A Fragment of Histidine-Rich Glycoprotein Is a Potent Inhibitor of Tumor Vascularization

Anna-Karin Olsson,1 Helena Larsson,1 Johan Dixellius,1 Irja Johansson,1 Chunsik Lee,1 Cornelia Oellig,2 Ingemar Björk,3 and Lena Claesson-Welsh1

1Department of Genetics and Pathology, Uppsala University, Rudbeck Laboratory, Uppsala; 2Innovetus Project AB, Uppsala; and 3Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Biomedical Center, Uppsala, Sweden

ABSTRACT

In this study, we show that recombinant human histidine-rich glycoprotein (HRGP) has potent antiangiogenic properties as judged from effects on a syngeneic tumor model in C57/bl6 mice. Growth of fibrosarcoma, a very aggressive tumor, was reduced by >60% by HRGP treatment, and tumor angiogenesis was dramatically decreased. Treatment with HRGP led to increased apoptosis and reduced proliferation in the tumors. In contrast, HRGP did not affect apoptosis or DNA synthesis in endothelial cells or tumor cells in vitro. The mechanism of action of HRGP involves rearrangement of focal adhesions and decreased attachment of endothelial cells to vitronectin and, as a consequence, reduced endothelial cell migration. By using truncated versions of HRGP, we demonstrate that the isolated 150 amino acid-residue His/Pro-rich domain, which is also released by spontaneous proteolysis from purified HRGP, mediates the inhibitory effect on chemotaxis. Moreover, the His/Pro-rich domain must be released from HRGP to exert its effect. This study shows for the first time inhibitory effects of HRGP on tumor vascularization in vivo, thus providing proof of concept that HRGP is an angiogenesis inhibitor.

INTRODUCTION

Angiogenesis, formation of new capillaries from already existing blood vessels, is essential during development and physiological conditions that require increased vascularization such as wound healing and the menstrual cycle (1). The resting vasculature is tightly regulated by a balance between pro- and antiangiogenic factors (2). This balance is disturbed in a number of pathological processes, resulting in deficient angiogenesis, as in ischemic conditions, or excess angiogenesis, as in rheumatoid arthritis, diabetic retinopathy, and tumor growth (3). It is well established that many types of tumors stimulate infiltration of new capillaries to grow and metastasize (2, 4–6).

Histidine-rich glycoprotein (HRGP) is a heparin-binding plasma protein (7), present in human plasma at a concentration of 100 μg/ml (8). The amino acid sequence of mouse, rat (9), rabbit (10), and human (11) HRGP has been resolved, and the protein is well conserved among these vertebrate species. Structurally, the HRGP molecule can be divided into three main domains. The NH2-terminal domain contains two cysteine protease inhibitor (cystatin)-like stretches (Fig. 1A), which allows the classification of HRGP as a member of the cystatin superfamily together with α2HS glycoprotein, cystatin, and kininogen. The central domain is very rich in proline and histidine residues, and the human form contains 12 more or less conserved tandem repeats of the pentapeptide HHPHG. Both the central domain and the COOH-terminal domain are disulfide bonded to the cystatin-like stretches in the NH2-terminal domain (10). HRGP is present in platelet α-granules and is released after thrombin stimulation (12), but mRNA can only be found in the liver (9). HRGP is sensitive to proteolytic cleavage and degradation products of the protein are present in purified fractions, giving rise to a characteristic pattern of smaller bands when resolved on polyacrylamide gels (13).

Multiple binding partners for HRGP have been reported such as heparin/heparan sulfates (14), divalent cations (15, 16), components in the coagulation-fibrinolysis system; plasminogen (17) and fibrinogen (18), components in the immune system such as T lymphocytes (19, 20), monocytes/macrophages (21) and immunoglobulins (22); and extracellular matrix components such as thrombospondin-1 and vitronectin (for a review, see Ref. 23). Suggested functions of HRGP include modulation of fibrinogenesis (13), inhibition of insoluble immune complex formation (22), and, recently, potentiation of the ingestion of apoptotic cells by macrophages (24). In this study, we demonstrate that human HRGP, purified from plasma or recombinantly produced, has potent antiangiogenic properties, mediated via its His/Pro-rich domain.

MATERIALS AND METHODS

Tissue Culture. The porcine aortic endothelial (PAE) cell line expressing fibroblast growth factor receptor-1 (FGFR-1; Ref. 25) was cultured in Ham’s F12/10% fetal calf serum (FCS); NIH 3T3 murine fibroblasts were cultured in DMEM/10% newborn calf serum. Media and serum were from Life Technologies, Inc. Primary bovine adrenal cortex capillary endothelial (BCE) cells were cultured on gelatin-coated dishes in DMEM/10% newborn calf serum, 2 ng/ml FGF-2 (Boehringer Mannheim). For chemotaxis assays, PAE and NIH 3T3 cells were serum starved over night in 0.1% BSA and BCE cells in 0.5% newborn calf serum. Human embryonic kidney 293-EBNA cells were cultured in DMEM/10% FCS. Approximately every second month, they were given 0.25 mg/ml G418 (Calbiochem) to ensure selection of the EBNA-1 expression.

HRGP Expression Vectors, Transfection, and Purification of Protein. Full-length cDNA encoding human HRGP, including the signal sequence (amino acid residues 1–18; Invitrogen, Carlsbad, CA) was cloned into the pCEP-Pu2 (26) expression vector. Expression vectors for His-tagged HRGP variants were also constructed using this vector. Truncations were produced by PCR-amplification. A His-tag was introduced NH2-terminally of the HRGP coding region to enable purification. An enterokinase cleavage site was introduced between the His-tag and the HRGP coding region but was never used because of enterokinase spuriously cleaving within the HRGP polypeptide chain. In the His-tag constructs, the HRGP signal sequence was excluded, and the PCR product was ligated in frame with the BM40 signal sequence in pCEP-Pu2.

Human embryonic kidney 293-EBNA cells were used to produce recombinant HRGP. These cells are stably transfected with the EBNA-1 gene, which is also expressed by the pCEP-Pu2 vector, thereby preventing chromosomal integration of transfected plasmid DNA. This allows an overall high yield of recombinant protein. The HRGP expression vectors were transfected using Lipofectamine (Invitrogen) and selected with 2.5 μg/ml puromycin (Sigma). To avoid contamination of bovine HRGP, a defined serum-replacement medium, TCM (ICN Biomedicals) was used instead of FCS.

Chromatography on phosphocellulose (13, 27) in the presence of protease inhibitors was used to purify HRGP from freshly collected human plasma (pHRGP) or recombinant untagged HRGP from conditioned medium (rHRGP). His-tagged HRGP was purified using Ni-NTA agarose (Qiagen). Protein-containing fractions were pooled and dialyzed against PBS (pH 7.4).
HRGP FRAGMENT HAS POTENT ANTIANGIOGENIC PROPERTIES

Fig. 1. Human histidine-rich glycoprotein (HRGP) inhibits chorioallantoic membrane angiogenesis. A, structurally HRGP can be divided into three main domains; the \( \text{NH}_2 \) terminus with two cystatin-like stretches, a histidine-proline-rich (His-Pro-rich) middle domain and the COOH terminus. Both the His-Pro-rich middle domain and the COOH terminus are disulfide bonded to the \( \text{NH}_2 \)-terminal part of the protein. B, HRGP was obtained from three different sources: either purified from human plasma (pHRGP) or recombinantly produced, with or without a His-tag (His-HRGP and rHRGP, respectively). Both Coomassie staining and Western blot (WB) with a HRGP-specific antibody revealed, except for the full-length protein at \( 75 \text{kDa} \), a number of smaller fragments. C, fibroblast growth factor (FGF)-2-induced angiogenesis in the chicken chorioallantoic membrane was effectively inhibited in the presence of HRGP.

Chorioallantoic Membrane (CAM) Assay. The conditions for the CAM assay were as described previously (28, 29), FGF-2 (Boehringer Mannheim) and vascular endothelial growth factor (VEGF)-A (Peprotech) was used at 0.5 ng/ml and the rabbit polyclonal peptide antibodies 0115, 0116 and 0119 (produced in-house) at 1:5000 dilution. The anti-penta His antibody (Molecular Probes) was used at 1:5000 dilution. The samples were separated using SDS-PAGE (12% gels) and visualized by silver staining (Invitrogen). Both Coomassie staining and Western blot (WB) with a HRGP-specific antibody revealed, except for the full-length protein at \( 75 \text{kDa} \), a number of smaller fragments. C, fibroblast growth factor (FGF)-2-induced angiogenesis in the chicken chorioallantoic membrane was effectively inhibited in the presence of HRGP.

Immunohistochemical Staining. Paraffin sections were immunohistochemically stained using a goat antimouse CD31 antibody (1506; Santa Cruz Biotechnology), diluted 1:500 and incubated at +4°C over night, and a rat antimouse Ki67 antibody (TEC-3/M7249; Dako) diluted 1:50 and incubated for 30 min at room temperature. Detection of apoptotic cells by the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) technique was performed using ApopTag (Intergen Company). Stereological quantification of vascular parameters was performed as described earlier (31, 32). The relative number of apoptotic cells in the tumors was calculated by counting all TUNEL-positive cells in two sections from the middle of three different tumors from each treatment group. The number of TUNEL-positive cells was set in relation to the mass of each tumor. The proportion of Ki67-positive cells was calculated from counting -3000 cells/tumor section in three tumors from each treatment group. Significance at the level of \( P < 0.05 \) was calculated using Student’s t test.

Immunofluorescence. BCE cells were seeded onto gelatin-coated Culture slides (Falcon), starved overnight (DMEM/1% newborn calf serum), and treated with HRGP (100 ng/ml), FGF-2 (10 ng/ml) and VEGF (10 ng/ml) for 10 min as indicated. The slides were put on ice, washed in ice-cold TBS [20 mM Tris-HCl (pH 7.5) and 150 mM NaCl] and fixed in Zn-fx [0.05% Ca-Ac, 0.5% Zn-Ac, 0.5% ZnCl in TBS (pH 6.6), and 0.2% Triton X-100] for 15 min at room temperature. Paxillin was detected using anti-paxillin antibody (0.25 
\[ \mu g/ml \], no. 13520; Transduction Laboratories) and a secondary Alexa 488-conjugated secondary antibody (no. A-11017; Molecular Probes). Nuclei were stained with Hoechst 33342 (1 
\[ \mu g/ml \]; Molecular Probes). The preparations were examined and microphotographed using a Nikon Eclipse E1000 microscope.

Adhesion Analysis. BCE cells were detached using nonenzymatic Cell Dissociation solution (Sigma), washed, and resuspended in DMEM/0.1% BSA and FGF-2 (2 ng/ml). The cells were seeded into wells precoated with BSA, collagen I, collagen IV, laminin-1, or vitronectin, respectively (CytoMatrix ECM 205 kit; Chemicon). The cells were incubated for 45 (collagen I, collagen IV, and vitronectin) or 60 min (laminin-1), washed three times in PBS, and stained with Hoechst 33342 (1 
\[ \mu g/ml \]; Molecular Probes). The cells were microphotographed using the x2 objective, and the number of attached cells was counted using the Easy Image Analysis software (Tekno Optik). Statistical analysis was performed by ANOVA and Tukey’s honestly significant difference test. The SD was based on the pooled variance.

Chemoattractant Assay. The migration assay was performed using a modified Boyden chamber as described earlier (33), using microtiter cell culture inserts (8.0 
\[ \mu m \] pore) coated with type-1 collagen solution (BCE) at 100 
\[ \mu g/ml \] (BD Biosciences). Cells were preincubated or not with HRGP (100 ng/ml) for 30 min, trypsinized, and resuspended at 5.0 
\[ \times 10^5 \] cells/ml. The cell suspension was added in the upper chamber and FGF-2 (5 ng/ml), VEGF-A (5 ng/ml), HRGP (100 ng/ml), the protein C inhibitor (100 
\[ \mu g/ml \]; Molecular Probes) and a secondary Alexa 488-conjugated secondary antibody (no. A-11017; Molecular Probes). The samples were separated using SDS-PAGE (12% gels) and visualized by silver staining (Invitrogen). Both Coomassie staining and Western blot (WB) with a HRGP-specific antibody revealed, except for the full-length protein at \( 75 \text{kDa} \), a number of smaller fragments. C, fibroblast growth factor (FGF)-2-induced angiogenesis in the chicken chorioallantoic membrane was effectively inhibited in the presence of HRGP.

Immunoprecipitation and Western Blotting. Purified HRGP was separated on 10% (pHRGP, hrHRGP, His-HRGP, and His 4) or 12.5% SDS-PAGE (His 5). The monoclonal mouse antihuman HRGP antibody M037 (Takara) was used at 0.05 
\[ \mu g/ml \] and the rabbit polyclonal antibody polyclonal antibodies 0115, 0116 and 0119 (produced in-house) at 1:5000 dilution. The anti-penta His antibody, directly conjugated to horseradish peroxidase (34460; Qiagen), was used at 1:5000 dilution.

FGFR-1 was immunoprecipitated from starved PAE/FGFR-1 cells, untreated or treated with 100 ng/ml HRGP in the presence and absence of 50 ng/ml FGF-2, as indicated, using phosphosyrosine antibodies 4G10. In parallel, total cell lysates were prepared by solubilization of cells in Triton X-100 lysis buffer [0.5% Triton X-100, 0.5% sodium deoxycholate, 20 mM Tris HCl (pH 7.5), 10 mM EDTA, 150 mM NaCl, 1 mM Na_3 VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 
\[ \mu g/ml \] leupeptin, 10 
\[ \mu g/ml \] aprotinin]. The samples were separated...
by 7.5–10% SDS-PAGE and transferred to nitrocellulose. The phosphoryrosine immunoprecipitates were blotted using a monoclonal antibody against FGFR-1, kindly made available by Dr. Pamela Maher (Department of Cell Biology, The Scripps Research Institute, La Jolla, CA). Total cell lysates were blotted with the FGFR-1 monoclonal antibody, with antibodies against phospho-extracellular-regulated kinase 1 and 2 and with antibodies against phospho-histone H1 (1b102/Tyr 204, New England Biolabs, Beverly, MA), as indicated.

For paxillin analyses, starved BCE cells, untreated or treated with 100 ng/ml full-length HRGP, were lysed in NP40 lysis buffer (1% NP40, 150 mM NaCl, 10% glycerol, 20 mM HEPES, 1 mM phenylmethylsulfonyl fluoride, 2.5 mM EDTA, 100 μM Na3VO4, and 1% aprotinin). For immunoprecipitation, cell lysates were incubated with anti-paxillin antibody (no. P13520; BD Biosciences) for 2 h on ice and precipitated with Protein G Sepharose (Amersham Biosciences). The samples were separated by 8% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated with phosphotyrosine antibody (no. SC-7020; Santa Cruz Biotechnology) and detected using horseradish peroxidase-conjugated secondary antibody. Immune complexes were visualized by enhanced chemiluminescence (Amersham Biosciences).

### RESULTS

**HRGP Sources.** HRGP from three different sources were used; pHRGP and recombinant HRGP produced in human embryonic kidney 293-EBNA cells, with or without a His-tag (His-HRGP and rHRGP, respectively). Recombinant untagged HRGP migrates with an apparent lower molecular mass on SDS-PAGE than HRGP purified from plasma (Fig. 1B), probably reflecting different levels of glycosylation. The 10 extra amino acid residues in His-HRGP decreased the molecular mass of the 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membranes were incubated with phosphotyrosine antibody (no. SC-7020; Santa Cruz Biotechnology) and detected using horseradish peroxidase-conjugated secondary antibody. Immune complexes were visualized by enhanced chemiluminescence (Amersham Biosciences).

**DNA Synthesis Assay.** BCE or T21 cells were serum starved in 0.1% BSA over night and then treated with 2 ng/ml (BCE) or 10 ng/ml (T241) FGF-2 and 0.5 μCi of [3H]thymidine for 20 h. Cells were washed in PBS and exposed to 10% trichloroacetic acid for 20 min on ice. The precipitates were washed in 99% ethanol and solubilized in 0.2 M NaOH and analyzed by liquid scintillation.

**HRGP Inhibits CAM Angiogenesis.** The effect of HRGP on angiogenesis was tested in the chicken CAM assay. A filter disk soaked in vehicle or growth factor, with or without HRGP, was placed on top of the CAM in the vicinity of a large vessel and incubated for 3 days. Treatment of the CAM with angiogenic growth factors such as FGF-2 and VEGF stimulated angiogenesis (Fig. 1C; Table 1). Application of FGF-2 or VEGF-A together with pHRGP on the CAM led to an efficient suppression of newly formed vessels, whereas preestablished vessels remained unaffected. The same result was obtained using rHRGP (data not shown).

**HRGP Inhibits Tumor Growth in Mice.** Because angiogenesis plays an important role in the growth of aggressive tumors, the effect of HRGP on progression of fibrosarcoma in mice was investigated, using both pHRGP (Fig. 2A) and His-HRGP (Fig. 2B). C57/bl6 mice were inoculated s.c. with T241 fibrosarcoma cells on their left flank and when the tumors were palpable (after 4–6 days), a daily treatment was initiated with HRGP or control (PBS, Fig. 2A; or human IgG, Fig. 2B). Endostatin was included in one study for comparison (Fig. 2B). The treatment was given as s.c. injections in the right flank until the size of the control tumors reached the upper limit of 2 cm3 (11 days, Fig. 2A; 14 days, Fig. 2B). Injections with both pHRGP (4 mg/kg/day) and His-HRGP (5 mg/kg/day) led to a drastic reduction in tumor growth and at the time of sacrifice, the size of the tumors was reduced by 67% (Fig. 2A) and 61% (Fig. 2B), respectively, compared with control-treated animals. Endostatin treatment (25 mg/kg/day) showed only a moderate inhibitory effect on tumor growth in this model (32% decrease).

**Reduced Vascularization in Tumors from HRGP-Treated Mice.** Sections of paraffin-embedded tumors from control (IgG)- or HRGP-treated mice were immunohistochemically stained for CD31 expression to visualize the vessels. Visual inspection revealed striking changes in the extent of vascularization of the treated tumors compared with control, in particular, with regard to the reduced vessel diameter (Fig. 2C). Stereological quantification of vascular parameters was performed as described earlier (31, 32). This method of quantifying tumor angiogenesis relates the length, volume, and surface area of the vessels to tumor volume. The results show that all six vascular parameters determined were reduced in tumors from HRGP-treated mice (Fig. 2D).

**Increased Apoptosis and Decreased Proliferation in Tumors from HRGP-Treated Mice.** To determine the mechanism whereby HRGP treatment reduced tumor growth, we quantified apoptotic and proliferating cells in tumor tissue from the two treatment groups. Apoptotic cells were visualized by TUNEL staining. The number of TUNEL-positive cells was increased ~2-fold in the HRGP-treated tumors (Fig. 2A). The proportion of proliferating cells was determined by immunostaining for the Ki67 antigen. As shown in Fig. 3B, there was a significant decrease in proliferating cells in the tumors from HRGP-treated mice compared with those from control-treated animals, 29 and 43%, respectively.

The extent of apoptosis of endothelial and tumor cells in vitro was very low (~1–2%) and remained unaffected by addition of HRGP for 24 h (data not shown). Similarly, DNA synthesis in endothelial and tumor cells in vitro was not affected by the inclusion of 100 ng/ml HRGP (Fig. 3, C and D).

**HRGP Inhibits Chemotaxis of Primary Endothelial Cells.** A common feature of the antiangiogenic molecules is their ability to inhibit chemotaxis of endothelial cells in vitro. In accordance, inclusion of 100 ng/ml rHRGP completely blocked FGF-2-induced chemotaxis of BCE cells (Fig. 4A). The same concentration of rHRGP also attenuated VEGF-A-induced chemotaxis (Fig. 4B). However, chemotaxis induced by FCS was not inhibited by rHRGP (Fig. 4C). To demonstrate specificity, another plasma protein of approximately the same mass as HRGP, the protein C inhibitor, was included for comparison. Protein C inhibitor had no effect on FGF-2-induced chemotaxis (Fig. 4D). The specificity of HRGP toward endothelial cells was implied by the fact that FGF-2-induced chemotaxis of NIH 3T3 fibroblasts was not affected by rHRGP (Fig. 4E).

**Mechanism of Action of HRGP.** HRGP is a heparin-binding protein. Possibly, the inhibition of endothelial cell chemotaxis by HRGP could be due to competition with FGF-2/FGFR-1 for binding to heparan sulfate, which is an important coreceptor for the FGFs. Fig. 5 shows that FGF-2-induced tyrosine phosphorylation of FGFR-1 in the PAE/FGFR-1 cells was not suppressed by HRGP. Furthermore,

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Table 1 Inhibitory effect of histidine-rich glycoprotein (HRGP) (3 μg/filter) on chicken chorioallantoic membrane angiogenesis induced by fibroblast growth factor-2 (FGF-2) or vascular endothelial growth factor (VEGF)-A (0.2 μg/filter)¢

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>Inhibitor</th>
<th>Angiogenesis score</th>
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<tbody>
<tr>
<td>Buffer</td>
<td>1.2</td>
<td></td>
</tr>
<tr>
<td>FGF-2</td>
<td>2.4</td>
<td></td>
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<tr>
<td>FGF-2</td>
<td>Plasma HRGP</td>
<td>1.3</td>
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<tr>
<td>VEGF</td>
<td>2.8</td>
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<tr>
<td>VEGF</td>
<td>Plasma HRGP</td>
<td>1.6</td>
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¢ The score, from 0 (low) to 3 (high) was based on the number of vessel branch points, as described previously (28, 29). Average values for five to six embryos were recorded. The variability was <15%.
FGF-2-induced extracellular-regulated kinase 1/2 activation was not affected by HRGP.

Migration of cells requires a continuous turnover of cell-matrix interactions (34). Cells interact with the underlying matrix at focal adhesions where specific integrins act as receptors for different extracellular matrix proteins. Ligation of the integrins leads to activation of focal adhesion kinase, a cytoplasmic kinase that is engaged in a multiprotein complex at the focal adhesions. We examined the influence of HRGP on the focal adhesions by examining paxillin, a substrate for focal adhesion kinase (for a review, see Ref. 35). HRGP treatment led to a transient increased solubility and increased tyrosine phosphorylation of paxillin in resting BCE cells (Fig. 6A). At 90 min of incubation, the amount of solubilized paxillin was reduced to below that in untreated cells. Furthermore, immunofluorescent staining of endothelial cells with anti-paxillin antibodies showed a rearrangement of focal adhesions in BCE cells treated with HRGP for 10 min in the absence or presence of growth factors (Fig. 6B). The focal adhesions were visualized as spots, which increased in number and intensity in the cells treated for a short time period with HRGP. This effect was transient, and after 90 min, the paxillin-staining was similar between HRGP-treated and untreated BCE cells (data not shown).

To identify consequences of the rearranged focal adhesions, the effect of HRGP on BCE cell adhesion to different purified matrices was tested. As seen in Fig. 7, inclusion of HRGP significantly de-
creased attachment of the cells to vitronectin. There was also reduced adhesion to collagen I and IV, but no effect on adhesion to laminin. These data indicate that in the presence of HRGP, endothelial cells fail to migrate due to changes in focal adhesions and decreased cell attachment.

His/Pro-Rich Domain of HRGP Inhibits Chemotaxis. To determine which part of HRGP that was responsible for the antiangiogenic effect, we produced recombinant truncated forms of the protein (Fig. 8A). Truncated versions containing the COOH-terminal part of HRGP, but lacking an intact NH2 terminus, were not possible to produce, probably because of instability. Of the four truncated proteins tested (His 2–5), only the His/Pro-rich domain (His 5) inhibited chemotaxis of PAE/FGFR-1 endothelial cells toward FGF-2 (see indications in Fig. 8, A and B).

His/Pro-Rich Domain Is a Degradation Product of HRGP. Domain-specific antibodies against HRGP were raised by immunizing rabbits with three different peptides; 0115, 0116, and 0119 (Fig. 8A). Western blotting of full-length HRGP with the three peptide-antibodies revealed, apart from the full-length protein, a specific pattern of reactivity with smaller HRGP-derived fragments (Fig. 8C). Interestingly, the apparent molecular weight of 30,000 of the His/Pro-rich fragment (His 5) equals that of a naturally occurring HRGP fragment recognized by the antibody 0119, directed against the His/Pro-rich domain (Fig. 8D). This indicates that the His/Pro-rich domain in human HRGP may be proteolytically released from the full-length protein in vivo. Moreover, immunoblotting of His 4 with the 0119 antibody showed that the Mr 30,000 His/Pro-rich fragment was not released from this truncated HRGP variant (Fig. 8E). This provides an explanation for why His 4 failed to inhibit chemotaxis and indicates that release of the His/Pro domain is critical for its antiangiogenic effect.

DISCUSSION

In this study, we show both in vitro and in vivo that human HRGP, purified from human plasma or recombinantly produced, is a potent inhibitor of angiogenesis. HRGP treatment of fibrosarcoma-bearing mice resulted in decreased tumor angiogenesis and a 60–70% reduction in tumor volume compared with control-treated mice. The smaller tumor volume in HRGP-treated animals was due to increased apopto-
sis of the tumor cells, in accordance to what has been reported for other endogenous angiogenesis inhibitors such as thrombospondin-1 (36) and angiostatin (37, 38). In addition, the proportion of proliferating tumor cells was significantly decreased in HRGP-treated animals. Both the increased apoptosis and the decreased proliferation probably reflect a limited supply of oxygen and nutrients to the tumor cells by the less extensive vasculature. In vitro, where oxygen and nutrient supply is unlimited, HRGP treatment failed to affect apoptosis as well as DNA synthesis in tumor and endothelial cells. The mechanism of action of HRGP involves inhibition of endothelial cell chemotaxis because of decreased adhesion of cells and rearrangement of focal adhesions. It is possible that HRGP or a fragment thereof binds directly to integrins, thereby modulating focal adhesions and attachment. Other angiogenesis inhibitors such as endostatin, a fragment of the α1 chain of collagen XVIII, and tumstatin, a fragment of α3 chain of type IV collagen, have been shown to bind to integrins α5β1 and α6β1, respectively (39, 40).

Loss of cell anchorage is known to lead to substrate-dependent apoptosis, also denoted anoikis (for a review, see Ref. 41). Although we cannot rule out that HRGP-induced endothelial cell anoikis contributed to the reduced vascularization in the tumors, it appears unlikely because we failed to record HRGP-induced apoptosis of endothelial cells in vitro. Thus, HRGP does not appear to induce vessel regression. Instead, the reduced migration in the presence of HRGP may act to disturb vessel formation. This is in agreement with the concept that angiogenesis requires migration of endothelial cells.

The physiological plasma concentration of HRGP is relatively high, 100 μg/ml. The dose used in the tumor studies represents a 100% increase in the amount of HRGP in the mice every day, which could accumulate and reach substantially higher concentrations than normal. This is supported by the fact that the turnover of HRGP is slow, with a plasma half-life of 3 days (42). HRGP could also acquire different properties during certain physiological circumstances. For instance, the heparin-binding affinity of HRGP can be modulated and is increased in the presence of Zn2+ and at low pH, a common environment in hypoxic tumors. However, we favor the notion that a fragment of HRGP is responsible for the angiogenic effect. This fragment could be proteolytically cleaved out from the full-length protein under conditions of increased angiogenesis, e.g., by proteolytic activity secreted from a tumor and/or be enriched for during purification of the protein. Such a fragment is likely to be derived from the His/Pro-rich domain, which inhibited chemotaxis as potently as the full-length protein. The corresponding fragment in rabbit HRGP can be produced by proteolytic processing of full-length HRGP by plasminogen. This might also apply to human HRGP because immunoblotting of a purified preparation of full-length HRGP, with an antibody directed against the His/Pro-rich domain, recognizes a fragment corresponding in size to the recombinantly produced His/Pro-rich domain. Another truncated fragment denoted His 4 did not inhibit endothelial cell chemotaxis, although it contains the His/Pro-rich domain. However, the His/Pro-rich domain was not released from His 4, possibly because of an improper folding of the protein that prevents the His/Pro-rich domain from being exposed/cleaved out. These results strengthen the concept that the angiogenic effect is mediated by the isolated His/Pro-rich domain.

High molecular weight kininogen is structurally related to HRGP; their genes, which may have evolved through a gene duplication event, are located in close proximity on chromosome 3q. High molecular weight kininogen has been shown to interfere with endothelial cell function via a part of the protein with sequence similarities to the His/Pro-rich domain of HRGP (43). Recently, Guo et al. (44) showed that a proteolytically processed form of high molecular weight kininogen, denoted as HKα, close to completely inhibited adhesion of human vein endothelial cells to vitronectin, leading to apoptosis of 75–80% of the culture. As shown in the present study, the effect of HRGP on BCE cell adhesion to vitronectin was only partial (Fig. 7); moreover, we have not observed increased apoptosis of cells exposed to HRGP. During preparation of this article, Juarez et al. (45) reported that the
His/Pro-rich domain of rabbit HRGP purified from plasma inhibits endothelial cell proliferation and vasoconstriction of Matrigel plugs. In a previous article by Simantov et al. (46), HRGP was suggested to also under certain circumstances promote angiogenesis and, furthermore, to attenuate the antiangiogenic effect of thrombospondin-1 by complex-formation between the two proteins. Juarez et al. (45) suggest that this reported effect is dependent on contamination of the HRGP preparation by plasminogen. These reported results, which may appear contradictory at a first glance, demonstrate the need for well-defined protein preparations. To avoid problems with serum contaminants, which may have influenced previous studies on the effects of HRGP (45, 46), we have produced wild-type as well as different truncated variants of recombinant human HRGP under serum-free conditions. Using these reagents, we now extend the data reported by Juarez et al. (45) and provide evidence for in vivo effects on tumor vasculization. With these data, we provide proof of the concept that HRGP, through the released His/Pro-rich domain, is an angiogenesis inhibitor, with potential clinical relevance for the treatment of cancer.

ACKNOWLEDGMENTS

We thank Dr. Johan Stenflo (Lund University) for kindly providing purified protein C inhibitor and Entremed Corp. for providing purified human endostatin. We also thank Dr. Lars Uppsalma (Upplands University) for the pCEP-Pu2 expression vector and valuable help regarding recombinant protein production. We thank Drs. Ulf Hellman and Åke Engström (Uppsala University), who provided valuable help with mass spectrometry and two-dimensional gel analysis and expertise in protein biochemistry.

REFERENCES

Corrections

In the article by A-K. Olsson et al., titled “A Fragment of Histidine-Rich Glycoprotein Is a Potent Inhibitor of Tumor Vascularization,” which appeared in the January 15, 2004 issue of Cancer Research (pp. 599–605), the color contrast in Figure 6B was insufficient to illustrate the authors’ findings. The correct figure appears below.

Fig. 6. Paxillin in focal adhesions is affected by histidine-rich glycoprotein (HRGP) treatment. A, cells were treated with HRGP (100 ng/ml), as indicated, and paxillin was immunoprecipitated from the cell lysate. Immunoblotting was performed for paxillin (top panel) and phospho-tyrosine (middle panel). To verify equal loading, the cell lysate was blotted for β-actin (bottom panel). IP, immunoprecipitation. B, bovine adrenal cortex capillary endothelial cells treated with vascular endothelial growth factor (VEGF; 10 ng/ml), fibroblast growth factor (FGF)-2 (10 ng/ml), and HRGP (100 ng/ml) as indicated were fixed after 10 min and stained with anti-paxillin antibody (green). Nuclei (blue) were stained by Hoechst 33342. Bar indicates 20 μm.

In the article by D. Wiener et al., titled “Correlation between UDP-Glucuronosyltransferase Genotypes and 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanone Glucuronidation Phenotype in Human Liver Microsomes,” which appeared in the February 1, 2004 issue of Cancer Research (pp. 1190–1196), the titular phenotype should have been identified as butanol. The correct title is “Correlation between UDP-Glucuronosyltransferase Genotypes and 4-(Methylnitrosamino)-1-(3-Pyridyl)-1-Butanol Glucuronidation Phenotype in Human Liver Microsomes.”
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*Cancer Res* 2004;64:599-605.