Minimal Active Domain and Mechanism of Action of the Angiogenesis Inhibitor Histidine-Rich Glycoprotein

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Abstract

Histidine-rich glycoprotein (HRGP) is an abundant heparin-binding plasma protein that efficiently arrests growth and vascularization of mouse tumor models. We have shown that the antiangiogenic effect of HRGP is dependent on its histidine/proline-rich domain, which needs to be released from the mother protein to exert its effects. Here we identify a 35-amino-acid peptide, HRGP330, derived from the histidine/proline-rich domain as endowed with antiangiogenic properties in vitro and in vivo. The mechanism of action of HRGP330 involves subversion of focal adhesion function by disruption of integrin-linked kinase (ILK) and focal adhesion kinase (FAK) functions, inhibition of vascular endothelial growth factor (VEGF)–induced tyrosine phosphorylation of the FAK substrate α-actinin, and, as a consequence, an arrest in endothelial cell motility. The disturbed focal adhesion function is reflected in the ability of HRGP as well as of HRGP330 to prevent endothelial cell adhesion to vitronectin in a manner involving α5β3 integrin. In conclusion, HRGP330, which we define as the minimal antiangiogenic domain of HRGP, exerts its effects through signal transduction targeting focal adhesions, thereby interrupting VEGF-induced endothelial cell motility. (Cancer Res 2006; 66(4): 2089-97)

Introduction

Angiogenesis, formation of new blood vessels from preexisting ones, is a physiologic process that occurs during development, as a result of exercise, in wound healing and during the menstrual cycle. Angiogenesis is tightly regulated by a number of proangiogenic and antiangiogenic factors acting in concert to adjust the distribution of oxygen and nutrients to meet the demands of the tissue. A number of pathologic conditions including rheumatoid arthritis, diabetic retinopathy, tumor growth, atherosclerosis, and diabetic loss of peripheral circulation are characterized by deregulated angiogenesis (1, 2). The consequences range from inadequate tissue growth to ischemia.

Vascular endothelial growth factor (VEGF; first identified as vascular permeability factor; refs. 3, 4) acts as a proangiogenic factor by promoting endothelial migration, proliferation, differentiation, and tissue degradation. These effects are primarily mediated by the receptor tyrosine kinase VEGF receptor 2 (VEGFR-2; also known as KDR in the human and Flk-1 in the mouse). Most, if not all, tumors express VEGF, which induces angiogenesis in neighboring VEGFR-2-expressing vessels and which promotes endothelial cell permeability, leading to extravasation of provisional matrix components such as fibrin, fibronectin, and vitronectin. Binding of endothelial cell integrins to these matrix components further modulates the angiogenic response by inducing downstream signaling pathways converging with those induced by activation of VEGFR-2. Targeting the vasculature by neutralizing the action of VEGF with antibodies (Avastin/bevacizumab) prolonged the lives of patients with metastatic renal or colorectal cancer in phase III clinical trials (5, 6). This provides evidence that targeting the vasculature is a valid approach in cancer treatment. A number of endogenous antiangiogenic factors, derived from naturally occurring proteins, have been identified and shown to inhibit growth of mouse tumors (see ref. 7 for a review).

A common theme for inhibitors such as endostatin, tumstatin, and kininostatin, fragments of collagen XVIII, collagen IV, and kininogen, respectively, is their interference with integrin function either by using integrins as receptors (8, 9) or by specifically binding to different matrix components (10). Endostatin is reported to act via α5β1 integrins and tumstatin via α,β3. Binding of endostatin to endothelial cells also involves heparan sulfates (11, 12) and the leukocyte adhesion antigen E-selectin (13), indicating that the receptor mechanism may be complex and dependent on several components.

We and others have identified histidine-rich glycoprotein (HRGP; also known as HRG/HPRG, for review see ref. 14) as a potent inhibitor of angiogenesis, an effect mediated via the histidine/proline-rich (His/Pro–rich) domain of the protein (15, 16). We have shown that HRGP reduces endothelial cell migration and tumor vascularization and growth in mice. HRGP is a member of the cystatin superfamily together with α2-HS glycoprotein/fetuin, fetuin-B, cystatin, and kininogen (17, 18). The central domain of HRGP is rich in histidine and proline residues and contains multiple more or less conserved tandem repeats of the pentapeptide GHHIP. We have shown that this domain can be proteolytically released from the full-length protein, which is required for the antiangiogenic activity (16). HRGP preparations always contain proteolytic fragments of distinct masses (16, 19) and one of these fragments corresponds to the His/Pro–rich domain (16). In the present study, we further define the minimal active domain within the His/Pro–rich fragment of HRGP and describe its mechanism of action.

Materials and Methods

Antibodies. The antibodies used were neutralizing anti-α,β3 (MAB 1976z, Chemicon, Temecula, CA); anti–focal adhesion kinase (FAK; Santa Cruz Biotechnology, Santa Cruz, CA); anti-paxillin (Transduction Lab, Franklin Lakes, NJ); anti-α-actinin; horseradish peroxidase (HRP) anti-goat

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).
HRGP and HRGP-derived synthetic peptides. Recombinant His-tagged HRGP was produced and purified as previously described (16). In preparations of purified HRGP, there are always proteolytic fragments of distinct masses present (16, 19). One of these fragments corresponds to the His-Pro-rich domain of HRGP (16). The designation HRGP in this study refers to the purified preparation consisting of the mix of full-length HRGP and proteolytic fragments thereof. HRGP-derived synthetic peptides HRGP330, HRGP396, and HRGP398 were purchased from Innovagen AB (Lund, Sweden). The peptides were analyzed by reverse-phase high-performance liquid chromatography and mass spectral analysis and were dissolved in TBS [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl] containing 1 mmol/L ZnCl2. The peptides have the following sequences: HRGP330 (amino acids 330-364), DLHIPKIHISEHGHPIPHPIAIHPDHTHQR-HPH-COOH; HRGP396 (amino acids 365-397), GHIPHPHGGHPHIH-GHIPHPGHHPHCDFDQYG-COOH; and HRGP398 (amino acid 398-439), PCDPIMPQNHQHCQGHIPHPGPHLBRRGPKGPPRPHFCRHGQIGS-COOH.

Cell culture. Bovine capillary endothelial cells, a kind gift from Dr. R. Christofferson (Department of Medical Cell Biology, Uppsala University), were cultured on gelatin-coated dishes in DMEM (Life Technologies, Inc., Rockville, MD), 10% newborn calf serum, and 2 mg fibroblast growth factor/2 mL (complete medium). Telomerase immortalized endothelial cells, derived from human dermal microvascular endothelial cells, were a kind gift from Dr. Martin McMahon (Cancer Research Institute, University of California San Francisco, San Francisco, CA; ref. 20). Telomerase immortalized endothelial cells were cultured in complete endothelial cell basal medium (EBM MV2, PromoCell, Heidelberg, Germany) without antibiotics on gelatin-coated cell culture plastic.

Conditions for microscopy and immunoblotting. For all microscopy and immunoblotting experiments, cells were seeded on vitronectin-coated (V8379, Sigma-Aldrich) Petri dishes or cell culture slides in complete medium, incubated for 24 hours, washed, and incubated in DMEM/1% newborn calf cell (serum starvation medium) for 20 to 24 hours. To prevent contribution from serum-derived HRGP, the medium was replaced with fresh EBM/0.1% bovine serum albumin (BSA), 20 mmol/L HEPES (pH 7.2), and incubated for 90 minutes. The cells were then stimulated as indicated.

Microphotography. The slides were put on ice, washed in ice-cold TBS [20 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl], and fixed in Zn-fix [0.05% Ca-acetate, 0.5% Zn-acetate, 0.5% ZnCl2 in TBS (pH 6.6), and 0.2% Triton X-100] for 15 minutes at room temperature. The preparations were washed in TBS, blocked in TBS/10% FCS, and stained using standard protocols. The 60×/NA 1.4 or 10×/NA 0.45 objectives of a Nikon E1000 microscope and a Nikon DXM1200 camera were used.

Immunoblotting. Cells were put on ice and quickly washed once in ice-cold TBS/100 mmol/L Na2VO3 then lysed in NP40 lysis buffer [1% NP40, 20 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 10% glycerol, 2.5 mmol/L EDTA, 10 kallikrein inhibitory units (K) (Kie) antiprotease/ml, 1 mmol/L phenylmethylsulfonyl fluoride, and 100 mmol/L Na2VO3]. Lysates were clarified by centrifugation. Immunoprecipitation, SDS-PAGE, and immunoblotting were done using standard procedures. Immunoreactive sites on the filters were detected by enhanced chemiluminescence.

Time lapse. Telomerase immortalized endothelial cells were seeded on vitronectin-coated coverslips at a subconfluent density in complete EBME medium and incubated for 24 hours, washed, and starved in EBME/1% FCS for 20 hours. The coverslips were mounted in observation dishes in a 1:1 mixture of conditioned EBME/1% FCS and EBME/20 mmol/L HEPES (pH 7.2) in a temperature-controlled chamber set to 37°C on a Zeiss LSM 510 Meta confocal microscope. The cells were allowed to stabilize for 60 minutes; after which, one differential interference contrast image per minute was collected for 2 hours using the 40×/NA 1.3 objective. The image in Fig. 4.4 was processed in Adobe Photoshop software (San Jose, CA) using the “find edges” function. Coloring was done by manually outlining the cells using the “pen tool.”

Adhesion assay. Ninety-six-well cell culture plates were incubated overnight with vitronectin (1 μg/ml in PBS) or BSA (2% BSA in PBS), blocked for 2 hours with BSA in all wells, and washed thrice with PBS. Cells plated the previous day in complete medium were washed once and incubated for 60 minutes in medium [0.2% BSA, 20 mmol/L HEPES (pH 7.2), 2 ng FGF-2/ml; assay medium]. Cells were dissociated using nonenzymatic “Cell dissociation solution” (Sigma), washed once, and resuspended in assay medium. Reagents were added to the cell suspension at the following concentrations: HRGP, 100 ng/ml; HRGP330, 100 ng/ml; LM609, 10 μg/ml, and incubated for 15 or 5 minutes before seeding to allow complete or only partial inhibition of adhesion, respectively. After 45 minutes at 37°C, cells were washed thrice in TBS and fixed in Zn-fix for 10 minutes. Finally, cells were stained with Hoechst 33342 (1 μg/ml) washed once in TBS, photographed (2× objective), and cell number was quantified using the Easy Image software (Tekno Optik AB, Skärholmen, Sweden). The experiments were repeated at least three times and the mean value of at least four wells per condition was used. Error bars represent 1 SD. Statistical significance was calculated using Students t test. Differences with P < 0.05 were considered significant.

Chemotaxis assay. The chemotaxis assay was done using a modified Boyden chamber, as described earlier (16), with 8-μm micropore polycarbonate filters (FBF8-50; Neuro Probe, Inc., Gaithersburg, MD) coated with type I collagen solution at 100 μg/ml (Vitrogen 100, Collagen Corp., Palo Alto, CA). Bovine capillary endothelial cells that had been starved overnight in 0.5% NCS were trypsinized and resuspended at 7.5 × 104/ml in DMEM, 0.25% BSA, and trysylol (aprotinin) at 1,000 KE/ml. The cell suspension was added in the upper chamber and VEGF-A (Peprotech, Rocky Hill, NJ) at 10 ng/ml in the lower chamber. HRGP peptides (100 ng/ml) and α1β3 neutralizing antibody (LM609; 10 μg/ml) were added both in the upper and lower chambers. After 5 hours at 37°C, cells that had migrated through the filter were stained with Giemsa and counted using the Easy Image Analysis software (Tekno Optik). All samples were analyzed in at least six wells for each treatment and at several separate occasions. Error bars represent 1 SD. Statistical significance was calculated using Students t test. Differences with P < 0.05 were considered significant.

Animal studies. Animal work was approved by the Uppsala University board of animal experimentation and thus was done according to the United Kingdom Coordinating Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia (21). The mice were anesthetized with isoflurane (Forene; Abbott, Abbott Park, IL) during all manipulations. Six-week-old female Fox Chase severe combined immunodeficiency mice were injected s.c. with 2.5 × 106 BxPc3 pancreatic carcinoma cells on their left flank. When the tumors had reached a size of ~200 mm3, the animals were randomized into two groups (n = 7 per group) and treatment was initiated with daily s.c injections on the right flank with either 5 mg/kg/d HRGP330 (dissolved in 1 mmol/L ZnCl2, 68 mmol/L NaCl, 1.4 mmol/L KCl, 2.1 mmol/L NaHPO4, 7.1H2O, 0.7 mmol/L KH2PO4) or vehicle. The tumors were measured with a caliper, in a blind procedure, and volumes of the vessels to tumor volume (22). Error bars represent 1 SD. Statistical significance was calculated using Students t test. Differences with P < 0.05 were considered significant.

CD31 staining and quantification of vascular variables. Frozen sections of the tumor tissue were fixed in acetone and blocked in 2% rabbit serum before incubation with a rat anti-mouse CD31 antibody (Becton Dickinson), diluted 1:500, and incubated at 4°C overnight. Detection of the primary antibody binding sites was done using a biotinylated antirat antibody (BA-4001; Vector Laboratories), diluted 1:300, and streptavidin-HRP (SA-5004; Vector Laboratories) diluted 1:200. The enzymatic signal was visualized using AEC peroxidase substrate kit (SK-4200; Vector Laboratories). Hematoxylin staining was done to visualize all cells. Stereological quantification of vascular variables was done as described earlier (16) on at least three different tumors from each treatment group. This method of quantifying tumor angiogenesis relates the length, volume, and surface area of the vessels to tumor volume (22). Error bars represent 1 SD. Statistical significance was calculated using Students t test. Differences with P < 0.05 were considered significant.
Results

Identification of the minimal active domain of HRGP. We have shown that inhibition of endothelial cell chemotaxis by the angiogenesis inhibitor HRGP is mediated by the His/Pro–rich domain (16). HRGP is a 75-kDa multidomain molecule which engages in interactions with a broad spectrum of molecules (14). To avoid complications in our studies from such interactive partners (e.g., thrombospondins 1 and 2), we wished to identify the minimal active domain of HRGP. A series of synthetic peptides (HRGP330, amino acids 330-364; HRGP365, amino acids 365-397; and HRGP398, amino acids 398-439) derived sequentially from the His/Pro–rich region were screened for their ability to inhibit endothelial cell chemotaxis (Fig. 1A-C). Of these, only HRGP330 was able to inhibit VEGF-induced chemotaxis of primary endothelial cells (Fig. 1A). As seen in Fig. 1D, HRGP330 attenuated chemotaxis in a dose-dependent manner.

HRGP330 is antiangiogenic in vivo. We next tested whether HRGP330 retained the antiangiogenic properties of HRGP in vivo.

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**Figure 1.** Identification of the minimal active domain of HRGP. A to C, chemotaxis of primary endothelial cells induced by 10 ng/mL VEGF was inhibited by 100 ng/mL HRGP330 (H330; A) but not by HRGP365 (B) or HRGP398 (C). D, the 35-amino-acid residue peptide HRGP330 inhibited VEGF-induced endothelial cell chemotaxis in a dose-dependent manner. E, growth curves of BxPC3 tumors in HRGP330-treated (5 mg/kg/d; †) and control-treated (○) mice (n = 7 per group). F, visualization of the tumor vasculature by immunohistochemical staining against CD31 revealed a striking reduction in CD31-positive vessels in tumors from HRGP330-treated animals. Bar, 100 μm. G, stereological quantification of vascular parameters in tumors from control- or HRGP330-treated mice showed a significant reduction in vessel length, volume, and surface area. Bars, 1 SD. Significance at the *P* < 0.01 or *P* < 0.05 level is indicated.
Female severe combined immunodeficient mice (n = 7 per group) were injected with BxPC3 human pancreatic carcinoma cells on their left flank. The tumors were allowed to become well established and reach ~200 mm³ before treatment was initiated with daily s.c. injections on their right flank with HRGP330 (5 mg/kg/d in 1 mmol/L ZnCl₂) or vehicle. The tumor growth curves (Fig. 1E) showed no significant difference in tumor size between the groups although there was a tendency for reduced growth in the treatment group. After 31 days, the study had to be interrupted due to weight loss of animals in both groups. To determine whether HRGP330 exerted antiangiogenic effect in vivo, the degree of tumor vascularization was analyzed by immunohistochemical staining for expression of the vascular marker CD31. As seen in Fig. 1F, tumor vascularization of HRGP330-treated mice was dramatically reduced. The control mice tumor vasculature was composed of larger vessels and an even, dense capillary network. In contrast, tumors from the HRGP330-treated mice retained the large vessels whereas the small capillaries were essentially absent (Fig. 1F). Quantification of tumor vascularization according to the method by Gunderssen et al. (22) showed a striking reduction primarily in length and volume but also in surface area of the blood vessels in tumors from HRGP330-treated mice as compared with control-treated mice (Fig. 1G). These data show that HRGP330 possesses potent antiangiogenic properties in an in vivo setting.

**HRGP330 inhibits VEGF-induced complex formation of ILK and paxillin.** Migration requires coordinated signaling from growth factor receptors and integrins. The focal adhesion components ILK and FAK are instrumental integrators of these signals. ILK is a serine/threonine kinase; however, its contribution to focal adhesion function seems to be dependent on its role as an adapter for a large number of molecules (e.g., paxillin). Complex formation between ILK and paxillin is needed for subcellular localization of ILK to focal adhesions and for cellular migration (23). We have previously shown that HRGP induces rearrangement of focal adhesions in endothelial cells after 10 minutes of treatment. To seek the mechanism of action of HRGP330, we subjected endothelial cells to treatment with the peptide for 10 minutes in the presence and absence of VEGF. Immunoprecipitation of either

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**Figure 2.** HRGP330 disrupts ILK-paxillin complex formation. A, endothelial cells on vitronectin were treated with HRGP330 (100 ng/mL) and VEGF (50 ng/mL), individually or in combination, for 10 minutes. VEGF induced increased complex formation between paxillin and ILK as shown by immunoprecipitation (IP) of paxillin and immunoblotting (IB) for ILK (top; arrow, ILK). Cotreatment with HRGP330 and VEGF disrupted ILK-paxillin complex formation. Control immunoblotting for paxillin on the paxillin immunoprecipitates (middle) and immunoblotting for actin on total cell lysates (bottom), show equal protein amounts in cell lysates used for immunoprecipitation. B, the reciprocal experiment (IP:ILK, IB:paxillin) gave the same result. The broad paxillin band is indicated. Immunoblotting for ILK and actin showed equal amount of protein in total cell lysates. Control immunoblotting for the amount of immunoprecipitated ILK was not feasible due to comigration with the immunoglobulin heavy chain. C, immunocytochemical staining for paxillin (red) and ILK (green), using the same conditions as in (A), revealed colocalization of paxillin and ILK after VEGF stimulation (Merge; yellow, indicated by arrowheads), which was disrupted by cotreatment with VEGF and HRGP330. Bar, 4 μm.
paxillin (Fig. 2A) or ILK (Fig. 2B) followed by immunoblotting for the partner showed that ILK-paxillin complex formation was enhanced by VEGF whereas cotreatment with HRGP330 blocked the VEGF-induced increase. The reduced ILK-paxillin complex formation was not due to decreased protein expression levels; in contrast, there was a tendency for increased recovery of paxillin in the biochemical analysis of cells treated with HRGP330 (Fig. 2A). Because ILK migrates at the same position of the immunoglobulin heavy chain, immunoblotting for ILK was done on total cell lysates from stimulated cells (Fig. 2B, middle), which revealed similar recovery of ILK independent of treatment. Immunostaining of endothelial cells (Fig. 2C), using the same experimental conditions as above, confirmed increased colocalization of ILK and paxillin in focal adhesions in response to treatment with VEGF. The colocalization was blocked by inclusion of HRGP330 together with VEGF. Disruption of VEGF-induced complex formation between ILK and paxillin by HRGP330, visualized by immunoblotting or immunostaining of intact cells, provides a mechanistic explanation for the inhibition of migration by the angiogenesis inhibitor.

**HRGP330 inhibits VEGF-induced tyrosine phosphorylation of FAK and α-actinin.** The cytoplasmic tyrosine kinase FAK is crucial for focal adhesion function. Several angiogenesis inhibitors such as angiostatin and endostatin have been shown to activate FAK in endothelial cells (24, 25). Both VEGF and HRGP330 individually stimulated tyrosine phosphorylation (reflecting activation) of FAK in endothelial cells, with a slight increase after 10 minutes and a more pronounced effect after 2 hours of treatment (Fig. 3A). In contrast, in cells cotreated with VEGF and HRGP330, FAK tyrosine phosphorylation was attenuated. One important substrate for the FAK kinase is α-actinin, of which binding to actin is negatively regulated by its tyrosine phosphorylation (26, 27). Tyrosine phosphorylation of α-actinin was studied in cells treated with HRGP330 and VEGF for different time periods under the same conditions as above (Fig. 3B). Immunoblotting analyses showed that VEGF as well as HRGP330 induced a robust phosphorylation of α-actinin both at 10 minutes and 2 hours. However, cotreatment of the cells with VEGF and HRGP330 for 2 hours blocked α-actinin phosphorylation, indicating persistent binding to actin and reduced cellular motility.

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**Figure 3.** HRGP330 inhibits VEGF-induced activation of FAK and tyrosine phosphorylation of α-actinin and induces redistribution of α-actinin. Endothelial cells on vitronectin were treated with HRGP330 (100 ng/mL) and VEGF (50 ng/mL in A and B, 10 ng/mL in C), individually or in combination, for 10 minutes and 2 hours. A, both VEGF and HRGP330 added individually induced tyrosine phosphorylation of FAK at 10 minutes and more pronounced after 2 hours, as shown by immunoprecipitation of FAK and subsequent immunoblotting for phosphotyrosine (PY). Cotreatment of the cells with VEGF and HRGP330, both at 10 minutes and 2 hours, abolished FAK phosphorylation. Bottom, control immunoblotting for FAK. Levels of phosphorylated FAK normalized to total FAK levels are depicted graphically below the blot (PY/FAK). B, VEGF and HRGP330 added individually induced a pronounced increase in tyrosine phosphorylation of α-actinin (α-act), both at 10 minutes and 2 hours, as shown by immunoprecipitation for α-actinin, followed by immunoblotting for phosphotyrosine or α-actinin. Cotreatment of cells with VEGF and HRGP330 for 10 minutes allowed α-actinin phosphorylation whereas, at 2 hours, the level of phosphorylation had returned to basal, in contrast to treatment with the individual factors. Bottom, control immunoblotting for α-actinin. Levels of phosphorylated α-actinin normalized to total α-actinin levels are depicted graphically below the blot (PY/α-act). C, immunocytochemical staining for α-actinin (red) was done in endothelial cells on vitronectin treated with VEGF and HRGP330, individually or in combination, for 10 minutes and 2 hours (i.e., the same experimental setup as in A and B). FAK staining (green) was used to visualize focal contacts. No significant change in α-actinin staining pattern compared with control cells was detected after individual treatment with VEGF or HRGP330 or after the combined treatment for 10 minutes. Cotreatment with VEGF and HRGP330 for 2 hours resulted in accumulation of α-actinin along actinlike fiber structures. Bar, 8 μm.


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**HRGP330 and VEGF cotreatment induces redistribution of α-actin.** Migrating cells have a rapid turnover of focal adhesions and a dynamic actin cytoskeleton whereas nonmigrating cells are characterized by localization of α-actin to their stable focal adhesions (27). Further, α-actin incorporation into actin fibers is likely to promote a less dynamic cytoskeleton (28). The distribution of α-actin was investigated by immunofluorescent staining of endothelial cells treated with VEGF and HRGP330 as indicated (Fig. 3C). Untreated cells or cells treated with VEGF or HRGP330 alone, for 10 minutes or 2 hours, showed low α-actin immunoreactivity. However, in cells cotreated with VEGF and HRGP330 for 2 hours, α-actin-positive fibers were clearly visible, merging with focal adhesions visualized by FAK staining. These data reinforce the notion that HRGP330 acts to inhibit endothelial cell motility responses by subverting the function of focal adhesions.

**HRGP330 interferes with VEGF-induced polarization and lamellipodia formation but does not inhibit VEGFR-2 activation.** Cell migration requires the concerted regulation of growth factor–induced lamellipodia formation and cell polarization. Endothelial cells on coverslips were monitored by time-lapse microscopy to study the effect of HRGP330 on these cellular responses (Fig. 4A; Supplementary data). Untreated cells were nonpolarized with a basal lamellipodia activity. Treatment with HRGP330 alone (Supplementary data) did not affect the cell morphology whereas inclusion of VEGF resulted in a transient burst of increased formation of lamellipodia at 30 minutes and, within two hours, a shift towards a polarized phenotype with elongated cells, consistent with a migratory phenotype. Cotreatment of the cells with VEGF and HRGP330 abrogated the VEGF-induced changes, indicating that HRGP330 directly or indirectly interferes with signaling events induced by VEGF (Fig. 4A; Supplementary data).

To show that HRGP330 did not simply exert its effects by direct inhibition of activation of VEGFR-2, the main transducer of endothelial cell responses to VEGF, cells were treated with VEGF in the presence and absence of HRGP330 (i.e., similar conditions as those used above to show HRGP330 activation of FAK). As shown in Fig. 4B, VEGF-2 tyrosine phosphorylation, indicative of its activation, was induced by VEGF irrespective of whether HRGP330 was included in the treatment or not.

**Involvement of αvβ3 integrin in HRGP action.** We have previously shown that HRGP interferes with the ability of endothelial cells to adhere to vitronectin (16). As shown in Fig. 5A, HRGP330 also inhibited adhesion of endothelial cells to vitronectin, possibly implicating the αvβ3 integrin, the main vitronectin receptor on endothelial cells, as a target molecule for HRGP330. To address the role of αvβ3 in the adhesion assay, we compared the effect of treatment with the neutralizing anti-αvβ3 antibodies and HRGP/HRGP330 on endothelial cell adhesion to vitronectin. As shown in Fig. 5A, treatment with HRGP/HRGP330 and anti-αvβ3 antibodies inhibited the vitronectin-induced adhesion. Under conditions where adhesion was only partially inhibited by individual treatment with HRGP or by the neutralizing antibody LM609, we noted that the combined treatment did not further reduce adhesion of the endothelial cells (Fig. 5B). These data indicate an overlapping target for HRGP and the anti-αvβ3 antibody, suggesting that HRGP/HRGP330 may act via interference with αvβ3 function.

That αvβ3 integrin function was important for endothelial cell motility under the conditions used in the chemotaxis assay was shown by inclusion of a neutralization anti-αvβ3 antibody, which reduced VEGF-induced chemotaxis to basal levels (Fig. 5C). The chemotaxis experiment was done using collagen I–coated filters because vitronectin coating of the polycarbonate filters used in the chemotaxis assay did not support endothelial cell adhesion. It is conceivable that αvβ3 integrins used cryptic sites exposed in the partially denatured collagen I coat for movement and that these interactions were obliterated by the neutralizing antibody, thereby attenuating the migratory response (Fig. 5C). This result shows that migration of the endothelial cells in the present experimental setup is dependent on αvβ3 integrins and supports the notion that HRGP330 may act by interfering with integrin signaling.

**Discussion**

HRGP330 exerts its antiangiogenic effect by arresting the actin cytoskeleton in an immobile state and preventing growth factor–induced promigratory events, such as lamellipodia formation and cell polarization. The molecular mechanism involves disruption of VEGF-induced complex formation between paxillin and ILK, as well as...
as prevention of VEGF-induced phosphorylation of FAK and its downstream substrate α-actinin. VEGFR-2 activation is not affected by the presence of HRGP330. Instead, our data implicate αvβ3, the main vitronectin receptor on endothelial cells, in the mechanism of action of HRGP330. However, we do not exclude that the receptor mechanism involves one or more additional cell-surface components. A summary and possible model for the mechanism of action of HRGP330, based on the above findings, is depicted in Fig. 6.

HRGP interacts with a number of plasma proteins (14). Complex formation with thrombospondins 1 and 2, involving a COOH-terminal CLESH-1 domain of HRGP (29, 30), has received attention as it apparently leads to neutralization of the antiangiogenic effect of thrombospondin. Therefore, the identification of a 35-amino-acid residue peptide, HRGP330, which lacks the thrombospondin binding domain but retains the antiangiogenic properties, is important in further development of HRGP as an angiogenesis inhibitor (15, 16, 31). Although it is likely that the stability of HRGP330 is low in vivo, we achieved a significant reduction in BxPC3 vascularization with a virtual disappearance of small vessels in tumors in the treated animals. Donate et al. (31) recently reported on a multimerized pentapeptide based on the repetitive sequence GHHPH from the His/Pro–rich domain of HRGP, which was used to inhibit angiogenesis in s.c. Matrigel plugs and tumor growth in mice at 80 mg/kg/d. Five repeats of the sequence GHHPH are contained within the peptide HRGP365 that did not show any antichemotactic effect on endothelial cells in our hands (Fig. 1B) whereas HRGP330 contains one GHHPH repeat. Another difference between HRGP330 and HRGP365 and HRGP398 is that HRGP330 binds to heparin/heparan sulfate, showing that HRGP330 is dependent on sequences distinct from GHHPH for its biological properties.1

How does HRGP330 interact with the endothelial cell surface and transduce its effects? Considering the effect of HRGP/HRGP330 on endothelial cell adhesion, migration, and focal adhesions, integrins are obvious receptor candidates. The major vitronectin receptor on endothelial cells, αvβ3 integrin, is implicated in the mechanism of action of HRGP330. However, it is likely that the receptor mechanism involves one or several cell-surface molecules, such as heparan sulfates, or other thus far unidentified components (?). Vn, vitronectin; HSPG, heparan sulfate proteoglycan; pax, paxillin; α-act, α-actinin.

**Figure 6.** Mechanism of action of HRGP330. The antiangiogenic effect of HRGP330 is exerted through an arrest of the endothelial cell actin cytoskeleton and a consequent inhibition of VEGF-induced effects such as lamellopodia formation and polarization, events needed for migration, and an angiogenic response. HRGP330 disrupts VEGF-induced complex formation between paxillin and ILK in focal adhesions, as well as VEGF-induced phosphorylation/activation of FAK and its downstream substrate α-actinin. VEGFR-2 activation is not affected by the presence of HRGP330. The main vitronectin receptor on endothelial cells, αvβ3 integrin, is implicated in the mechanism of action of HRGP330. However, it is likely that the receptor mechanism involves one or several cell-surface molecules, such as heparan sulfates, or other thus far unidentified components (?). Vn, vitronectin; HSPG, heparan sulfate proteoglycan; pax, paxillin; α-act, α-actinin.

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**Figure 5.** Involvement of αvβ3 integrin in HRGP action. A, adhesion of endothelial cells to vitronectin was significantly reduced in the presence of HRGP, HRGP330, or neutralizing αvβ3 antibody (LM609). B, under conditions where adhesion was only partially decreased (compare extent of HRGP and HRGP330 block in relation to BSA between A and B), no further reduction was seen as a result of combined treatment with HRGP and LM609. C, chemotaxis of endothelial cells towards VEGF in a modified Boyden chamber was reduced to similar extents by inclusion of HRGP, HRGP330, or neutralizing αvβ3 antibody (LM609). Bars, 1 SD. All changes induced by HRGP, HRGP330, or LM609, compared with control in (A and B), or by the combination of VEGF and HRGP, HRGP330, or LM609, compared with VEGF in (C), were significant (P < 0.01; P < 0.05).

expressed on angiogenic endothelial cells in conjunction with vessel remodeling (32). We show that endothelial cell chemotaxis is dependent on αβ3; moreover, neutralizing anti-αβ3 antibodies block endothelial cell adhesion to vitronectin to the same extent as HRGP330. These data are compatible with the notion that the mechanism of action of HRGP330 involves interference with αβ3 function. Moreover, ongoing studies implicate heparan sulfate in the mechanism of action of HRGP330. It is of interest that αβ3 has been shown to occur in complex with VEGFR-2 and to be required for VEGFR-2 responsiveness (33). In this context, it is important to note that HRGP330 does not simply interfere with VEGFR-2 activation by binding either to heparin/heparan sulfate or to αβ3 (see Fig. 4B). Thus, we suggest that there may be more than one interactive partner for HRGP330 on the endothelial cell surface. The His/Pro-rich region of HRGP is able to bind Zn2+, which is also required for HRGP330 to exert its antiangiogenic function. Spectra obtained using far-UV technique indicate that HRGP belongs to the category of intrinsically unstructured proteins (34). A probable effect of Zn2+ binding is stabilization of the conformation of the His/Pro-rich domain, which may be required for its interaction with a receptor.

Other potential mechanisms of action of HRGP330 include the possibility of interaction with circulating vitronectin, which may promote homing of HRGP330 to sites of active angiogenesis. This type of mechanism has recently been proposed for the antiangiogenic molecules anginex and anastellin (10). Moreover, two recent reports from the same group (31, 35) suggest membrane-bound tropomyosin as a receptor for HRGP. Tropomyosins constitute a large family of widely expressed intracellular actin-binding molecules lacking transmembrane domain (36). It is noteworthy that tropomyosin has been suggested to also serve as a receptor for kininogen (37) as well as for endostatin (38). The current consensus is that endostatin requires α5β1 integrins and heparan sulfate to exert its effects on endothelial cells.

Our in vitro data show that HRGP330 potently inhibits chemotaxis of endothelial cells in the nanogram range. Cell motility is a complex process, which requires cells to polarize and concentrate lamellipodia and filopodia activity to the leading edge, where small focal adhesions are formed de novo. Actin stress fibers, microtubuli, and RhoA activity are confined to and necessary for the trailing end where focal adhesions are larger and need to be remodeled or disrupted. The chemotaxic signal emitted by receptor tyrosine kinases, such as VEGFR-2, converge with signals from integrin receptors to orchestrate the motility response. This integration may be achieved by direct interaction (e.g., between VEGFR-2 and αβ3) serving to amplify reciprocal signals (see ref. 39 for review). Furthermore, downstream signaling pathways integrate to transmit a permissive migratory signal. Such pathways include the cytoplasmic kinases FAK and ILK, both of which interact directly or indirectly with integrins as well as growth factor receptors. FAK has been ascribed a critical role in focal adhesion turnover (40) and FAK-dependent tyrosine phosphorylation of α-actinin blocks its association with actin, allowing increased actin dynamics (26). In FAK-/- cells, α-actinin fails to become tyrosine phosphorylated, indicating a key role for FAK in this signaling pathway (26). ILK has a structural role in linking integrins to the actin cytoskeleton (41). Endothelial cell–specific ablation of the serine/threonine kinase ILK leads to severe vascular defects and embryonic death at E11.5 (42) and inhibition of ILK kinase activity inhibits tumor angiogenesis in mice (43). The importance of ILK serine/threonine kinase activity, however, has been questioned as genetic data from C. elegans and D. melanogaster suggest that the kinase activity is dispensable in vivo (44, 44). Of particular relevance for interpretation of data in this study, overexpression of a mutant form of ILK that lacks the ability to engage in complex formation with paxillin leads to severely reduced motility (23). Moreover, cells overexpressing wild-type ILK fail to polarize and display a poorly spread phenotype similar to that of cells overexpressing RhoA. Forced expression of FAK, which negatively regulates RhoA (45), rescues this phenotype in wild-type ILK–transfected cells, suggesting that a balanced activation of FAK and ILK is required for polarization and migration. Our results on the effect of HRGP330 on FAK and ILK are in excellent agreement with the well-established and central role of these cytoplasmic kinases in cell migration.

Cancer is a major cause of premature death. Proof of the concept that angiogenesis inhibition has clinical benefits in tumor treatment is given by the VEGF-blocking antibody Avastin (bevacizumab). Recent reports indicate that VEGFR-2 expression and function is not restricted to angiogenic endothelium and effects on neuronal survival cannot be excluded in long-term anti-VEGF treatments (46). We have, at this point, no indications of adverse effects on normal vasculature by systemic HRGP/HRGP330 treatment but these studies will continue. It seems clear that members of the cystatin superfamily, to which HRGP belongs, present a number of interesting features that support our focus on HRGP as an angiogenesis inhibitor. Thus, fetuin-B has recently been implicated as a tumor suppressor (47) and kininogen possesses antiangiogenic properties, also mediated via a histidine-rich domain (37). Deletion of the proximal region of mouse chromosome 16, containing fetuin, fetuin-B, HRGP, and kininogen of the cystatin superfamily, as well as other genes, has been associated with the “angiogenic switch” in a transgenic model of multitissue tumorigenesis (48). A recent report on HRGP gene inactivation implicates HRGP in regulation of blood coagulation (49). The consequence of loss of HRGP expression for pathologic angiogenesis awaits further characterization. In conclusion, antiangiogenic drugs have a large potential, perhaps in particular when combined with cytotoxic drugs or surgery, and their development will be aided by mechanistic insights.

Acknowledgments


Grant support: Swedish Cancer Society (project no. 3820-B04-09XAC), Innovonics Project AB, and the Sixth European Union Framework Programme (Integrated Project “Angiotargeting”; contract no. 504743) in the area of Life Sciences, Genomics, and Biotechnology for Health (L. Claesson-Welsh; Magnus Bergvalls Foundation and Erik, Karin, and Gosta Selanders Foundation [A-K. Olsson]; and Swedish Society of Medical Research [J. Dixellus]).

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We thank Dr. Martin McMahon for the kind gift of telomerase immortalized endothelial cells.

References

4. Ferrara N, Henzel WJ. Putative follicular cell secretions secrete a novel heparin-binding growth factor specific for vascular
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