Microenvironment and Immunology

Genetic Deficiency in Plasma Protein HRG Enhances Tumor Growth and Metastasis by Exacerbating Immune Escape and Vessel Abnormalization

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Abstract
Histidine-rich glycoprotein (HRG) is a 75-kDa heparin-binding plasma protein implicated in the regulation of tumor growth and vascularization. In this study, we show that hrg−/− mice challenged with fibrosarcoma or pancreatic carcinoma grow larger tumors with increased metastatic properties. Compared with wild-type mice, fibrosarcomas in hrg−/− mice were more hypoxic, necrotic, and less perfused, indicating enhanced vessel abnormalization. HRG deficiency was associated with a suppressed antitumor immune response, with both increased infiltration of M2 marker–expressing macrophages and decreased infiltration of dendritic cells and cytotoxic T cells. Analysis of transcript expression in tumor-associated as well as peritoneal macrophages from hrg−/− mice revealed an increased expression of genes associated with a proangiogenic and immunoinhibitory phenotype. In accordance, expression arrays conducted on HRG-treated peritoneal macrophages showed induction of genes involved in extracellular matrix biology and immune responsiveness. In conclusion, our findings show that macrophages are a direct target of HRG. HRG loss influences macrophage gene regulation, leading to excessive stimulation of tumor angiogenesis, suppression of tumor immune response, and increased tumor growth and metastatic spread. Cancer Res; 72(8); 1953–63. ©2012 AACR.

Introduction
Cancer progression is influenced by the tumor microenvironment, in which tumor cells and different stromal constituents exchange regulatory cues that steer tumor growth and invasion. Tumor angiogenesis and tumor-promoting inflammation are hallmarks of cancer (1). Tumor vessels are morphologically and functionally abnormal, with deficient perivascular support and partially or completely obliterated lumens, leading to poor perfusion, high interstitial pressure, and hypoxia (2). Hypoxia regulates the production of cytokines that promote infiltration and activation of various populations of myeloid cells, which serve to further sustain the proangiogenic environment and to suppress antitumor immune responses (3). Thereby, the vicious circle of uncontrolled tumor growth, invasion, and metastatic dissemination is propagated. Thus, inflammation is a critical factor in every step of tumorigenesis, from initiation to tumor progression and metastatic dissemination (4–6). The tumor-associated macrophage (TAM), a highly adaptive myeloid-derived cell that exhibits different activation states depending on the tumor microenvironment (7, 8), has emerged as an attractive target in cancer therapy. Two main TAM phenotypes denoted M1 and M2 coexist in tumors. The M1 (classically activated) TAMs secrete tumoricidal agents that promote T-helper 1 responses, whereas the more abundant M2 (alternatively activated) TAMs promote exaggerated, dysfunctional angiogenesis and suppress antitumor immunity by preventing infiltration and activation of dendritic cells (DC), cytotoxic T lymphocytes (CTL), and natural killer (NK) cells (5, 9). TAMs and their released factors thereby influence different steps of invasion and metastasis (6).

Histidine-rich glycoprotein (HRG) is a 75-kDa heparin-binding plasma protein exclusively synthesized by hepatocytes (10, 11). HRG is stored in platelet α-granules but is also present free in plasma at concentrations of 100 to 150 μg/mL in humans (see ref. 12 for a review). Tumor angiogenesis is inhibited by HRG and peptides derived from its central His/Pro-rich domain, leading to reduced vascularization and growth of different syngeneic tumor models (13–16). HRG inhibits adhesion and chemotaxis of primary endothelial cells in vitro (17), providing a mechanism for the effect of HRG in attenuation of
tumor angiogenesis. Furthermore, HRG inhibits tumor growth and metastasis by inducing TAM polarization from M2 to M1, leading to antitumor immunity and vessel normalization (11).

Through its many interactions, HRG has been implicated in diverse functions. Thus, HRG binds to different components of both the coagulation and fibrinolytic systems (18), components of the immune system such as complement proteins and Fcγ receptors (19), immunoglobulins (IgG; ref. 18), immunocomplexes (20), and other types of ligands such as heme, divalent cations (21), and glycosaminoglycans (22). Through certain of these interactions, HRG modulates immune responsiveness (12). For example, HRG facilitates the clearance of immune complexes and dying/dead cells via an FcγRI-dependent mechanism (12, 19, 23, 24). HRG moreover enhances the ability of immune complexes to activate complement, allowing faster clearance of necrotic cells (18). HRG has also been implicated in the defense at the local site of bacterial infection (25).

We now further validate and extend the concept that HRG is instrumental in the tumor immune response. Using an hrg gene–inactivated mouse model, we show that lack of HRG leads (i) to increased tumor growth rate and metastatic dissemination, (ii) to enhanced tumor vessel abnormalization, (iii) to critical changes in the activation state of macrophages that lead to a tumor-promoting immune response, and (iv) to a constitutive M2-like polarity of peritoneal macrophages (pMO). Furthermore, the immune-stimulatory transcript profile induced by HRG in pMOs ex vivo is in accordance with the inhibitory immune profile in the HRG-deficient tumors. Thus, through direct and indirect effects, lack of HRG leads to alterations in several molecular and cellular components within the tumor microenvironment.

Materials and Methods

See Supplementary Materials and Methods for lentiviral production, retinal vascularization, leukocyte count, chemotaxis, tumor studies, immunofluorescence and immunohistochemistry, hypoxia, necrosis assessment and tumor perfusion, flow sorting analysis, immunoblotting, microarray expression analysis, and for primer sequences.

Tissue culture

Murine T241 fibrosarcoma cells (American Type Culture Collection; ATCC) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen), 10% FBS (Sigma Aldrich). The murine pancreatic Panc02 tumor cell line (provided by Dr. B. Wiedenmann, Charité, Berlin, Germany) was cultured in RPMI (Invitrogen), 10% FBS. Where indicated, T241 and Panc02 cells were starved overnight in medium with 0.5% FBS. Where indicated, T241 and Panc02 cells were starved overnight in medium with 0.5% FBS before being treated with HRG (1 μg/mL) for 6 or 24 hours.

Human embryonic kidney HEK-293 cells (ATCC) were cultured in DMEM, 10% fetal calf serum and given 0.25 mg/mL G418 (Calbiochem) to ensure selection of EBNA-1 expression. The 293-EBNA was used to produce recombinant HRG as described previously (15).

A subclone of the human monoclastic cell line U-937, U-937-1 (26), a kind gift from Dr. Kenneth Nilsson (Uppsala University, Uppsala, Sweden), was maintained in RPMI-1640 supplemented with 10% FBS. Cells were treated with 25β-dihydroxycholecalciferol (Vitamin D3) for 4 days before stimulation with recombinant hHRG (1 μg/mL) alone or in combination with lipopolysaccharide (LPS; 100ng/mL) for 24 hours.

Cell lines were not authenticated after purchase (T241, 293-EBNA) or after transfer from other laboratories (Panc02, U-937-1) but were routinely tested negative for mycoplasma using the Mycoplasma Detection Kit (Lonza).

Animals

All animal work was carried out according to the guidelines for animal experimentation and welfare provided by Uppsala University and approved by a regional ethics committee. Mice deficient for HRG (hrg−/−) have been described previously (27). C57BL/6 wild-type (wt) mice (8–10 weeks) were obtained from Taconic M&B.

Isolation of naive pMOs

pMOs were isolated from naive hrg−/− and age-matched C57BL/6 wt mice. Mice were anesthetized with ketamine/xylazine and injected intraperitoneally with 5 mL RPMI (Invitrogen) + 5% FBS. Cells were extracted by peritoneal lavage, counted, plated overnight, and stimulated with HRG (1 μg/mL) for 24 hours.

Statistical analysis

Data were analyzed with GraphPad Prism 5.0 (GraphPad Software Inc.). Bars represent mean values ± SEM. For comparison of 2 groups, Student unpaired t test was used. Two-way ANOVA followed by Bonferroni posttests was conducted to assess for significant differences in tumor volume. P value < 0.05 was considered statistically significant.

GEO accession number

Microarray results were submitted to the Gene Expression Omnibus (28) and given the accession number (GSE34164).
express hHRG as a result of lentivirus-mediated transduction (11, 15). Importantly, expression of mouse HRG (mHRG) in T241 fibrosarcoma suppressed tumor growth (Fig. 1A and B) to the same extent as reported earlier for hHRG (11).

To test the effects of endogenous HRG on tumor growth, wt and hrg−/− mice were inoculated with T241 tumors as in A; n = 15 per group. D, histogram shows tumor endpoint weights (g) at day 21; n = 15 per group. E, Panc02 endpoint weight, day 11 after implantation; n = 20 per group. F, Panc02 peritoneal lymph node metastasis; number of lymph nodes/mesentery; n = 20 per group. Data in A and C were analyzed by two-way ANOVA followed by Bonferroni posttests. * , P < 0.05; ** , P < 0.01; *** , P < 0.001. Data are mean values ± SEM.

HRG deficiency is accompanied by dysfunctional tumor angiogenesis

Analysis of the properties of T241 neovascularization in hrg−/− mice by immunostaining for the endothelial cell marker vascular endothelial (VE)-cadherin displayed significantly increased area in tumors from hrg−/− mice versus wt mice
T241 vessel coverage

T241 vessel perfusion

Panc02 vessel coverage

Panc02 vessel perfusion

Figure 2. Tumor properties in HRG-deficient mice. A, immunostaining for VE-cadherin in T241 fibrosarcoma from wt and hrg–/– mice. Quantification to the right shows percentage of VE-cadherin-positive area/tumor area; n = 15 per group. B, percent of αSMA-positive area/CD31 vessel area in T241 tumors; quantification to the right; n = 15 per group. C, vessel functionality in T241 tumors determined by perfusion with FITC-lectin. Quantification to the right, percentage of FITC-lectin-perfused vessel area/CD31 vessel area; n = 15 per group. D, quantification shows percentage of VE-cadherin-positive area/tot al tumor area in Panc02 tumors; n = 15 per group. E, quantification shows percentage of αSMA-positive area/total CD31 area in Panc02 tumors; n = 15 per group. F, quantification shows percentage of FITC-lectin-perfused vessel area/CD31 area in Panc02 tumors; n = 15. Data are mean values ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05. Data are mean values ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05. Data are mean values ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05. Data are mean values ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05. Data are mean values ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05. Data are mean values ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05. Data are mean values ± SEM. ***P < 0.001, **P < 0.01, *P < 0.05.
Importantly, compared with HRG-deficient T241 tumors, mHRG-overexpressing T241 tumors showed the reversed pattern with a clear trend toward decreased infiltration of MRC1-positive macrophages and significantly increased the number of CD8 T cells (Supplementary Fig. S4B and S4C).

Also, Panc02 tumors showed increased infiltration of F4/80-positive cells in the absence of HRG (Fig. 4E). However, Panc02 TAMs from hrg−/− and wt mice expressed similar levels of MRC1 (Fig. 4F). On the other hand, infiltration of CD11c-positive cells was decreased in the Panc02 tumors from hrg−/− mice (Fig. 4G), in agreement with the pattern in the T241 tumors. Around 25% to 30% of the CD11c-positive cells coexpressed F4/80 in both genotypes (data not shown). Moreover, Panc02 tumors from hrg−/− mice showed reduced numbers of CD8 T cells (Fig. 4H).

Overall, these data indicate that HRG deficiency was accompanied by altered infiltration of several important immune cell types, potentially contributing to the increased tumor burden and metastatic dissemination observed in HRG-deficient mice.

**TAMs and pMOs from hrg−/− mice display a proangiogenic, immunoinhibitory phenotype**

Transcript profiling was used to explore the mechanisms underlying the increased tumor angiogenesis and altered immune cell profile in HRG-deficient mice. As shown in Fig. 5A, tumors from hrg−/− mice expressed increased levels of the proangiogenic growth factors platelet growth factor (PIGF) and VEGF, whereas the angiostatic members of the CXC chemokine family CXCL10 and CXCL11 (31) were downregulated. CXCL10 and CXCL11 are T-cell chemoattractants, and their reduced expression is in keeping with the reduced number of tumor-infiltrating CD8 T cells in HRG-deficient mice.

Profiling of TAMs isolated from T241 fibrosarcoma growing in hrg−/− mice compared with TAMs from wt mice showed that HRG deficiency was accompanied by increased expression of the M2-associated gene arginase-1 (Arg-1); of angiopoietin-2 (Ang-2), a Tie-2 ligand that promotes tumor angiogenesis and infiltration of Tie-2-expressing TAMs (32); and of CCL12, a
potent chemoattractant for peripheral blood monocytes (33). The matrix metalloproteinase (MMP8), which has been linked to inflammation and cancer progression (34), was also upregulated. TAMs from hrg–/– mice further contained reduced levels of CXCL10 and CXCL11 and of the costimulatory molecule CD80, important for T-cell priming during DC activation (Fig. 5B). Taken together, these results suggest that HRG deficiency leads to important alterations in the activation state of TAMs.

M2 marker expression was further examined in pMOs isolated from naïve hrg–/– and wt mice. pMOs from HRG-deficient mice expressed about 10-fold higher levels of IL-10, Arg-1, and CCL22 in comparison with wt pMOs, indicating that under conditions of constitutive HRG deficiency, macrophages are strongly skewed toward a proangiogenic and immunoinhibitory M2 phenotype (Fig. 5C). In agreement, immunoblotting showed a 3.5-fold increase in phospho-STAT6 levels in pMOs from hrg–/– mice compared with wt (Supplementary Fig. S5). STAT6 is a known regulator of several M2 genes such as Arg1 and MRC1 (35). On the other hand, activation of the stress kinase p38MAPK was markedly downregulated in the hrg–/– pMOs compared with wt (Supplementary Fig. S5).

HRG-induced gene regulation

We detected decreased expression of the antitumor chemokines CXCL10 and CXCL11 in tumors from hrg–/– mice (Fig. 5A), as well as in freshly isolated TAMs (Fig. 5B) and tumor cells (Supplementary Fig. S6A) derived from T241 tumors grown in hrg–/– mice. Further analyses showed that treatment of pMOs or T241 tumor cells in vitro with HRG did not alter expression of CXCL10 (Supplementary Fig. S6B), whereas CXCL11 was undetectable, overall suggesting that their regulation is not directly mediated by HRG. Furthermore, we excluded potential direct effects of HRG on Panc02 tumor cells, as treatment of tumor cells with HRG did not affect the migration of these cells in vitro (Supplementary Fig. S7A). Loss or gain of HRG also did not significantly affect the expression of different genes involved in epithelial–mesenchymal transition (EMT), a process known to confer an invasive and metastatic phenotype in tumor cells (ref. 36; Supplementary Fig. S7B).

To determine global HRG-induced gene regulation in relation to macrophage activation and function, we isolated pMOs from wt mice, treated them ex vivo with HRG, and assessed for differences in gene expression by oligonucleotide microarrays. Interestingly, treatment of pMOs with HRG resulted in...
significant upregulation of 96 genes and downregulation of 55 (defined as fold change ± 1.8; P < 0.05), which clustered in different functional categories. Overall, validation analyses by quantitative real-time PCR showed results consistent with the data obtained in microarray experiments (Supplementary Fig. S8). As shown in Table 1, the most significant changes occurred for genes involved in regulation of the extracellular matrix and immune responsiveness. The latter category includes humoral immune response genes such as components of the complement system and Fcγ receptors, several chemokines regulating chemotaxis and activation of monocytes and T cells, and genes implicated in antigen presentation and priming of T cells. The HRG-induced upregulation of genes related to DC maturation and T-cell priming (CD11c, CD83, and MHC class II; see Table 1) is compatible with an immunocompetent antitumor microenvironment in the presence of HRG, and, conversely, with the immunosuppressed pattern described here for tumors growing in HRG-deficient mice.

To predict which transcriptional factors are engaged downstream of HRG to regulate the listed target genes in pMOs, we used the tool TFactS. This approach resulted in a list of factors including POU2F1 (Oct-1), POU2F2 (Oct-2), SP1 (PU.1), NF-E2-related factor 2 (Nrf2), JUN, Forkhead box protein 01 (FoxO1), and GLI1, which thus may be, at least in part, responsible for the gene expression profile in the HRG-treated pMOs (Table 1).

To address the mechanisms of the effects of HRG on macrophages, we exposed different cell lines to HRG in vitro. Mature macrophage cell lines such as Monomac (37) or RAW 264.7 (38) were unresponsive to HRG and failed to show gene regulatory effects such as those observed in pMOs (Supplementary Fig. S8). We further analyzed the monoblastic cell line U-937 (39), which differentiates toward the monocytic/macrophage lineage when treated with vitamin D3. As shown in Fig. 6A, combined treatment with vitamin D3 and HRG significantly increased expression of the monocyte macrophage antigen CD14. Importantly, treatment with HRG in the presence of vitamin D3 led to significantly increased expression of FcγRI and CD11c (Fig. 6B), responses that were further strengthened in the presence of LPS. These genes were upregulated by HRG also in pMOs (Table 1). These data show that HRG acts on monocytes/macrophages in a specific differentiation window, where it exerts its effects, at least in part, by inducing certain markers of proinflammatory macrophages.
Table 1. Functional categories of genes regulated by HRG in pMOs

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*Log fold change HRG-treated relative to untreated cells.
bleeding, and necrosis in the tumor tissue. The role of hypoxia in promoting tumor angiogenesis and inflammation is generally accepted. Moreover, hypoxia is a well-known instigator of tumor aggressiveness and promoter of EMT, which is associated with increased local invasiveness (36). There were no significant changes in expression of EMT-related genes either in tumor cells treated with HRG in vitro or in tumors from wt and hrg−/− mice.

Oxygen deficiency alters the expression of a wide range of genes implicated in inflammation. In agreement, we detected elevated expression of hypoxia-sensitive genes such as Arg-1, Ang-2, Mmp8, and Ccl12 in TAMs isolated from hrg−/− mice. In accordance with the established function of these genes, HRG deficiency was accompanied by a tumor-promoting immune response, characterized by increased infiltration of MRC1-expressing TAMs and decreased infiltration of CD11c-positive cells and cytolytic CD8 T cells. Notably, isolated PMs that had never been in contact with HRG showed an activated phenotype reminiscent of M2 TAMs. Thus, HRG deficiency seems to prime macrophages to a constitutive M2 polarity favoring tumor growth and immunosuppression. It must be stressed that the definition of M1 and M2 genotypes needs to take the context into consideration, in particular as tumors were growing in a constitutively HRG-deficient environment. Phenotypically, the M2 classification is highly relevant, as we noted M2-type changes in infiltration of several immune cells (decreased DCs and T cells), which may account for the increased growth and dissemination of the tumor. M2 TAMs are also known to be proangiogenic (5,9), promoting formation of dysfunctional and excess tumor vasculature, which agrees with the altered vessel morphology and impaired function that we detected in the 2 tumor models in hrg−/− mice, studied here.

T241 and Panc02 tumors from hrg−/− mice contained reduced numbers of CD11c-positive DCs, which expressed lower levels of CD86, a costimulatory molecule from the B7 family important for antigen presentation and T-cell priming (30). Tumor progression is, as a rule, accompanied by reduced DC recruitment and maturation, and DC depletion and dysfunction are critical in the escape from immune surveillance (42). Immunosuppressive factors, such as interleukin (IL)-6, VEGF, TGF-β, and IL-10, have been shown to inhibit DC recruitment and/or functions (43). The decreased DC recruitment and maturation in the HRG-deficient condition presents a mirror image of our previous report on more active DCs in HRG-expressing T241 fibrosarcoma in wt mice (11). Hrg−/− mouse T241 and Panc02 tumors furthermore showed reduced infiltration of CD8 T cells. Decreased expression of the antiangiogenic chemokines Cxcl10 and Cxcl11 both by tumor cells and by TAMs, combined with decreased numbers and function of DCs in the HRG-deficient condition, could account for the reduced T-cell infiltration.

There were clear similarities in the overall effects of HRG deficiency on T241 and Panc02 tumor properties. The T241 tumors from hrg−/− mice displayed a more deteriorated condition probably due to the larger size of these tumors (see Fig. 1D vs. E), which grew in a relatively avascular anatomic location (intradermally) compared to the orthotopic Panc02

Figure 6. Effects of HRG on human U-937-1 monoblasts. A, expression of CD14 in U-937-1 cells treated with vitamin D3 (VitD3) and hHRG (1 μg/mL) for 4 days. B, transcript expression of FCγR and Cd11c in VitD3-differentiated U-937-1 cells treated with HRG or LPS for 24 hours. *, P < 0.05; **, P < 0.01. Data are mean values ± SEM.

Discussion

We report that lack of HRG promotes increased tumor growth and dissemination accompanied by an altered gene expression profile of infiltrating TAMs, leading to a tumor-promoting microenvironment and dysfunctional tumor vasculature. Our data extend a recent report on enhanced tumor growth but unaffected angiogenesis in established pancreatic tumors in HRG-deficient Rip1-Tag2 mice (40), which were sensitive to treatment with a platelet-depleting antibody. HRG negatively affected the angiogenic switch as shown by the increased incidence of hemoglobin-containing pancreatic islets in the absence of HRG in the RipTag2 model (41). The potential contribution of platelets to the immunomodulatory role of HRG remains to be addressed.

The T241 fibrosarcomas growing in hrg−/− mice presented several features of impaired vessel function. The vessel area was increased, probably through increased production of proangiogenic VEGF and PIGF and reduced production of the angiostatic chemokines Cxcl10 and Cxcl11. Vessels were also more immature, as indicated by the reduced pericyte coverage, and poorly perfused, resulting in increased hypoxia,
tumors. There may be additional aspects of the microenvironments of the 2 cancer models due, for example, to differences in lymphatic drainage, different interstitial composition and so on, which may have influenced the tumor–host interactions. That HRG deficiency led to increased metastatic dissemination in the Panc02 orthotopic model is important in relation to our previous finding of decreased metastatic spread from HRG-expressing Panc02 tumors (11). However, the blood vessel area, vascular perfusion, and hypoxia were similar in Panc02 tumors from wt and hrg−/− mice. On the other hand, tumor vessels from HRG-deficient mice were poorly covered by αSMA+ cells and the tumor tissues were more hemorrhagic and necrotic. Therefore, vessel parameters were affected also in the Panc02 model, which in turn might contribute to the increased metastatic spread. The changed immune profile of Panc02 tumors from hrg−/− mice with increased infiltration of TAMs and decreased numbers of CD11c-positive cells and CD8 T cells may also have contributed to tumor aggressiveness. Of note, the metastatic index (nodules/gram tumor weight) did not differ between wt and hrg−/− genotypes (data not shown), indicating that increased dissemination could be due to the combined effects on protumorigenic immunity and dysfunctional blood vessels and therefore enhanced tumor growth in the absence of HRG.

The biologic consequence of HRG and HRG deficiency prompts the question of the identity of a potential HRG receptor. FcγRs are important candidates and well-known HRG binders. However, it has still not been possible to tie HRG-induced gene regulation to FcγR expression in vivo (data not shown), perhaps due to the redundancy within this family of immunoglobulin receptors (44). Constitutive HRG deficiency affects signaling, as we noted clear differences in the activity states of STAT6 and p38MAPK when comparing wt and hrg−/− pMOs. To better understand HRG effects, we studied gene regulation in pMOs. The range of genes regulated in pMOs in response to HRG (humoral immune response, IFN-inducible proteins, T-cell/monocyte chemotaxis and activation, antigen presentation, and regulation of extracellular matrix) agrees with the consequence of loss of HRG in the tumor microenvironment. The HRG-induced upregulation of genes related to DC maturation and T-cell priming (CD11c, CD83, and MHC class II) is of particular interest, as they would favor a more efficient, antitumoral immunostimulatory tumor microenvironment. HRG-induced upregulation of M1 markers was also reproduced in vitro in monoblastic U-937 cells. It is essential to further identify the mechanism of action of HRG to successfully apply HRG to therapeutic steering of tumor macrophage activity in cancer forms characterized by proangiogenic and protumor inflammation.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interests were disclosed.

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