Activated Platelets Provide a Functional Microenvironment for the Antiangiogenic Fragment of Histidine-Rich Glycoprotein

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Abstract

The angiogenesis inhibitor histidine-rich glycoprotein (HRG) constitutes one of several examples of molecules regulating both angiogenesis and hemostasis. The antiangiogenic properties of HRG are mediated via its proteolytically released histidine- and proline-rich (His/Pro-rich) domain. Using a combination of immunohistochemistry and mass spectrometry, we here provide biochemical evidence for the presence of a proteolytic peptide, corresponding to the antiangiogenic domain of HRG, in vivo in human tissue. This finding supports a role for HRG as an endogenous regulator of angiogenesis. Interestingly, the His/Pro-rich peptide bound to the vessel wall in tissue from cancer patients but not to the vasculature in tissue from healthy persons. Moreover, the His/Pro-rich peptide was found in close association with platelets. Release from in vitro–activated platelets promoted binding of the His/Pro-rich domain of HRG to endothelial cells, an effect mediated by Zn2+.

Previous studies have shown that zinc-dependent binding of the His/Pro-rich domain of HRG to heparan sulfate on endothelial cells is required for inhibition of angiogenesis. We describe a novel mechanism to increase the local concentration and activity of an angiogenesis inhibitor, which may reflect a host response to counteract angiogenesis during pathologic conditions. Our finding that tumor angiogenesis is elevated in HRG-deficient mice supports this conclusion. (Mol Cancer Res 2009;7(11): 1792–802)

Introduction

Local regulation of angiogenesis—formation of new capillary blood vessels—is essential during development and physiologic conditions such as wound healing and the female menstrual cycle. However, prolonged and excessive angiogenesis has been implicated in several pathologic processes, for instance, rheumatoid arthritis, retinopathy, and tumor growth (1). The normal vasculature is tightly regulated by naturally occurring proangiogenic and antiangiogenic factors. Data from mice either lacking or overexpressing positive or negative regulators of angiogenesis show their importance during development, physiologic processes, or disease. The most well known proangiogenic factor to date is vascular endothelial growth factor (VEGF), which is required for the development of a vascular system during embryogenesis and is also a central regulator of adult neovascularization (2). Many endogenous factors negatively regulating angiogenesis have also been described, and their function was supported by data from gene targeting in mice (3). For example, mice lacking the angiogenesis inhibitor thrombospondin-2, a basement membrane protein, show enhanced skin carcinogenesis and increased tumor angiogenesis (4). Several of the identified inhibitors gain their antiangiogenic properties as a result of proteolytic processing (5).

Platelets contain a large number of both proangiogenic and antiangiogenic factors, and regulation of angiogenesis by
platelets was suggested as early as 1968 (6). Examples of positive regulators of angiogenesis found in platelets are VEGF-A, VEGF-C, platelet-derived growth factor, and fibroblast growth factor-2, whereas negative regulators include thrombospondin, platelet factor 4 (PF4), and plasminogen activator inhibitor type-1 (7, 8). Platelets are anucleated cellular fragments, derived from megakaryocytes in the bone marrow, and play a crucial role in regulating blood hemostasis. At sites of blood vessel injury, platelets are activated and aggregate at the site of the damaged endothelium to prevent hemorrhage. Besides their role in hemostasis, platelets contribute to nonhemostatic processes such as immunity, tumor metastasis, and angiogenesis (9-11). Despite their content of both positive and negative regulators of blood vessel formation, platelets have in several different experimental settings been shown to stimulate angiogenesis (11-15). This apparent contradiction may be explained by recent data from Italiano and coworkers (16). They show that proangiogenic and antiangiogenic factors may be stored in separate α-granules in the same platelet and that these granules can release their content differentially depending on the stimuli the platelets are exposed to. These data show a more fine-tuned regulation of platelet degranulation than was previously known.

Histidine-rich glycoprotein (HRG; alternatively, HRGP/HPRG) has been identified as an angiogenesis inhibitor in vitro and in vivo by us and others (17, 18). HRG is a 75-kDa single-chain heparin-binding plasma protein produced by the liver (19). Structurally, HRG consists of three distinct domains: an NH₂-terminal part with two cystatin (cysteine proteinase inhibitor)–like domains, which classifies HRG as a member of the cystatin superfamily together with, for example, kininogen and fetuin; a central histidine/proline-rich (His/Pro-rich) domain organized in tandem repeats of a consensus GHHPH motif; and a COOH-terminal domain. Multiple binding partners for HRG have been reported, such as heparin/heparan sulfate, divalent cations, and components in the coagulation/fibrinolysis system; plasminogen and fibrinogen; as well as components in the immune system such as T lymphocytes, monocytes/macrophages, and immunoglobulins (19). Monocytes were previously believed to express HRG because HRG binds to the cell surface of monocytes, but more recent data show that RNA is found only in the liver (20). In addition, HRG has been found within platelets and megakaryocytes (21). HRG constitutes one of several examples of molecules regulating both angiogenesis and hemostasis. Mice lacking the HRG gene are viable and fertile but have an enhanced coagulation resulting in decreased bleeding times (22). It is unknown whether HRG is involved in physiologic angiogenesis during development, but it seems not crucial for proper development. Moreover, no significant difference in time required for wound healing in adult wild-type and HRG knockout animals was recorded (22), indicating that physiologic angiogenesis is not regulated by HRG.

Proteolytic fragments of discrete mass are consistently found in purified fractions of HRG from human plasma (23). We have previously shown that one of these fragments, a 30-kDa peptide derived from the histidine- and proline-rich (His/Pro-rich) domain of HRG, mediates the antiangiogenic effect of HRG and that this peptide must be released from the full-length protein to exert its effect as an inhibitor of angiogenesis (18). Moreover, in a screen for the minimal active antiangiogenic domain of HRG, using synthetically produced peptides covering the His/Pro-rich domain, we identified a 35–amino acid peptide (HRGP330) that retained the antiangiogenic properties in vitro and in vivo (24).

In the present study, we address whether the His/Pro-rich domain of HRG is proteolytically released from the full-length protein in vivo in human tissue. This is an important aspect because the presence of a proteolytically released His/Pro-rich domain of HRG in vivo (and not only in in vitro preparations) would significantly strengthen its role as an endogenous regulator of angiogenesis. Our data show that a proteolytic fragment of HRG, corresponding to the antiangiogenic peptide HRGP330, is present in vivo. Moreover, elevated tumor angiogenesis in HRG-deficient mice support a role for HRG as a regulator of pathologic angiogenesis. Based on these data, we provide a model for how activated platelets can provide a microenvironment that promotes activity of the antiangiogenic fragment of HRG.

Results

A Proteolytic Fragment Derived from the His/Pro-Rich Domain of HRG Is Present In vivo

To address the presence of proteolytic fragments of HRG in human tissue, three rabbit polyclonal peptide antibodies (0115, 0116, and 0119), directed toward distinct regions of HRG (Fig. 1A), were used for immunohistochemical staining of human tissue. As described above, HRG is only expressed by the liver but present in the circulation. The antibody raised against amino acids 321 to 344 within the His/Pro-rich domain of HRG (0119) generated a patch-like staining along the blood vessels in sections of frozen human kidney tumor tissue, which was not seen with either the antibody raised against a COOH-terminal peptide (0115) or the antibody directed against an NH₂-terminal peptide (0116) in HRG (Fig. 1B). Blood vessels were visualized by binding to the FITC-conjugated lectin Ulex europaeus agglutinin 1 (UEA-1). Because the antibody directed toward the His/Pro-rich domain recognized structures that were not detected by either the COOH-terminal or the NH₂-terminal antibody, this finding indicates that a fragment corresponding to the His/Pro-rich domain of HRG is proteolytically released in vivo.

An alternative explanation for the discrepancy in staining pattern generated by the three antibodies—0115, 0116, and 0119—could be that the epitopes in HRG recognized by 0115 and 0116 are masked in vivo. However, when immunohistochemistry was done on tissue with a high amount of infiltrating monocytes, which are known to carry HRG on their cell surface (25), the antibodies recognized HRG equally well (Supplementary Fig. S1A). In addition, human liver tissue, where HRG is actually expressed, was positively stained by all three antibodies (Supplementary Fig. S1B). This finding shows that epitope masking is not the reason why the antibodies directed against the NH₂-terminal and COOH-terminal regions did not generate any staining.

To map the location of the 0119 staining more precisely, we used paraffin-embedded tissue, where the structures are more preserved compared with frozen tissue. Using confocal microscopy analysis, we were able to show that the staining was located along the luminal side of the vessel wall (Fig. 1C).
Specificity in the staining was shown by preincubation of the 0119 antibody with the peptide it was raised against, which completely abolished the staining (Fig. 1D). Moreover, immunoprecipitation of native HRG with 0115, 0116, and 0119 showed that all three antibodies were capable of recognizing native HRG (Fig. 1E).

The His/Pro-Rich Fragment Is Present in Tissue from Cancer Patients but not in Healthy Individuals

To investigate if the staining pattern detected in Fig. 1 was specific to kidney tumor tissue or present also in other organs, human colon tumor and glioma tissue were included in the analysis. The patch-like staining generated by the 0119 antibody was present along the vasculature also in tumors from these organs (Fig. 2A, left). Untransformed tissue from kidney, colon, and brain, removed at the same time as the tumor, was immunostained in the same way to investigate whether this staining pattern was specific for tumor tissue (Fig. 2A, right). We could not detect any apparent differences between the tumor and the corresponding untransformed tissue from the same organ, with respect to the staining pattern generated by 0119.

To address this question in more detail in a large collection of samples, we used a tissue array containing 83 colon samples from untransformed (n = 18) and transformed (n = 65) tissue from 47 individuals. The tissue array was costained using the 0119 antibody and an antibody against the vascular marker CD31. The amount of 0119 staining in the colon samples was quantified by manual counting of individual 0119-positive patches and related to the number of vessels in the tissue. This was done to compensate for varying degree of vascularization in the analyzed tissues because the 0119-specific staining was found only in connection to vessels. No significant difference between untransformed and transformed tissue, with respect to the amount of staining for the His/Pro-rich domain of HRG, was detected in this large collection of samples (data not shown).

A tumor may not only have local effects but can also induce systemic changes in an affected individual. Therefore, we did immunohistochemistry on placenta, one of the few tissues available from a healthy person, using CD31 to detect vessels and the 0119 antibody. Placentas collected from 20 healthy individuals were used for the analysis. Interestingly, no 0119 staining could be detected along the vessel wall in these tissues (Fig. 2B).
The His/Pro-Rich Fragment of HRG Is Found in Association with Platelets

HRG has been reported to be present in platelets and megakaryocytes and to be released from platelets on thrombin-induced degranulation (21). To determine if the patch-like structures recognized by the antibody 0119 in tissue from cancer patients were associated with platelets, we costained human kidney tumor sections with the antibody 0119 and the platelet markers CD41 and CD42b. As can be seen in Fig. 3, areas positively stained by the 0119 antibody contained platelets as judged by CD41 or CD42b expression. Confocal microscope analysis of kidney tumor tissue triple stained for blood vessels (CD31), platelets (CD41), and the His/Pro-rich fragment of HRG (0119) shows a close association of all three structures (Supplementary Fig. S2).

A possible explanation for the association of the His/Pro-rich fragment with platelets could be that this HRG-derived peptide is present in platelets and released on activation. To investigate this alternative, platelets were purified from human plasma under conditions that prevent them from becoming activated and thereby release the content in their granules. The platelets were lysed and protein extracts were analyzed by two-dimensional gel electrophoresis, by Western blot using the 0119 antibody, and by mass spectrometry (MS), but we could not detect any degradation product of HRG in platelets. In addition, releasate (secreted material) from platelets activated by thrombin \textit{in vitro} was analyzed as above, with the same result. Full-length HRG could, however, be detected both in platelets and in releasate by immunoblotting, as previously reported, but at low levels (data not shown).

The Identified His/Pro-Rich Fragment Contains the Antiangiogenic Domain of HRG

A second possibility is that the His/Pro-rich fragment is generated in the vicinity of activated platelets because they release several proteases. To address this, blood from healthy persons was collected in citrated vials and two types of plasma were prepared: platelet-poor plasma (PPP) and platelet-rich plasma (PRP). To investigate whether a fragment from the His/Pro-rich domain of HRG was generated in the presence of activated platelets, half of the PPP and PRP samples were stimulated with thrombin at 0.1 unit/mL for 20 minutes. The samples were subsequently treated with lysis buffer and separated by reducing PAGE, and Western blot was done using the antibody 0119. Full-length HRG was clearly visible at 75 kDa (Fig. 4A). In addition, a strongly immunoreactive band at 50 kDa could be detected, as well as two weaker bands at approximately 30 and 33 kDa. Immunoblotting for thrombospondin, a protein present at high levels in platelets, was done to confirm release of platelet content after lysis. Activation of platelets with thrombin did not affect the size or intensity of the 0119-immunoreactive bands, arguing against a role for platelet proteases in the generation of a His/Pro-rich fragment derived from HRG.

To enrich for histidine-containing peptides, the PPP and PRP samples were incubated with nickel agarose and bound material was eluted with imidazole. Western blot of these samples with 0119 showed that full-length HRG, as well as several immunoreactive peptides between 75 and 55 kDa, increased in intensity (Fig. 4B), as well as the 30- and 33-kDa bands detected in Fig. 4A. In addition, a band of ~38 kDa appeared, which was not detected by 0119 before incubation with nickel agarose. Again, there was no visible difference between samples treated with thrombin or not, indicating that secreted platelet proteases are not involved in generating the His/Pro-rich fragment of HRG.

The 0119-immunoreactive peptides in the lane containing thrombin-treated PRP (Fig. 4B, right) were analyzed by trypsin digestion (cleavage after lysine and arginine) and MS, and they were all identified as HRG derived. Except for full-length HRG at 75 kDa and two bands between 65 and 70 kDa, the only smaller band that contained a tryptic peptide identified as

FIGURE 2. The His/Pro-rich fragment is present in tissue from cancer patients but not in healthy individuals. A. Frozen sections of human kidney tumor, colon tumor, or glioma tissue (left), as well as untransformed tissue from the same organ (right), were coimmunostained using the antibody 0119, recognizing the His/Pro-rich domain of HRG (red), together with either anti-CD31 or the FITC-conjugated lectin UEA-1 to detect blood vessels (green). B. Immunohistochemical staining of placenta tissue from a healthy person using the antibody 0119 (red) did not generate any staining. Green, blood vessels are detected with an antibody against CD31. Scale bars, 20 μm.
The His/Pro-rich Domain of HRG binds to endothelium in the presence of platelet releasate.

The data presented above show the presence of a 33-kDa His/Pro-rich fragment of HRG in human plasma. What is the reason for its localization around platelets in human tissue? We have previously shown that HRGP330 binds to heparan sulfate on endothelial cells in a zinc-dependent manner (27). Platelets contain several molecules such as growth factors, chemokines, and proteases, but they also contain 30 to 60 times higher concentrations of Zn²⁺ than the surrounding plasma (28). When activated, the local concentration of Zn²⁺ is elevated above the ∼10 μmol/L normally present in plasma. We hypothesized that the reason for the His/Pro-rich fragment to localize in the vicinity of platelets is because the local environment, perhaps due to the release of Zn²⁺, favors its binding to the vessel wall. To test this hypothesis, we analyzed binding of biotinylated HRGP330 to confluent human telomerase immortalized endothelial (TIME) cells in the presence or absence of platelet releasate. Bound HRGP330 peptide was detected using streptavidin-conjugated Alexa680 and an IR fluorescent scanner. As can be seen in Fig. 5, platelet releasate induced an ∼3.5-fold increased binding of HRGP330 to the endothelial cells. ZnCl₂ (50 μmol/L) was included as a positive control. A concentration of Zn²⁺ corresponding to that in plasma (10 μmol/L) was not sufficient to induce a significantly increased binding of HRGP330 to endothelial cells in this assay. Platelet releasate preincubated with 5 mmol/L EDTA, which chelates divalent cations, lost the ability to induce binding of HRGP330 to the endothelial cells (Fig. 5). Other divalent cations that are chelated by EDTA and relevant in a physiologic setting are Ca²⁺ and Mg²⁺. However, we know from previous studies that Ca²⁺ and Mg²⁺ are unable to induce a conformational change of the His/Pro-rich domain of HRG and subsequent binding to cell surface heparan sulfate even at a concentration of 1.5 mmol/L (data not shown). Taken together, these data show that platelet releasate mediates the binding of HRGP330 to endothelial cells by providing a local increase in Zn²⁺ concentration.
releases promotes binding of the His/Pro-rich domain of HRG to endothelium and that Zn\(^{2+}\) most likely is the factor mediating this effect.

We have previously shown that inhibition of VEGF-induced chemotaxis of TIME cells by HRGP335, a 25–amino acid synthetic peptide derived from the same region as HRGP330, is dependent on the presence of zinc (18, 27). This assay was repeated using HRGP330 and human umbilical vein endothelial cells. VEGF stimulation induced an ∼2-fold increase in migration in a Boyden chamber chemotaxis assay, which was prevented by HRGP330 in the presence of zinc (Supplementary Fig. S3). However, in the absence of zinc, HRGP330 did not inhibit VEGF-induced migration of human umbilical vein endothelial cells, a result in agreement with previously published data. This finding shows that direct binding of HRGP330 to endothelial cells is required for the antiangiogenic effect.

Enhanced Pathologic Angiogenesis in HRG-Deficient Mice

To address whether lack of HRG would affect tumor angiogenesis in vivo, we have crossed HRG-deficient mice with a transgenic model of insulinoma: the Rip1-Tag2 mouse (29). These mice carry the SV40 T antigens under the control of the insulin promoter, which is expressed in the islets of Langerhans in the pancreas. The Rip1-Tag2 is an orthotopic model of multistage carcinogenesis and is believed to better reflect the stepwise process of tumor development via distinctive stages than conventional subcutaneous models with injected tumor cells. One of these stages represents the “angiogenic switch.” The islet capillaries are normally quiescent, but the angiogenic islets are characterized by endothelial proliferation, vascular dilation, and microhemorrhaging. The extent of the angiogenic switching can be quantified by pancreatic dissection and manual counting of blood containing islets.

HRG heterozygotes (HRG\(^{+/−}\)) were mated to enable analysis of HRG wild-type (HRG\(^{+/+}\)) and HRG knockout (HRG\(^{−/−}\)) litters. At 7 weeks of age, the pancreases of these mice were analyzed. As can be seen in Fig. 6, HRG\(^{−/−}\) mice had significantly elevated numbers of angiogenic islets compared with their HRG\(^{+/+}\) littermates, thus supporting the conclusion that HRG regulates pathologic angiogenesis. This is the first report describing an effect on angiogenesis in the HRG knockout mouse.

Discussion

Using a combination of immunohistochemistry and MS, we here show the presence of a proteolytic fragment, corresponding to the antiangiogenic His/Pro-rich domain of HRG, in human tissue. In our previous studies, we identified a proteolytically released 30-kDa fragment, derived from the His/Pro-rich domain of HRG, as mediator of its antiangiogenic effect.
The fragment must be released from the full-length protein to be active and can be found in purified fractions of HRG in vitro. We were therefore very excited to find a HRG fragment of ~30 kDa in human plasma under nonreducing conditions, which contained the sequence HHSHEQHPHGHH-PHAHPPHEHDTHR. This sequence is present within the 35–amino acid synthetic peptide HRGP330, previously identified as the minimal active antiangiogenic domain of HRG (24). The same sequence also mediates zinc-dependent binding of HRG to endothelial cell heparan sulfate, which is required for inhibition of angiogenesis (27). These findings strongly support a role for this HRG-derived peptide as an endogenous regulator of angiogenesis.

Using amino acid sequencing, we now aim to define the biochemical structure of the identified His/Pro-rich fragment. Sequencing of the naturally occurring His/Pro-rich peptide should provide additional data on which protease is involved in its in vitro generation and hence additional information on its regulation. Plasmin and kallikrein are two serine proteases previously shown to have the capacity to cleave purified HRG (30). One possibility is also that platelet activation creates an environment that favors activation of a proteolytic enzyme involved in generation of the His/Pro-rich fragment. For instance, matrix metalloproteinases are a group of proteases that require zinc for their activation. Because platelets contain substantially higher amounts of zinc than the surrounding plasma (28), they induce a local increase in the zinc concentration when activated and possibly also a local increase in matrix metalloproteinase activity. If matrix metalloproteinases can cleave HRG is not known. However, we cannot rule out that zinc has a dual function here—both to activate a proteolytic enzyme generating the fragment and to induce binding of the fragment to the endothelium.

In healthy persons, we identified the His/Pro-rich fragment in plasma using MS, but we could not detect any binding to the endothelium. This is in contrast to tissue from cancer patients, where the His/Pro-rich fragment of HRG was consistently found to bind the vessel wall in the presence of platelets. Cancer patients commonly have increased numbers of activated platelets in their circulation and problems with thrombotic occlusion of vessels (31, 32). Indeed, tumors have been described as “wounds that do not heal.” These effects are systemic and will also affect untransformed tissues in the same individual. Our finding that normal tissue from a healthy person or from a person affected with cancer, differed with respect to binding of the HRG fragment to the endothelium, highlights the importance of carefully considering which tissue should be referred to as “normal” in different types of studies.

Increased binding of the His/Pro-rich domain to endothelial cells in vitro was observed in the presence of platelet releasate and the factor mediating this effect is most likely zinc. Because direct interaction of the His/Pro-rich domain of HRG with endothelial cells is required for its antiangiogenic effect, the enhanced binding of the His/Pro-rich fragment to the endothelium in environments with increased platelet activation may confer specificity in the antiangiogenic effect of HRG for situations with increased coagulation and platelet activation, such as in tumor angiogenesis. We believe that this process reflects a host response to counteract angiogenesis during pathologic conditions. The fact that HRG-deficient mice display increased tumor angiogenesis in an orthotopic model of insulinoma supports this conclusion. A summary of the proposed mechanism is shown in Fig. 7. Interestingly, recent support for our conclusion that the HRG fragment binds to endothelium in situations with increased coagulation and platelet activation has emerged. As shown in Fig. 2B, the His/Pro-rich fragment of
HRG does not bind to the vasculature in placenta from a healthy person. There is a condition called preeclampsia that can affect pregnant women with a late or early onset. The severity of the condition varies but can cause both maternal and fetal mortality. In preeclampsia, the function of the placental microvasculature is compromised and displays increased clot formation and activation of platelets. In a recent study, placentas from preeclamptic women were analyzed by immunohistochemistry with the aim to find prognostic markers (33). Interestingly, the placenta vasculature was positively stained with the antibody 0119 (used in our study), which is directed against the His/Pro-rich domain of HRG. These data further support the model where increased platelet activation creates a microenvironment that favors retention of the His/Pro-rich fragment of HRG.

What is the underlying reason for the preferential localization of the HRG fragment together with platelets? There is an increasing awareness of the relationship between regulation of angiogenesis and hemostasis. Both processes are normally quiescent but are consistent host responses to vessel injury or tumor growth. Interestingly, several inhibitors of angiogenesis are found within the hemostatic pathway (7, 8), one of them being HRG. The reason for this dual function is thought to be the requirement for strict regulation of the angiogenic process during wound healing. At the early stages of vascular injury and platelet adhesion, angiogenesis must be counteracted and the endothelial cells lining the wound are immobile. As the clot stabilizes, angiogenesis is required for tissue regeneration and a new vessel wall is formed from activated endothelial cells. The different steps in this process need to be carefully orchestrated to prevent hemorrhage.

PF4 was the first hemostatic protein shown to possess antiangiogenic properties (34, 35). PF4 is released from activated platelets and can bind to heparin-like glycosaminoglycans on the surface of endothelial cells. It is believed that PF4 acts as an angiogenesis inhibitor by blocking access to these sites for vascular growth factors (34). Angiostatin, another inhibitor of angiogenesis present in platelets, is generated from proteolytic cleavage of plasminogen. Platelets contain plasminogen and enzymes such as matrix metalloproteinases and urokinase-type plasminogen activator, capable of generating angiostatin. Platelets release functional angiostatin, but the inhibitor is also generated from plasminogen during platelet activation and aggregation (36, 37). PF4 and angiostatin exemplify two different mechanisms used by platelets to increase the local concentration of an angiogenesis inhibitor: release from α-granules or generation of the inhibitor during platelet activation. In this study, we describe a third mechanism, namely, creation of a microenvironment that favors retention of the antiangiogenic molecule at sites of platelet activation. A local increase in zinc concentration after platelet degranulation can induce a conformational change of the His/Pro-rich domain and hence promote its binding to endothelial cells via heparan sulfate. However, we do not rule out that other platelet granule molecules may also be involved. Similar to PF4, the His/Pro-rich domain of HRG is capable of binding to heparan sulfate on the endothelial cell surface (27) and to interfere with VEGF-induced signaling and angiogenesis (24). However, the inhibition exerted by HRG is not due to a direct competition between HRG and the growth factor for access to heparan sulfate (18, 24), as in the case of PF4. Instead, HRG targets focal adhesion function in endothelial cells and thereby prevents an angiogenic response to VEGF (38).

In summary, our data provide solid biochemical evidence for the presence of a proteolytic peptide, corresponding to the antiangiogenic fragment of HRG, in human tissue. We show that tumor angiogenesis is increased in HRG-deficient mice. These findings support a role for HRG as an endogenous regulator of pathologic angiogenesis. Moreover, we describe a novel mechanism for how specificity of an angiogenesis inhibitor toward tumor angiogenesis can be achieved.

Materials and Methods

Biobank Material

Human tissue sections were obtained from the Uppsala University Hospital Biobank with no link to donor information and with permission from the local ethical committee.

Cell Culture

Human umbilical vein endothelial cells and TIME cells (39), a kind gift from Dr. Martin McMahon (Cancer Research Institute, University of California at San Francisco), were cultured on gelatinized dishes in the endothelial cell culture medium EBM MV2 (C-22121, Promocell) with supplements (5% FCS, 5 ng/mL epidermal growth factor, 0.2 μg/mL hydrocortisone, 0.5 ng/mL VEGF-A, 10 ng/mL fibroblast growth factor-2, 20 ng/mL insulin-like growth factor-1, and 1 μg/mL ascorbic acid). The cell line has not been tested and authenticated by us during the last 6 mo.

Antibodies

The following primary antibodies were used in this study: anti-HRG (MO37, Takara), anti-CD41 (CBL130, Chemicon), anti-angiostatin (BD Transclusion), anti-α-granule (Mayo Clinic, Düsseldorf), anti PF4 (BD Transclusion), anti-His/Pro-rich (neoEP, Oxford, UK), anti-Zn2+ (Zn2+ Bio), anti-VEGF-A (Abcam), anti-activated platelet (BD Transclusion), and anti-endothelium (BD Transclusion).
anti-CD42b (CBL480, Chemicon), anti-thrombospordin (ab1823, Abcam), anti-CD31 (JC70A, DakoCytomation), and anti-vitronectin (MAB1945, Chemicon). The domain-specific HRG antibodies 0115, 0116, and 0119 were produced in house by immunization of rabbits with the following peptides: SESCPGKFGQPSMVFTHTTPK (0115), VSPTDCSAVEPEAKALDLINKR (0116), and CRISHNNSSDLHIPKKHSHEQPH (0119). The peptides were produced at Proteomics Resource Center, Uppsala University. The following secondary antibodies were used: anti-rabbit IRDye800 (Rockland), anti-rabbit Alexa555 (Molecular Probes), anti-mouse Alexa488 (Molecular Probes), and anti-mouse Cy5 (Jackson ImmunoResearch).

**Immunoprecipitation of Native HRG**

Varying amounts (5 and 20 μg) of HRG purified from human plasma were immunoprecipitated using equal amounts (2 μg) of 0115, 0116, or 0119 in a total volume of 1 mL PBS during 4 h at +4°C. Protein A-Sepharose (GE Healthcare) was added during 30 min and the precipitates were subsequently washed five times in PBS. The samples were separated on 12% SDS-PAGE electrophoresis and transferred to a nitrocellulose filter, which was blocked in 3% bovine serum albumin in PBS with 0.1% Tween 20. Immunoprecipitated HRG was detected by Western blot using the commercial mouse monoclonal HRG antibody MO37 (Takara) at 0.05 μg/mL. Immunoreactivity was visualized by enhanced chemiluminescence (GE Healthcare). The antibody 0116 displayed a somewhat lower affinity for HRG and was therefore used at a higher concentration (8 μg/mL) than 0115 and 0119 (2 μg/mL) in immunohistochemistry.

**Immunohistochemical Staining of Frozen Human Tissue: Kidney/Kidney Tumor, Colon/Colon Tumor, and Liver**

Frozen sections of human tissue were fixed in cold methanol, washed in PBS, and blocked in 3% bovine serum albumin in PBS before incubation with primary antibody diluted in blocking buffer as follows: CD41, 1:200; CD42b, 1:100; and CD31, 1:50. HRG antibodies produced in house were used at the following concentrations: 0115 and 0119 at 2 μg/mL and 0116 at 8 μg/mL. Based on immunoprecipitation of native HRG (Fig. 1E), the antibody 0116 displayed a somewhat lower affinity and was therefore used at a higher concentration than 0115 and 0119 in immunohistochemistry. Sections were incubated with primary antibodies at +4°C overnight. The FITC-conjugated lectin UEA-1 (FL-1069, Vector Laboratories), which specifically binds human endothelial cells, was used at a 1:200 dilution. Detection of primary antibody binding sites was done by using the following fluorescently labeled secondary antibodies: anti-mouse Alexa488 for CD41, CD42b, CD31, and vitronectin; anti-rabbit Alexa555 for 0119, 0115, and 0116; and anti-mouse Cy5 for CD41. The secondary antibodies were all diluted 1:1,000 in blocking buffer and incubated at room temperature for 1 h. Hoechst 33342 at 1 μg/mL was used to visualize cell nuclei. Stained sections were mounted with Fluoromount-G (Southern Biotech). Preincubation of antibodies 0115 and 0119 with the peptides they were raised against was done with 2 μg peptide/μg antibody during 10 min in room temperature.

**Immunohistochemical Staining of Paraffin-Embedded Human Tissue: Kidney Tumor, Normal Brain/Glioma Tissue, Colon Tissue Array, and Placenta**

Tissue samples were fixed in 4% paraformaldehyde in PBS for 24 h, embedded in paraffin, and sectioned in 6-μm sections. The colon tissue array containing both normal and transformed colon tissue from a large number of individuals was assembled in the research group of Prof. Taina Pihlajaniemi (Oulu University, Oulu, Finland; ref. 40). The array contains 83 samples from 47 patients, of which 18 were from normal parts of colon and 65 from transformed tissue of different stages. Sections were fixed with glass slide by incubation at 37°C overnight and subsequently deparaffinized with xylene and rehydrated according to standard histologic procedures. Antigen retrieval was achieved by boiling 5 to 10 min in 0.01 mol/L sodium citrate buffer (pH 6.0). Sections were washed in PBS and blocked in 2% to 5% bovine serum albumin in PBS for 1 h followed by incubation in room temperature for 2 h with the primary antibodies 0119 diluted 1:500 and CD31 diluted 1:50, respectively, in blocking buffer. Detection of primary antibody binding sites was done by incubation with anti-rabbit Alexa555 for 0119 and anti-mouse Alexa488 for CD31 diluted 1:1,000 in blocking buffer. Nuclei were visualized with Hoechst 33342 at 1 μg/mL. Sections were subsequently washed and mounted with Fluoromount-G. Quantification of the 0119 staining in the colon tissue array was done by manual counting of the number of anti-His/Pro-positive patches and CD31-positive vessels in all samples.

**Western Blot of Plasma HRG**

PRP was generated from blood samples from healthy persons (n = 3) by centrifugation at 140 × g for 10 min. For PPP, an additional centrifugation of 1,100 × g was done to remove platelets. PPP and PRP were incubated at 37°C for 20 min with or without 0.1 unit/mL thrombin (Haematologic Technologies, Inc.) and 5 mmol/L Pefabloc FG (Pefab-6003, PentaPharm) to activate platelets without inducing fibrin clot formation. Lysis buffer [1% Triton X-100, 40 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 10% glycerol, 10 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 μg/mL aprotinin] was subsequently added 4:1 to lyse platelets and stop protease activity. To enrich for histidine-containing peptides, lysates were cleared by centrifugation and incubated with nickel agarose (Qiagen) for 3 h at +4°C. The agarose was subsequently washed and packed on a column, and bound material was eluted with 100 mmol/L imidazole in fractions. The samples were separated on 4% to 12% gradient Bis-Tris NuPAGE (Invitrogen), and for reducing conditions, 0.05 mol/L DTT was added. Separated protein samples were transferred to Immobilon-FL membranes (Millipore), optimized for low background fluorescence, and incubated with blocking buffer (LI-COR). Western blot was done with the anti-HRG antibody 0119 directed toward the His/Pro-rich domain. Immunoreactive sites were detected by the fluorescently labeled anti-rabbit IRDye800 antibody (Rockland) diluted 1:10,000 using the Odyssey IR imaging system (Westburg).
Mass Spectrometry

Plasma samples enriched for histidine-containing peptides (as described above) were separated on two parallel 4% to 12% gradient Bis-Tris NuPAGE gels under reducing conditions. One of the gels was Coomassie stained to visualize proteins and the other was used for transfer of proteins to nitrocellulose and Western blot with 0119. Protein bands corresponding to HRG-immunoreactive peptides were cut out and analyzed by matrix-assisted laser desorption/ionization time-of-flight MS at the Proteomics Resource Center, Uppsala University.

HRGP330 Binding to Endothelial Cells

To obtain platelet releasates, PRP was centrifuged at 1,100 × g for 10 min and the supernatant was removed. The platelet pellet was washed with HEPES-buffered saline [145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO4, 10 mmol/L HEPES (pH 7.4)], dissolved in endothelial cell culture medium EBM MV2, and activated with 0.1 unit/mL thrombin for 10 min at 37°C. The thrombin-activated PRP was subsequently centrifuged at 1,100 × g for 15 min to remove platelets and the supernatant (= releasate) was stored at −20°C until use. As control, EBM MV2 with 0.1 unit/mL thrombin, treated the same way as platelet releasates, was used. TIME cells were seeded at confluency in gelatin-coated 96-well plates and allowed to adhere overnight. The next day, the cells were plated in serum-free medium for 5 h, before incubation with 100 ng/mL biotinylated HRGP330 on ice for 30 min, in the presence or absence of either platelet releasate, 10 or 50 µmol/L ZnCl2, or platelet releasate pre-incubated with 5 mmol/L EDTA. Cell-bound HRGP330 peptide was detected by incubation with streptavidin-conjugated Alexa680 using the Odyssey IR imaging system. Careful washing with TBS was carried out between incubations. Fluorescence background corresponding to signal in wells that had not received biotinylated HRGP330 was subtracted. Data are expressed as mean ± SD, where the signal intensity from control-treated cells was set to 100%. Statistical significance was calculated using a two-tailed Student’s t test.

Endothelial Cell Chemotaxis

The chemotaxis assay was done using a modified Boyden chamber with 8-µm micropore polycarbonate filters (PF8-50, Neuro Probe, Inc.) coated with type 1 collagen solution at 100 µg/mL (Vitrogen 100, Collagen Corp). Human umbilical vein endothelial cells that had been starved overnight in 0.5% FCS/EBM MV2 without additions were trypsinized and resuspended at 4 × 10^7/mL in EBM MV2 without additions, 0.25% bovine serum albumin, and Trasylol at 1,000 KIE/mL to stop trypsin activity. The cell suspension was added in the upper chamber and growth factor (VEGF-A at 10 ng/mL; PeproTech) in the lower chamber. HRGP330 was added both in the upper and in the lower chamber at 100 ng/mL. ZnCl2 was used at 50 µmol/L. After 5 h at 37°C, cells that had migrated through the filter were stained with Giemsa and counted. Data are expressed as mean ± SD, where the number of cells that had migrated through the filter without VEGF-A or HRG present was set to 100%. Statistical significance was calculated using a two-tailed Student’s t test.

Rip1-Tag2 HRG+/+ and HRG−/− Mice

Animal work was approved by the Uppsala University board of animal experimentation and thus done according to the United Kingdom Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia (41). All mouse strains were on a pure C57BL/6 genetic background. HRG-deficient female mice were mated with Rip1-Tag2−/− positive males to produce founder mice for breeding of Rip1-Tag2−/−/HRG+/+ and Rip1-Tag2−/−/HRG+/+ litternates. DNA extracted from tail biopsies was used as the template for genotyping by PCR. The following primers were used: forward HRG primer, 5′-CCTGGGTCAAGTGAA-CATGC-3′; reverse HRG wild-type primer, 5′-CGCTCTGTC-CAAGTGGGCCTCA-3′; reverse knockout HRG primer (located in neomycin cassette), 5′-TTGTGATCCG-CAAGTGCACGG-3′; forward Tag2 primer, 5′-GGACACAACACAATAGAATGGCAG-3′; and reverse Tag2 primer, 5′-CAGAGCCAGAATTTGGAGTG-3′.

Analysis of the Angiogenic Switch in Rip1-Tag2 Mice

Seven-week-old Rip1-Tag2−/− positive female mice were anesthetized by i.p. injection of 2% avertin. Heart perfusion was done with 10 mL of PBS (pH 7.4) followed by 10 mL of 2% paraformaldehyde in PBS (pH 7.4). Pancreases were removed from the abdominal cavity and angiogenic islets were dissected away from exocrine pancreas under a stereo dissection microscope at ×10 magnification. Angiogenic islets were defined as blood containing hyperplastic islet with a diameter of <1 mm. Data are expressed as mean ± SEM. Statistical analysis was done using a two-tailed Student’s t test.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Molecular Cancer Research

## Activated Platelets Provide a Functional Microenvironment for the Antiangiogenic Fragment of Histidine-Rich Glycoprotein

Åsa Thulin, Maria Ringvall, Anna Dimberg, et al.


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