastatin and APTT-SP were from instrumentation Laboratory.

HRG was isolated by a modification of a previously published method. 

Citrated plasma was supplemented with 1 μM Val-Phe-Lys-chloromethyl ketone (EMD Chemicals), 0.1% protease inhibitor cocktail, and approximately 50 kallikrein inhibitory units (KIU) aprotinin (American Diagnostica). After centrifugation at 10 000g for 30 minutes at 4°C, any visible lipid layer was removed and solid imidazole was added to 5mM. Plasma was subjected to chromatography on a 50-mL nickel-nitrilotriacetic acid agarose column (QIAGEN). The column was washed with 5 volumes of 20mM Tris-HCl, 150mM NaCl, pH 7.4 (Tris-buffered saline [TBS]) containing 5mM imidazole. Successive washes with TBS contained 5mM imidazole, 5mM imidazole plus 10mM ε-aminocaproic acid, 80mM imidazole, and then 100mM imidazole. The ε-aminocaproic acid was present because plasminogen is a frequent contaminant. Aprotinin was added to the 80 and 100mM imidazole wash buffers at 10 KIU/mL. HRG was eluted with TBS containing 250mM imidazole, pH 7.5. The concentration of HRG was determined by absorbance at 280 nm, using a molecular weight of 67 000 and an extinction coefficient of 0.39 mL/mg/cm. As reported previously, HRG appeared as a closely migrating doublet on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing and reducing conditions (not shown). The identity of HRG was confirmed by NH$_2$-terminal sequence analysis (Hospital for Sick Children: Advanced Protein Technology Center Peptide Sequencing Facility, Toronto, ON) and by mass spectrometric analysis of tryptic fragments (Alphalyse Canada).

HRG-deficient plasma was prepared by repeated chromatography over an immobilized polyclonal antibody against HRG. A second sample of plasma was subjected to chromatography on unmodified Sepharose for use as a control. Removal of HRG from plasma was verified by Western blot analysis using the same antibody.

**Influence of HRG on FXII autoactivation**

FXII autoactivation, stimulated by polyphosphate (Sigma-Aldrich type 65), was evaluated at 23°C in the absence or presence of HRG. A total of 100nM FXII was incubated in 20mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid-OH, 150mM NaCl, pH 7.4 (HBS), containing 2mM CaCl$_2$, 12.5μM ZnCl$_2$, and 100μM polyphosphate (monomer concentration). Samples were mixed in the presence of increasing concentrations of HRG for 15 minutes at 37°C. After addition of 500μM Biopen CS11–22, absorbance was measured at 405 nm in a plate reader (Molecular Devices). Rates of cleavage of the chromogenic substrate were normalized relative to that obtained in the absence of HRG, and the IC$_50$ was determined.

**Influence of HRG on FXI activation by FXIIa**

The effect of HRG on FXI activation by FXIIa was examined in the presence of APTT-SP and HK. Samples containing 10nM FXIIa, 100nM HK, 120nM FXI, 10% APTT-SP, and 2μM HRG in HBS containing 2mM CaCl$_2$ and 12.5μM ZnCl$_2$ were incubated at 37°C. After 60 minutes, aliquots were removed and the extent of FXI activation was determined by monitoring FXIa-mediated hydrolysis of 600μM S-2366. Values were normalized relative to that obtained in the absence of HRG and the IC$_50$ was determined.

**Influence of HRG on FXI activation by kallikrein**

SDS-PAGE was used to monitor the activation of 2.5μM FXII by 100nM kallikrein in a mixture of 2 μg/mL dextran sulfate, 12.5μM CTI, 2mM CaCl$_2$, and 12.5μM ZnCl$_2$ in the absence or presence of HRG. At time points ranging from 0 to 2 hours, 10-μL aliquots containing 2 μg of FXII were removed and subjected to SDS-PAGE under reducing conditions. In the reciprocal experiment, the influence of HRG on prekallikrein activation by FXIIa was monitored in the absence or presence of 600nM HK. After incubation of 120nM prekallikrein with 2nM FXIIa in TBS containing 2mM CaCl$_2$, 12.5μM ZnCl$_2$, and 0.5 μg/mL dextran sulfate for 2 minutes, kallikrein generation was monitored using S-2302.

**Biotinylation**

Biotin labeling was carried out for 1.5 hours at 23°C. HRG was diazylized against 0.1M phosphate buffer, pH 7.5, and incubated with a 10-fold excess of sulfosuccinimidyl-6-biotinamidohexanolate. To stop the reaction, 16μM glycine, pH 8.0, was added and free biotin was removed by passage over a PD-10 column (GE Healthcare) equilibrated with HBS. Biotinylated-hydrazide was reacted with deaminated heparin at a 10:1 molar ratio. The sample was diazylized against water and the biotin-labeled heparin was lyophilized and its concentration determined by mass. FXIIa was biotinylated at its active site by reaction with biotin-Phc-Pro-Arg-chloromethyl ketone (b-FPR; Haematological Technologies).

**Surface plasmon resonance**

Biomolecular interaction analysis was performed on the Biacore T100 biosensor system (BIACore). Biotinylated-HRG was adsorbed to 800 response units (RU) onto a Series S sensor chip SA in HBS containing 0.05% Tween 20 (HBS-T) at a flow rate of 5 μL/min. An unmodified flow cell served as control. First, using dual injection mode, 10nM FXIIa was injected together with 0 to 20μM ZnCl$_2$, at 10 μL/min followed by a second injection of ZnCl$_2$ alone. A second dissociation phase was monitored with ZnCl$_2$-free buffer, and then flow cells were regenerated with 250mM imidazole and 5mM ethylenediaminetetraacetic acid. RU values were obtained from the instrument software, and association and dissociation rate constants and RU$_{max}$ values were used for determination of the dissociation constant (Kd). Second, to determine the affinity of Zn$^{2+}$ for HRG, ZnCl$_2$, in concentrations from 0 to 10μM, was injected at 30 μL/min over flow cells containing immobilized HRG and RU values were determined. Next, to confirm the Kd values for FXIIa for HRG, b-HRG was immobilized to 50 to 100 RU. FXIIa (0-256nM) was injected at a flow rate of 45 μL/min for 10 minutes in the absence or presence of 12.5μM ZnCl$_2$, followed by buffer alone. Kd values were determined from equilibrium values or calculated from rate constants using a 1:1 Langmuir analysis by the instrument software, and κ$^2$ values were calculated to assess closeness of fit.

**Clotting assays**

In a multwell plate, 90-μL aliquots of citrated, normal pooled human plasma were incubated with 0 to 5μM HRG for 10 minutes at 37°C before addition of 10 μL of 260mM CaCl$_2$ and 10 μL of APTT-SP. The plate was then read at 405 nm for 30 minutes at 37°C, and the time to reach half-maximal absorbance was determined by Soft Max Pro Version 5.4 (clot
time). To examine tissue factor-mediated clotting, Recombiplastin, at one-tenth dilution, was substituted for APTT-SP. Additional assays were performed in HRG-deficient plasma that was first dialyzed against TBS to remove citrate. In these assays, clotting was initiated with 8nM FXIIa, 1.5nM FIXa, or 2nM FXIa, which was added to 100 μL of plasma together with 2mM CaCl2, 15μM ZnCl2, and 0 to 5μM HRG in a total volume of 160 μL. FXIIa-mediated clotting of normal plasma was also assessed in the absence or presence of 3.5 mg/mL sheep anti-HRG antibody or control sheep IgG (Sigma-Aldrich).

Determination of plasma HRG concentration

The concentration of HRG in normal plasma was determined to be 1.6μM by surface plasmon resonance (SPR; supplemental Methods, available on the Blood Web site; see the Supplemental Materials link at the top of the online article).

Influence of HRG on inhibition of FIXa by C1 inhibitor or CTI

This information is contained in supplemental Methods.

Detection of HRG-FXIIa complexes in plasma

This information is contained in supplemental Methods.

Results

Effect of HRG on plasma clotting times

HRG was added to normal citrated plasma, and clotting was initiated with tissue factor or APTT-SP to examine the effect of HRG on the extrinsic and intrinsic pathways, respectively. HRG had little effect on tissue factor–induced clotting, where the clotting times in the absence and presence of 3.2 nM HRG were 79.7 ± 2.1 and 83.8 ± 4.9 seconds, respectively (P = .47). In contrast, the activated partial thromboplastin time of control plasma (248 ± 4 seconds) was 4-fold longer in the presence of 2μM HRG (1057 ± 127 seconds; P = .008). These data suggest that HRG affects clotting through the intrinsic pathway and does not influence the extrinsic pathway.

To examine this more closely, the effect of added HRG on clotting of HRG-deficient plasma was examined. To minimize the impact of plasma processing, control plasma was subjected to chromatography on unmodified Sepharose. In the APTT assay, HRG-deficient plasma clotted 2-fold more rapidly than control plasma (216 ± 24.1 and 426 ± 10.1 seconds, respectively; P < .001). These data confirm that HRG attenuates the contact pathway of clotting. When HRG was added back to the deficient plasma, a dose-dependent and saturable increase in clotting time was observed (Figure 1).

To identify where HRG was affecting the contact system, we next examined its effect on clotting of HRG-deficient plasma initiated by FXIIa, FXIa, or FIXa. HRG prolonged the FXIIa-mediated clotting time by 81% and 102% at 2.5 and 5μM, respectively (Figure 2). In contrast, HRG had little effect on FXIa- or FIXa-mediated clotting times. These results suggest that HRG prolongation of the APTT reflects an effect on FXIIa.

Binding of Zn2+ to HRG

Plasma-based assays were performed largely in the absence of Zn2+ because citrate was used as the anticoagulant. However, because HRG is a Zn2+ binding protein and Zn2+ modulates its properties, Zn2+ was included in our characterization of the molecular interactions of HRG with contact pathway proteins. To identify the optimal concentration of Zn2+ for these studies, we first determined its affinity for HRG using SPR. Biotin-HRG was adsorbed to a streptavidin chip, and increasing concentrations of ZnCl2 were injected with buffer regeneration between runs. RU values increased in a dose-dependent fashion reflecting Zn2+ binding (Figure 4). Analyses by kinetic and equilibrium parameters yielded K_d values of Zn2+ for HRG of 225 and 281nM, respectively (χ^2 = 1.2). Because saturable binding was observed at concentrations greater than 5μM Zn2+, subsequent experiments were performed at 10 to 20μM Zn2+.

Figure 1. Effect of HRG on the activated partial thromboplastin time in HRG-deficient plasma. HRG-deficient plasma was incubated with 0 to 6μM HRG for 10 minutes at 37°C. Clotting was initiated by the addition of CaCl2 to 26mM and one-tenth volume of APTT-SP. Absorbance was monitored at 405 nm, and the time to half-maximal value was defined as the clot time. Data are the mean ± SE of 3 determinations. The control was plasma subjected to chromatography on unmodified Sepharose.

Figure 2. Effect of HRG on clotting induced by FIXa, FXIa, or FXIIa in HRG-deficient plasma. HRG-deficient plasma was dialyzed to remove citrate and supplemented with 2mM CaCl2, 12.5μM ZnCl2, and increasing concentrations of HRG. Clotting at 37°C was initiated by the addition of 1.5nM FIXa, 0.5nM FXIa, or 5nM FXIIa and monitored by turbidity. Clotting times are plotted versus HRG concentration. Data are mean ± SE of 3 determinations.
Effect of HRG on the autoactivation of FXII

FXII (100nM) was incubated with 0 to 2μM HRG in the presence of 12.5μM ZnCl₂, 2mM CaCl₂, and 100μM polyphosphate. After 10 minutes of incubation, FXIIa activity was monitored by chromogenic assay. Rates were plotted versus HRG concentration and analyzed by nonlinear regression (Figure 5A). HRG inhibited FXII autoactivation by more than 95% and inhibition was concentration-dependent with an IC₅₀ of 115nM. HRG also inhibited autoactivation mediated by APTT-SP, silica, or dextran sulfate in a similar fashion (not shown). Although this activity could reflect the capacity of HRG to bind and neutralize the activator, further investigation indicated that HRG binds dextran sulfate but not silica, the active agent in APTT-SP (not shown). These data suggest that HRG does not attenuate coagulation simply by compromising the contact activator.

To begin to investigate the mechanism by which HRG inhibits FXII activation, control experiments were first performed to determine whether HRG binds to the active site of FXIIa in the presence of Zn²⁺. HRG had variable effects on FXIIa chromogenic activity, depending on the substrate (not shown). With S-2222, no effect of HRG was observed. In contrast, with Pefachrome XIIa, 2μM HRG promoted FXIIa activity by 35%, raising the possibility that HRG binds FXIIa and allosterically modifies its activity. The observation that HRG did not decrease FXIIa chromogenic activity makes it unlikely that HRG obscures the active site of FXIIa. To verify this concept, the effect of HRG on FXIIa inhibition by C₁ inhibitor or CTI was examined in the presence of 5μM HRG. The second-order rate constant for FXIIa inhibition by C₁ inhibitor in the absence of 5μM HRG was similar to that in its presence (1.3 ± 0.05 × 10⁵ and 1.1 ± 0.07 × 10⁵ M⁻¹ min⁻¹, respectively). Likewise, HRG had minimal effect on the inhibition of FXIIa by CTI, with second-order rate constants of 4.5 ± 0.5 × 10⁶ and 2.9 ± 1.29 × 10⁶ M⁻¹ min⁻¹ in the absence or presence of 5μM HRG, respectively. These findings suggest that HRG does not interact directly with the active site of FXIIa nor does it sterically hinder access of macromolecular inhibitors to the active site of FXIIa.

Effect of HRG on FXIIa-mediated activation of FXI

Additional intrinsic pathway reactions were examined to reveal whether the effect of HRG was specific for FXII autoactivation. Thus, FXIIa-mediated activation of FXI in the presence of HK was assessed in the absence or presence of HRG. As with autoactivation, HRG caused dose-dependent inhibition of FXIIa-mediated activation of FXI in the presence of Zn²⁺ (Figure 5B). Activation was reduced by more than 95% with an IC₅₀ of 287nM.

Effect of HRG on the kallikrein system

Because prekallikrein and FXIIa comprise an autolytic feedback loop, reactions involving these factors in their zymogen or active forms were examined. The effect of HRG on the activation of FXII by kallikrein in the presence of Zn²⁺ was examined first. To avoid autoactivation of FXII by FXIIa, CTI was added to inhibit any FXIIa that was formed and SDS-PAGE analysis was used to monitor FXII activation. In the presence of 2μM HRG, activation of FXII by kallikrein was reduced by 50% (not shown). These results demonstrate that HRG has only a modest inhibitory effect.
on kallikrein activity. In the reciprocal experiment, the effect of HRG on the activation of PK by FXIIa in the presence of Zn²⁺ was investigated in the absence or presence of HK. In the absence of cofactor HK, HRG inhibited PK activation by 75% with an IC₅₀ of 160nM. When HK was present, the magnitude of inhibition was similar, but the IC₅₀ value was 23-fold higher. These findings suggest that HRG inhibits FXIIa-mediated activation of PK, an effect that is attenuated in the presence of HK. Taken together, the results of these experiments suggest that the major effect of HRG is on FXIIa activity and not FXII activation. This concept was tested in binding experiments.

**Binding of HRG to components of the intrinsic pathway**

The affinity of HRG for FXIIa was determined by SPR where immobilized b-HRG was titrated with increasing concentrations of FXIIa in the absence or presence of 12.5μM Zn²⁺ (Figure 6A-B). Zn²⁺ had little effect on association but considerably slowed dissociation. Analysis of the rates indicated a K₅₀ of 21nM (χ² = 2.6) in the presence of ethylenediaminetetraacetic acid and 7.5μM (χ² = 7.0) in the presence of Zn²⁺. Binding of HRG to FXIIa was reversible because the interaction was abrogated by injection of imidazole. To investigate the Zn²⁺ dependence of binding, FXIIa was injected in the presence of increasing concentrations of ZnCl₂ (Figure 6C). With increasing concentrations of Zn²⁺, dissociation of FXIIa gradually slowed. When Zn²⁺ was removed, samples originally containing Zn²⁺ dissociated more rapidly. Kinetic analyses of the rates indicated a dose-dependent decrease in the K₅₀, from 1.6nM without Zn²⁺ to 9pM in the presence of 20μM Zn²⁺ (χ² = 3.7). These results demonstrate that Zn²⁺ increases the affinity of HRG for FXIIa by 1000-fold and this effect is exerted on the dissociation phase. Inspection of the Zn²⁺ dependence indicated that the half-maximal effect occurred at 140nM Zn²⁺, comparable with the K₅₀ of Zn²⁺ for HRG of 250nM, as determined directly.

Because of the extremely high affinity of HRG for FXIIa in the presence of Zn²⁺, the interaction of these 2 proteins was reassessed in a reciprocal experiment. In this experiment, FXIIa, labeled at its active site with b-FPRck, was bound to the SA chip. Using HRG as the analyte, K₅₀ values of 450 ± 40nM and 376 ± 68pM were obtained in the absence or presence of Zn²⁺, respectively (not shown). Retention of the high-affinity interaction in the reverse binding experiment verifies the initial observation of high-affinity FXIIa binding to HRG in the presence of Zn²⁺. Further SPR analyses indicated no interaction of HRG with zymogen FXII or biotin-FPR-FXIIaβ (not shown).

To ensure that modification or adsorption of either protein to the SA chip did not lead to spurious results, an additional control was performed in which both proteins were unmodified. SPR experiments indicated that HRG binds heparin with a K₅₀ value of 2nM in the presence of Zn²⁺, whereas FXII and FXIIa do not bind heparin (not shown). Therefore, biotin-heparin was adsorbed to an SA chip.

**Figure 5. Influence of HRG on FXI activation.** (A) FXII was incubated with 100μM polyphosphate for 15 minutes in HBS buffer containing 2mM CaCl₂ and 12.5μM ZnCl₂. After 10 minutes, aliquots were removed and FXII genera
tion was determined by monitoring the hydrolysis of 500μM biophen CS11–22. The data points represent the mean ± SE of 2 separate experiments. Data were analyzed by nonlinear regression (line) to determine the IC₅₀. (B) To examine the influence of HRG on FXI activation, 10nM FXIIa was incubated with 0 to 1μM HRG, 100nM HK, 10% APTT-SP, and 120nM FXI in HBS buffer containing 12.5μM ZnCl₂ and 2mM CaCl₂. Aliquots were removed, and FXI generation was determined by monitoring the hydrolysis of 600μM S-2366. Rates of chromogenic substrate cleavage in the presence of HRG were normalized relative to that obtained in its absence. The data were fit to a rectangular hyperbola equation (line) to determine the IC₅₀. The data points represent the mean ± SE of 2 experiments.

**Figure 6. Effect of Zn²⁺ on binding of FXIIa to HRG monitored by SPR.** Biotin-HRG was adsorbed onto a streptavidin-coated sensor chip to 50 RU. Increasing concentrations of FXIIa (0-256nM) were injected at a flow rate of 45 μl/min in the presence of 2mM ethylenediaminetetraacetic acid (A) or 12.5μM ZnCl₂ (B). FXIIa concentrations are indicated adjacent to each tracing, with the exception of 0.5 and 0.25nM, which were omitted for clarity. FXIIa association to the chip surface was monitored for 8 minutes followed by injection of HBS to monitor dissociation. RU values were corrected for background and plotted versus time. (C) b-HRG (800 RU) was immobilized on a streptavidin chip, and 10nM FXIIa was injected in the presence of 0 to 20μM ZnCl₂ for 200 seconds (a), followed by Zn²⁺-containing buffer lacking FXIIa for 600 seconds (b). Dissociation was then observed in buffer lacking Zn²⁺ for 400 seconds (c). The micromolar concentrations of ZnCl₂ are noted on each tracing.
FXIIa is 21nM in buffer systems without Zn²⁺. Kallikrein binds HRG with a high affinity and binding of HRG was examined in the absence or presence of FXIIa. Injection of HRG in the presence of Zn²⁺ indicated high-affinity binding to immobilized heparin (Figure 7). In the presence of increasing concentrations of FXIIa, binding of HRG to heparin was reduced in a dose-dependent fashion. At 100nM FXIIa, binding of HRG to heparin was completely inhibited. This result confirms the interaction of HRG with FXIIa and suggests that heparin and FXIIa share the same binding site on HRG. In contrast, FXIIa did not affect the binding of HRG to heparin (not shown). In additional SPR experiments, it was observed that neither FXI nor FXII binds to immobilized HRG, whereas kallikrein binds HRG with a $K_d$ value of 340nM (not shown).

Modulation of HRG activity by fibrinogen

It is apparent that the concentration of HRG required to modulate plasma clotting assays is higher than expected, given that the $K_d$ for FXIIa is 21nM in buffer systems without Zn²⁺. One potential explanation is that the interaction of HRG with other plasma proteins attenuates its effect on FXIIa. To explore this, the effect of fibrinogen on HRG modulation of FXIIa autoactivation was examined. Although polyphosphate-mediated autoactivation of FXII was inhibited by 50% at 350nM HRG, the $IC_{50}$ increased 5-fold to 2000nM in the presence of 3µM fibrinogen (not shown). Thus, considering the number of plasma proteins that bind HRG,²² it is likely that higher concentrations of HRG are required to attenuate FXIIa activity in plasma than predicted solely by its affinity for FXIIa determined in a purified system.

HRG-FXIIa complexes in plasma

An enzyme immunoassay was used to assess the formation of HRG-FXIIa complexes in plasma activated with APTT-SP. Activated plasma yielded an FXIIa-HRG complex concentration of 5 µg/mL, whereas no complexes were detected in unactivated normal plasma or FXII-deficient plasma. The concentration of FXIIa-HRG complexes detected in plasma after APTT-SP addition represents 10% to 20% of the normal plasma concentration of FXII.

Discussion

Emerging evidence suggests that the intrinsic pathway of coagulation contributes to thrombosis at sites of vascular injury.²³,²⁴ This has prompted the development of novel anticoagulants that target this pathway.²⁴,²⁵ The success of such an approach depends on an understanding of how the intrinsic pathway is regulated. Because the intrinsic pathway is initiated by the activation of FXII, an understanding of FXIIa regulation is particularly important. The current study was prompted by our initial observation that HRG addition to plasma prolongs the activated partial thromboplastin time but has no effect on the prothrombin time. In accordance with this observation, HRG attenuated clotting initiated by FXIIa, but not by FXIa or FIXa. We showed that HRG binds FXIIa with high affinity and inhibits its capacity to autoactivate FXII or to activate FXI. We also detected HRG-FXIIa complexes in contact-activated plasma. These findings raise the possibility that HRG modulates the intrinsic pathway of coagulation. In support of this concept, immunodepletion of HRG from plasma or addition of an HRG-directed antibody resulted in a shorter activated partial thromboplastin time, whereas HRG supplementation of HRG-deficient plasma prolonged this clotting time.

The available data suggest a mechanism by which HRG and FXIIa interact. Because Zn²⁺ heightens the affinity of FXIIa for HRG by more than 3 orders of magnitude, the histidine-rich region may mediate this interaction. However, the Zn²⁺-dependent interaction of heparan sulfate with HRG is mediated by its NH₂-terminal cystatin domains and not its histidine-rich domain, suggesting that Zn²⁺ binding to the histidine-rich domain modulates other domains in HRG.²⁶-²⁹ Because heparin disrupts the interaction of HRG with FXIIa, it is possible that FXIIa also binds to the cystatin domains and that the affinity of this interaction is heightened by Zn²⁺ binding to the histidine-rich domain. With respect to FXIIa, the catalytic domain of FXIIa can be excluded because β-FXIIa does not bind HRG. This leaves the heavy chain of FXII as the binding site for HRG. The conformational change in FXII zymogen evoked by Zn²⁺ is insufficient to promote HRG binding. Therefore, the HRG binding site must be cryptic on FXII and only exposed when it is converted to FXIIa. This is analogous to the interaction with fibronectin and a monoclonal antibody against the kringle domain of FXII; these proteins bind FXIIa, but not zymogen FXII.³¹,³² Chromogenic assays indicate that HRG binds to a site distinct from the active site of FXIIa. Furthermore, HRG does not impair the capacity of either CTI or C1 inhibitor to inhibit FXIIa. Therefore, it is likely that HRG binds to an exosite on FXIIa that mediates FXIIa binding to macromolecular substrates.

The specificity and avidity of HRG binding to FXIIa and the inhibitory effects of HRG on FXIIa activity imply a physiologic role for the interaction. Current thinking is that C1 inhibitor is the physiologic inhibitor of FXIIa, although the second-order rate constant suggests that inhibition is slow.³³,³⁴ Given the high affinity of HRG for FXIIa and the abundance of HRG in plasma, HRG is more likely to bind FXIIa than is C1 inhibitor. The affinity of FXIIa for HRG is increased in the presence of Zn²⁺. Although Zn²⁺ also binds and modulates FXIIa activity,³⁵,³⁶ the effect of HRG is not Zn²⁺-dependent because HRG binds FXIIa and modulates clotting even in the absence of Zn²⁺. However, FXIIa will bind HRG even more avidly at sites of vascular injury where platelet activation leads to release of Zn²⁺ from α-granules.³⁶ The HRG-FXIIa interaction would serve to attenuate FXIIa-mediated activation of the contact pathway, thereby limiting the procoagulant stimulus.
Although it has not been demonstrated, it is possible that dissociation of FXIIa from HRG restores its proteolytic activity, raising the possibility that under certain conditions, FXIIa inhibition may be reversible. This could occur in response to a change in the local pH or Zn2+ concentration, or possibly through alternate ligand binding to HRG. Despite the potential for further complexity, our data suggest that HRG modulates FXIIa-mediated initiation of the contact pathway, thereby limiting systemic FXIIa activation and localizing the procoagulant response.

HRG is proposed to play a role in numerous processes, including angiogenesis, apoptosis, and immune regulation. In a knockout model, mice lacking HRG exhibited a procoagulant phenotype, although involvement of the contact pathway was not examined. Furthermore, HRG deficiency in humans has been associated with a thrombotic phenotype, at least in some individuals. The current description of modulation of the contact system reveals a potential new function for HRG. In support of this concept, a genome wide association study aimed at investigating reveals a potential new function for HRG. In support of this proposed role in fibrinolysis, HRG may also serve as an effector FXIIa-mediated activation of coagulation. Thus, in addition to its HRG is retained within the fibrin clot where it is poised to modulate its plasma levels are relatively high and do not fluctuate widely, except in pathologic states because HRG is a negative acute phase protein. Therefore, rather than acting as a dynamic inhibitor of FXIIa, HRG is more likely to serve as a reservoir for FXIIa formed in situ or for FXIIa that has escaped from the site of injury. In this way, HRG may compensate for the low rate of FXIIa inhibition by C1 inhibitor. Because HRG binds fibrin, it is conceivable that HRG is retained within the fibrin clot where it is poised to modulate FXIIa-mediated activation of coagulation. Thus, in addition to its proposed role in fibrinolysis, HRG may also serve as an effector of coagulation. The homology of HRG with HK, a key modulator of the contact system, provides further support for this proposal. Both proteins have cystatin-like and histidine-rich domains and bind glycosaminoglycans, Zn2+, and various enzymatic forms of the contact factors. However, HK promotes activation of contact factors and protects FXIIa and kallikrein from inhibition by antithrombin, whereas HRG inhibits activation of FXII and has no effect on FXIIa inhibition. Thus, HRG may serve to offset the action of HK and limit propagation of the contact system. In conclusion, with emerging evidence for the role of the contact system in the pathogenesis of thrombosis at sites of vascular injury, our data suggest that HRG may be an important regulator of thrombogenesis.

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Authorship

Contribution: J.L.M. designed and performed research, analyzed and interpreted data, and wrote the manuscript; A.R.S. designed research, contributed vital new reagents, performed research, and analyzed and interpreted data; J.W.Y., B.A.L., and T.T.V. performed research and analyzed and interpreted data; and J.C.F. and J.I.W. designed research, analyzed and interpreted data, and wrote the manuscript.

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References

19. Borza DB, Shipulina NV, Morgan WT. Effects of


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