[CANCER RESEARCH 62, 5344–5350, September 15, 2002]

Histidine-Proline-rich Glycoprotein Has Potent Antiangiogenic Activity Mediated through the Histidine-Proline-rich Domain[1]

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ABSTRACT

Histidine-proline-rich glycoprotein (HPRG) is an abundant multidomain plasma protein evolutionarily related to high-molecular-weight kininogen. The cleaved form of high-molecular-weight kininogen has recently been demonstrated to exhibit antiangiogenic activities *in vitro* (J. C. Zhang *et al.*, FASEB J., *14:* 2589–2600, 2000), mediated primarily through domain 5. HPRG contains a histidine-proline-rich (H/P) domain with sequence and functional similarities to HKa-D5. We hypothesized that HPRG may also have antiangiogenic properties, localized within its H/P domain. The H/P domain is highly conserved among species, and because rabbit H/P domain is more resistant to internal proteolytic cleavage than the human domain, the rabbit HPRG (rbHPRG) was primarily used to assess the antiangiogenic activity of HPRG. Rabbit HPRG inhibited human umbilical vein endothelial cell (HUVEC) tube formation stimulated by fibroblast growth factor-2 (FGF-2) or vascular endothelial growth factor on a Matrigel surface as well as cell proliferation of FGF-2 stimulated HUVECs. The antiangiogenic activity of rbHPRG was localized to the H/P domain by use of proteolytic fragments of rbHPRG and was further confirmed and characterized in two *in vivo* models of angiogenesis: the chorioallantoic membrane of the chick assay and the mouse Matrigel plug assay. Caspase-3 activation was observed in HUVECs stimulated with FGF-2 in the presence of rbHPRG, suggesting that apoptosis of activated endothelial cells may be one of the mechanisms underlying its antiangiogenic activity. Finally, the H/P domain of rbHPRG reduced tumor cell number when tumor cells were co-inoculated in the Matrigel plug assay. In conclusion, the H/P domain within HPRG induces the apoptosis of activated endothelial cells leading to potent antiangiogenic effects.

INTRODUCTION

HPRG[3] (also known as HRG or HRGP) is an abundant (125 μg/ml, or 1.5 μM) multidomain plasma glycoprotein with an unusually high percentage of Pro and His residues (1). HPRG contains two cystatin-like domains at the NH_2 terminus and a H/P domain between two Pro-rich domains at the COOH terminus. The COOH-terminal domain is tethered to the NH_2 terminus by a disulfide bond as in HK (Fig. 1*A*). HPRG binds an array of ligands: (*a*) those belonging to the coagulation/fibrinolysis cascades and extracellular matrix, such as heparin, plasminogen, fibrinogen, and thrombospondin (1–5); (*b*) small ligands, such as heme and transition metal ions (zinc, copper and nickel; Refs. 6, 7); and (*c*) components of the immune system (8–10). The functions of HPRG may include modulating coagulation and fibrinolysis and regulation of the immune system. However, no hypothesis has been able to explain and integrate the apparent "promiscuity" of binding of this multidomain protein (11). The modular structure of HPRG suggests that each domain may bind different ligands independently of the other modules. Indeed, the H/P domain has been reported to interact with heme, metals, and heparan sulfate, whereas the NH_2-terminal domain appears to interact with immune system components and the COOH-terminal domain with plasminogen and plasma proteins involved in clotting (1).

It is estimated that 50% of plasminogen in plasma circulates associated to HPRG (12). In this context, HPRG plays a profibrinolytic role. Plasminogen binds to a lysine (K518) at the COOH terminus of HPRG ($K_d \sim 1$ μM) and is more rapidly converted to plasmin by tPA when HPRG is immobilized on the cell surface (3). Plasmin plays a prominent role in angiogenesis (13, 14) and is considered essential for the growth and metastasis of tumors. HPRG also binds to TSP-1 (5), and it has recently been suggested that binding through regions in the cystatin 1 and COOH-terminal domains inhibits the potent antiangiogenic activity of TSP-1 (15).

HPRG is evolutionarily related to HK, which is a multidomain protein (Fig. 1*B*) that is activated and converted into the antiangiogenic molecule HKa by kallikrein proteolysis and subsequent release of bradykinin from domain 4 (16, 17). The antiangiogenic activity of HKa has been localized to domain 5 (HKa-D5; Ref. 17). HKa-D5 and the H/P domain of HPRG appear to have similarities in function, such as binding to heparin and metals, and in sequence, such as an abundance of positive charges and lack of disulfide bonds. We hypothesized that because of these similarities, HPRG could also have antiangiogenic activity. In this report, we identify HPRG as a novel antiangiogenic protein and localize this activity to within the H/P domain. We also show that the H/P domain reduces the number of tumor cells when they are grown in a Matrigel Plug *in vivo*. Finally, we provide evidence suggesting that the recently reported proangiogenic activity of human HPRG (15) is at least partially attributable to plasminogen contamination and that appropriately treated human HPRG inhibits angiogenesis in a manner similar to that of rabbit HPRG.

MATERIALS AND METHODS

Proteins and Materials. FGF-2 and VEGF were purchased from Research Diagnostics Inc. (Flanders, NJ). HKa was obtained from Enzyme Research Laboratories (South Bend, IN). Heparin was acquired from Sigma (St. Louis, MO). Mouse endostatin was purchased from Calbiochem (San Diego, CA). Domains and fragments of rabbit HPRG were obtained as follows (18): rabbit HPRG was partially digested by stirring with plasmin-Sepharose, and the digestion was followed by SDS-PAGE (see Fig. 1 for a schematic of HPRG domains). Partial reduction and carboxymethylation of plasmin-cleaved HPRG was carried out by incubating the protein with 25 mM DTT in 0.1 M phosphate (pH 7.3)-5 mM EDTA for 60 min at 4°C in the dark, followed by alkylation

Received 4/17/02; accepted 7/10/02.

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[3] The abbreviations used are: HPRG, histidine-proline-rich protein; H/P, histidine-proline-rich; HK, high-molecular-weight kininogen; tPA, tissue plasminogen activator; TSP-1, thrombospondin-1, HKa, activated HK; FGF-2, fibroblast growth factor-2; VEGF, vascular endothelial growth factor; HUVEC, human umbilical vein endothelial cell; FBS, fetal bovine serum; CAM, chorioallantoic membrane; 3LL, Lewis lung carcinoma; MLL, MatLyLu; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; GFP, green fluorescent protein.

with 55 mM iodoacetamide for 30 min. The reaction mixture was dialyzed extensively against 20 mM sodium phosphate (pH 7.3) and applied to a DEAE-cellulose column equilibrated with the same buffer. The H/P domain (see Fig. 1) passed through the column unretarded. The N/C fragment, which is composed of the disulfide-linked NH_2- and COOH-terminal domains, was eluted with a sodium chloride gradient.

Tube Formation Assay. The wells of a 96-well microtiter plate were coated with 60 μl of ice-cold Matrigel and incubated overnight at 37°C. The next day, 12,000 single-donor human HUVECs (Clonetics, San Diego, CA) were layered on top of the gel in EGM-2 (Clonetics), 5% serum, 10 μM $ZnCl_2$ supplemented with either 10 ng/ml FGF-2 or 20 ng/ml FGF-2 and VEGF plus 40 ng/ml phorbol 12-myristate 13-acetate. Control medium or the compound to be tested was added to individual wells. Each treatment was carried out in triplicate. Pictures were taken 48 h later.

Endothelial Cell Proliferation Assay. HUVECs were serum-starved overnight (2% FBS). The next day, 3000 cells were plated per well in gelatin-coated 96-well plates. The cells were allowed to adhere and spread for 4–6 h, the medium was removed, and fresh medium containing 2% serum, 1 ng/ml FGF-2 as a proangiogenic stimulator, 10 μM $ZnCl_2$, and various concentrations of the compounds to be tested was added. Cells were then cultured for an additional 48 h, and relative cell numbers in each well were determined by the Cell Titer Aqueous Cell Proliferation Assay (Promega, Madison, WI) by measuring the $A_{490\ nm}$. As a positive control, the antiangiogenic molecule activated (two-chain) HKa was also included (17). Cell proliferation was also investigated in selected experiments by measuring the fluorescence of control and treated HUVECs that had been previously loaded (5-min incubation) with the fluorescent dye Calcein-a.m. (2.5 μM; Molecular Probes, Eugene, OR). The fluorescent dye becomes activated once inside the cell.

CAM Assay. Fertilized chicken (White Leghorn) embryos were cracked on day 3 into sterile containers and subsequently incubated (37°C in 3.5% CO_2) for 4 days. On day 7, Whatman filter discs impregnated with either 30 ng of FGF-2 or 30 ng of FGF-2 and the indicated amount of agent to be tested were placed on an avascular area of the chorioallantoic membrane. On day 10, neovascularization around the disk was quantitated by determination of the number of angiogenic vessels within the CAMs that touched the disk by use of image analysis software (MetaVue, version 4.5).

***In Vivo* Assessment of Angiogenesis Using the Matrigel Plug Assay.** This assay was performed essentially as described (19). Matrigel (500 μl; Collaborative Biomedical Products, Inc., Bedford, MA) on ice was mixed with heparin (50 μg/ml), FGF-2 (400 ng/ml), and the proteins to be tested. The Matrigel mixture was injected s.c. into 4–8-week-old female BALB/c nude mice at sites near the abdominal midline, three injections per mouse. Injection sites were chosen such that each animal received a positive control plug (FGF-2 and heparin), a negative control plug (heparin plus buffer), and a plug containing the treatment to be tested (FGF-2, heparin, and protein to be tested). All treatments were tested in triplicate. Animals were sacrificed 5 days after injection. The mouse skin was detached along the abdominal midline, and the Matrigel plugs were recovered and scanned immediately at high resolution. Plugs were then dispersed in water and incubated at 37°C overnight. Hemoglobin levels were determined using Drabkin's solution (Sigma) according to the manufacturer's instructions.

Determination of Plasmin and Plasminogen Activity. Plasmin activity of HPRG samples (1 μM) was measured spectrophotometrically using the plasmin-specific peptidyl substrate d-Val-Leu-Lys-p-nitroanilicle (S-2251; Sigma) at 0.3 mM in PBS-1.5 mg/ml BSA (pH 7.4) in a 96-well plate in a final volume of 100 μl (3). The kinetics of peptide hydrolysis were followed at 405 nm for 5 min, and initial velocities (V_i) were determined. A plasmin (Research Enzyme Laboratories) titration curve correlating concentration and V_i was obtained as described above and used to determine plasmin levels in HPRG samples. As a control, human HPRG (56 μM) was incubated with 4 mM Pefabloc SC (AEBSF; Roche Molecular Biochemicals, Indianapolis, IN) for 30 min at room temperature, followed by extensive dialysis against PBS for 24 h. The levels of plasminogen in the HPRG samples were measured by following the hydrolysis of S-2251 by plasmin generated by activation with tPA (2 nM) for 2 h (American Diagnostica, Greenwich, CT; Ref. 3). The $A_{405\ nm}$ was also measured at the end of the 2-h incubation.

Caspase-3 Activity. HUVECs (Clonetics) were serum starved overnight (2% FBS). The next day, 500,000 cells were plated in a gelatin-coated 10-cm dish in medium containing 2% serum, 10 ng/ml FGF-2, 10 μM $ZnCl_2$ (FGF-2 treated sample), and various concentrations of rbHPRG (10 or 100 nM) or HKa (100 nM). As a positive control, cells were also treated with 10 μM camptothecin (Calbiochem, San Diego, CA). After 6 h, adherent and nonadherent cells were harvested, pelleted down, and resuspended in 100 μl of lysis buffer. Caspase-3 activity was measured by the EnzCheck Caspase-3 assay kit 2 from Molecular Probes according to the manufacturer's instructions. As a negative control, 1 μl of the 1 mM caspase-3 inhibitor Ac-DEVD-CHO stock solution was added to an aliquot of the camptothecin-treated cells to show that the signal was specific to caspase-3 activity. The level of apoptosis was calculated by considering the levels of caspase-3 activity in the HKa-treated sample minus the FGF-2-treated sample to be 100%.

Modified Matrigel Plug Assay. In this assay, 2×10^6 cells of the mouse lung carcinoma cell line 3LL or the rat prostate tumor cell line MLL were mixed with Matrigel and the protein to be tested and then injected into the flank of a mouse according to the protocol described for the Matrigel plug assay. After 7 days, the animals were euthanized, and the plugs were removed, weighed, and scanned at high resolution. The plugs were then minced and homogenized with a tissue homogenizer, and total GFP fluorescence was measured in a fluorescent plate reader. Hemoglobin levels were determined after 18 h of incubation at room temperature using Drabkin's solution according to the manufacturer's instructions.

RESULTS

Homology of Rabbit and Human HPRG. The domain structure of HPRG is shown in Fig. 1. To investigate the role of HPRG in angiogenesis, we tested both human and rabbit HPRG. Optimal alignment of the two proteins gives 63.5% sequence identity and 68.6% homology (18). The highest homology is at the NH_2 and COOH termini. The apparent lower homology in the H/P domain is attributable to substitutions of Pro for His in the rabbit molecule. The human protein contains 15 repeats of the sequence His-His-Pro-His-Gly, whereas the rabbit protein has 2 repeats of His-His-Pro-His-Gly, 6 repeats of His-Pro-Pro-His-Gly, and 7 repeats of Pro-Pro-Pro-His-Gly. Rabbit HPRG is easily purified from rabbit plasma, and the different domains can be isolated by controlled plasmin digestion (Fig. 1*A*) and have been characterized previously (18). The isolation of the different domains of human HPRG is significantly more problematic because of an internal plasmin cleavage site within the human H/P domain (18). In addition, as demonstrated in Fig. 6, human HPRG binds a significant amount of plasminogen during purification, which can actually produce an apparent stimulation of angiogenesis. For

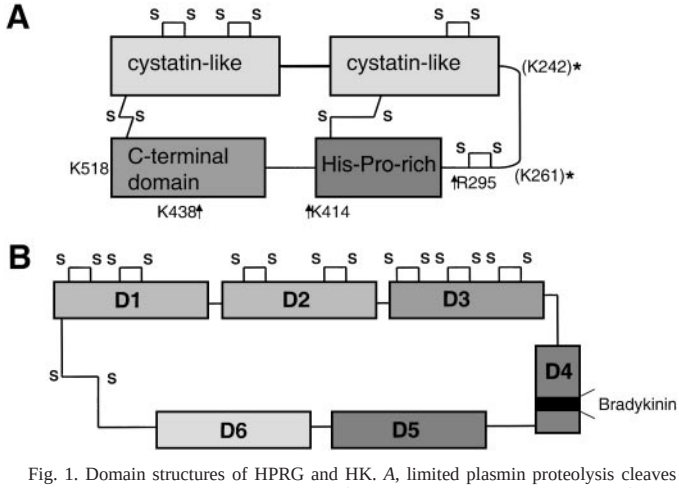

Fig. 1. Domain structures of HPRG and HK. *A*, limited plasmin proteolysis cleaves HPRG at the sites indicated by *up arrows*, followed by sites indicated by * on further incubation. Subsequent treatment with DTT after controlled digestion (↑) releases the H/P domain. The remaining molecule is the N/C fragment, which is held together by a buried disulfide bond between the NH_2- and COOH-terminal domains. *B*, HK is converted to HKa by kallikrein cleavage and release of bradykinin from domain 4. HK also contains two cystatin-like domains (*D1* and *D2*), and a COOH- to NH_2-terminal disulfide bond.

these reasons, studies evaluating the antiangiogenic effects of HPRG and its domains were carried out with HPRG purified from rabbit plasma.

HPRG Inhibits HUVEC Tube Formation on Matrigel. A Matrigel tube formation assay was used to investigate the possible antiangiogenic activity of rbHPRG. Matrigel is a mixture of basement membrane obtained from Engelbretch-Holm-Searm mouse sarcoma cells. The tube formation assay has been used extensively in the literature as a measure of the pro- or antiangiogenic properties of a molecule. HUVECs that are plated on Matrigel and stimulated with FGF-2 form tube-like structures (Fig. 2*A*). This process involves migration, invasion, and differentiation, but not proliferation (20). Nevertheless, in the presence of rabbit HPRG the formation of tubes is severely impaired (Fig. 2*B*). Similar inhibitory effects were observed for HPRG when the HUVECs were plated on Matrigel and stimulated with VEGF, FGF-2, and phorbol 12-myristate 13-acetate (data not shown).

HPRG Inhibits HUVEC Proliferation, and the Activity Is Localized to the H/P Domain. rbHPRG was tested for its ability to inhibit HUVEC proliferation *in vitro*. rbHPRG reproducibly inhibited FGF-2 stimulated proliferation in a dose-dependent manner (Fig. 3*A*). IC_{50} values for this inhibition were typically in the range of 50 nM. rbHPRG was not directly cytotoxic on 3LL, MLL, or HeLa tumor cells in culture at concentrations up to 2 μM (data not shown). The inconsistency of the data obtained for hHPRG (data not shown) precluded an assessment of its activity in this assay. This apparent inconsistency between rabbit and human HPRG is addressed below.

The H/P domain can be excised from rbHPRG by partial plasmin digestion, reduction, and alkylation of the disulfide bond linking it to the second cystatin domain, followed by chromatography on DEAE-cellulose (18) as described in "Materials and Methods." This procedure yields a completely intact (data not shown) rabbit H/P domain. The residual fragment, designated the N/C fragment, is also isolated during this procedure. Both the H/P domain and the N/C fragment were tested in the endothelial cell proliferation assay. The H/P domain inhibited HUVEC proliferation with a potency comparable to intact rbHPRG and HKa (Ref. 17; Fig. 3*B*). In contrast, the N/C fragment had no significant activity in this assay. Consistent with the results obtained with HKa and HKa-D5 (17), the antiproliferative activity of the H/P domain was dependent on the presence of Zn^{2+} (data not shown). Taken together, these results indicate that HPRG is antiangiogenic for HUVECs stimulated with proangiogenic factors.

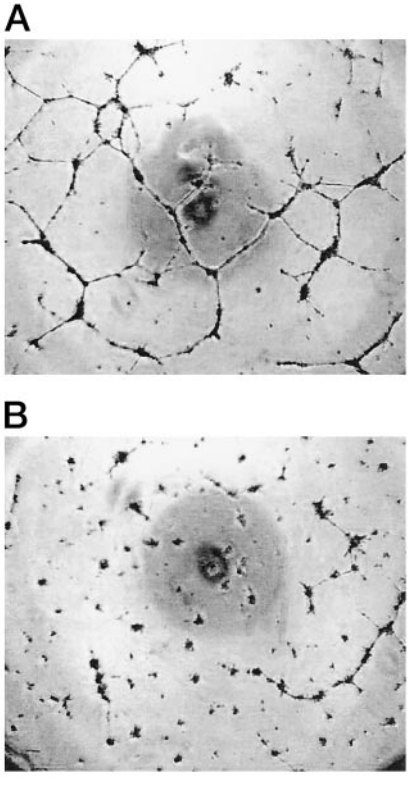

Fig. 2. Inhibition of endothelial tube formation assay by rbHPRG. A total of 12,000 HUVECs were plated on Matrigel-coated wells in EGM-2, 5% serum, 10 μM $ZnCl_2$ supplemented with 10 ng/ml FGF-2. Control medium or 500 nM rbHPRG was added to individual wells. All treatments were assayed in triplicate.

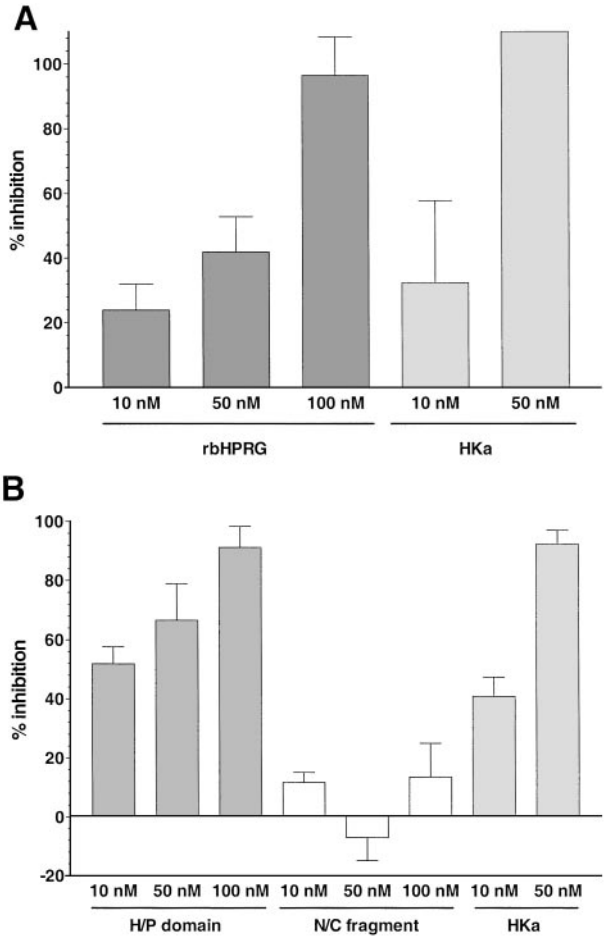

Fig. 3. Inhibition of HUVEC proliferation by rbHPRG and domains. HUVECs were starved in 2% FBS for 24 h and plated on gelatin-coated 96-well plates in EGM-2 medium containing 2% FBS, 1 ng/ml FGF-2, 10 μM $ZnCl_2$, and the indicated concentrations of rbHPRG or HKa (*A*). Proliferation was measured 48 h later as indicated in "Materials and Methods." *B*, the H/P domain and N/C fragment were isolated from rbHPRG by limited plasmin digestion, and their effects on FGF-2 HUVEC proliferation were determined as in *A*. *Bars*, SD.

HPRG Inhibits Angiogenesis in the CAM Assay. Filter disks containing FGF-2 were placed on an avascular area of the chorioallantoic membrane of a fertilized chicken embryo (21). Inhibition of angiogenesis (70%) was observed in the presence of the H/P domain (20 μg; Fig. 4, *A* and *B*). These preliminary results were extended to a larger study ($n = 12$/group; Fig. 4*C*). In this study, the H/P domain (10 μg) inhibited FGF-2-induced neovascularization (50%) at levels similar to those of rbHPRG (55%). Consistent with previous reports showing only moderate proangiogenic activity (15), human HPRG did not significantly inhibit angiogenesis in this assay (data not shown). As with the HUVEC proliferation experiments, this apparent inconsistency between rabbit and human HPRG is addressed below.

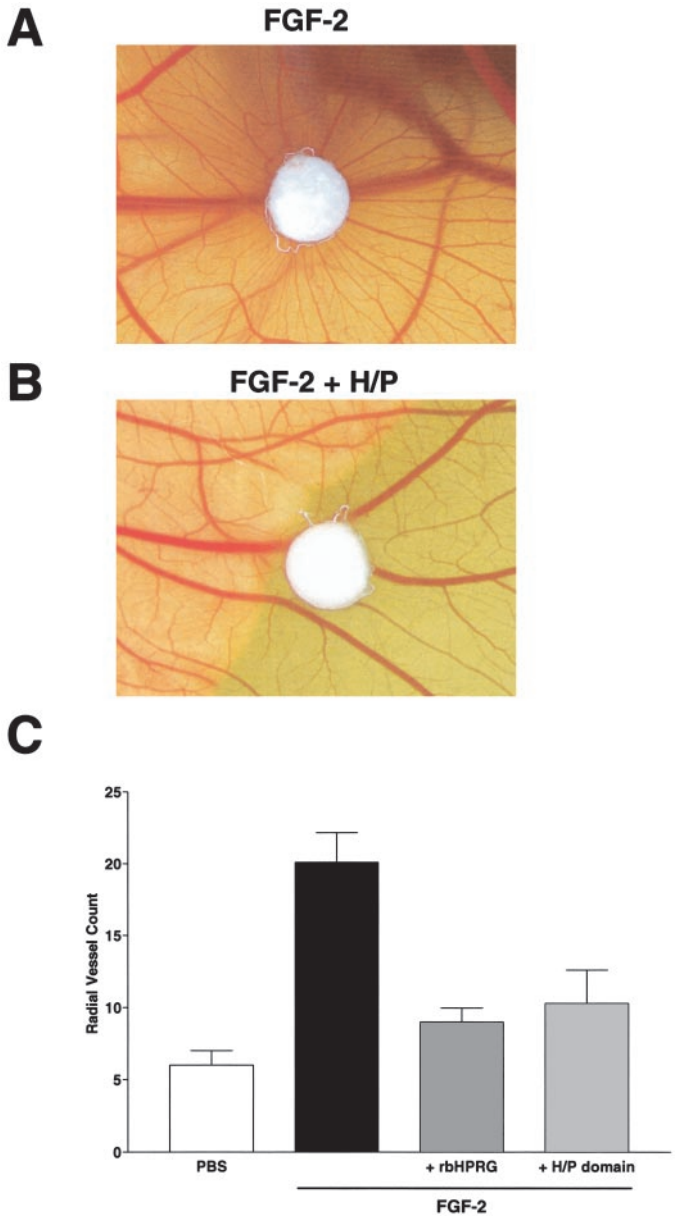

Fig. 4. HPRG and the H/P domain inhibit angiogenesis in the CAM assay. Whatman filter discs impregnated with FGF-2 with and without treatment were placed on an avascular area of the chorioallantoic membrane of fertilized eggs. A strong angiogenic response is characterized by the radial vessels contacting the disk. *Panel A* shows a picture of a representative egg containing a disk with 30 ng of FGF-2, whereas in *panel B* the disk contains 30 ng of FGF-2 plus 20 μg of the H/P domain. *Panel C* shows the resulting number of radial vessels ($\pm$SE; *bars*) for eggs with discs containing control ($n = 3$), FGF-2 ($n = 7$), FGF-2 + 10 μg of rbHPRG ($n = 11$), and FGF-2 + 10 μg of H/P domain ($n = 12$).

HPRG Inhibits Angiogenesis in the Matrigel Plug Model. The antiangiogenic activity of HPRG was also assessed in a second *in vivo* study, the Matrigel plug model (19). rbHPRG, the H/P domain, or the N/C fragment was added to Matrigel together with FGF-2 and heparin. HPRG and the H/P domain, but not the N/C fragment, inhibited the angiogenic response stimulated by FGF-2 (Fig. 5*A*). The inhibition of angiogenesis by rbHPRG (250 nM) or H/P (600 nM) was essentially 100%. The potency of the H/P domain in the Matrigel plug assay was evaluated by carrying out a dose titration (Fig. 5*B*). The H/P domain inhibited angiogenesis in this model with an IC_{50} of ~100 nM.

Plasminogen Contamination of Human HPRG Mediates Its Previously Observed Proangiogenic Activity. We have consistently observed antiangiogenic activity for rbHPRG and the rabbit H/P domain. However, human HPRG does not show similar levels of inhibition and has previously been reported to have moderate proangiogenic activity (15). We suspected that traces of plasminogen/plasmin impurities bound to hHPRG, but not rbHPRG, could explain the observed differences. Plasmin amidolytic activity in human HPRG was measured using the chromogenic substrate S-2251. Human HPRG contained an average of 1.8 ng plasmin/μg of HPRG, whereas plasmin was undetectable in our preparations of rbHPRG. A sample of hHPRG was then treated with the low-molecular-weight serine protease inhibitor AEBSF and extensively dialyzed over a 24-h period. Free AEBSF hydrolyzes quickly in aqueous solutions, which would destroy any residual AEBSF not removed by dialysis. Plasmin amidolytic activity was undetectable in human HPRG after treatment with AEBSF.

To determine the levels of plasminogen, AEBSF-treated human HPRG was incubated with tPA for 2 h, which would convert the plasminogen into the enzymatically active plasmin. Human HPRG, but not rbHPRG, contained significant levels of plasminogen as measured by an increase in amidolytic activity after tPA activation (data not shown). The presence of plasmin and plasminogen can induce angiogenesis directly (14). Thus, we hypothesized that the

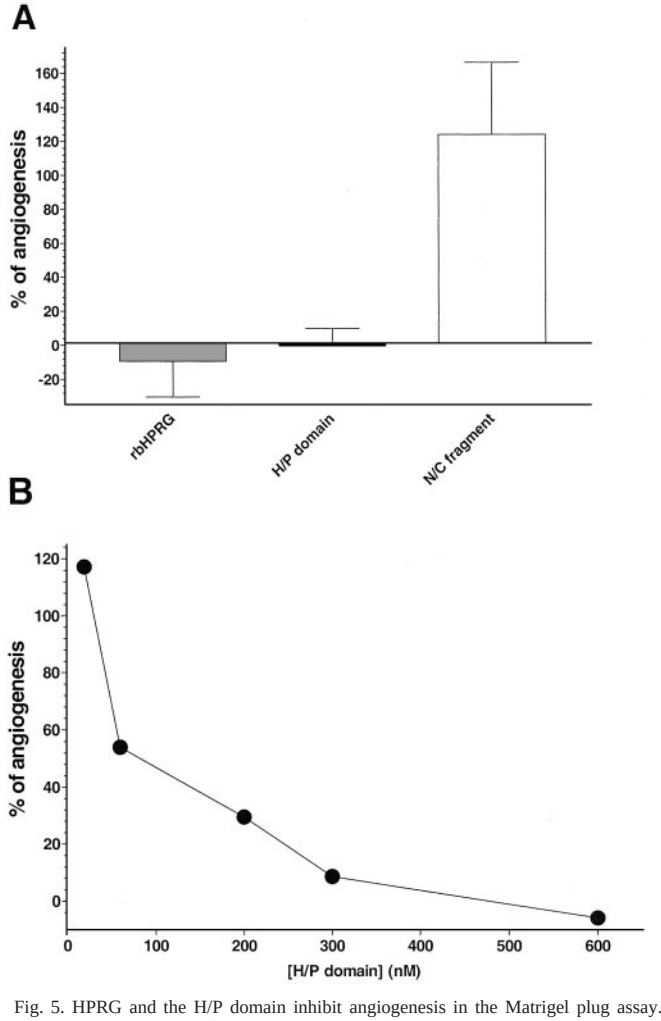

Fig. 5. HPRG and the H/P domain inhibit angiogenesis in the Matrigel plug assay. *Panel A* shows a summary of Matrigel plug hemoglobin determinations as indication of the level of angiogenesis (100% angiogenesis is defined as the hemoglobin level determined by the Drabkin's method in the FGF-2-containing plug minus the plug lacking FGF-2). For rbHPRG, $n = 6$ (250 nM); for the H/P domain, $n = 12$ (600 nM); and for the N/C fragment, $n = 3$ (500 nM). *Bars*, SD. *Panel B* shows a titration in the Matrigel plug of the H/P domain *versus* the percentage of angiogenesis.

plasminogen/plasmin bound to hHPRG may interfere with the antiangiogenic activity of the H/P domain.

To address this issue, we compared the activity of hHPRG with and without treatment with AEBSF in the Matrigel plug assay. hHPRG exhibited a modest stimulatory effect on angiogenesis consistent with previous results (15), whereas the AEBSF-treated hHPRG significantly inhibited angiogenesis (Fig. 6). The magnitude of inhibition of angiogenesis by AEBSF-treated hHPRG (~45%) was less than that of rbHPRG at the same concentration (100%; see Fig. 5*A*), possibly because residual plasminogen bound to rbHPRG is not affected by AEBSF treatment. It would be extremely difficult, if not impossible, to remove plasminogen completely. These results suggest that inhibition of hHPRG-bound plasmin restores, at least partially, antiangiogenic activity to hHPRG.

rbHPRG Induces Apoptosis in HUVECs. HKa has been demonstrated to induce apoptosis in activated endothelial cells (17). To assess the proapoptotic activity of rbHPRG, caspase-3 activity was measured in HUVECs treated with FGF-2 together with rbHPRG or HKa (used as a positive control). Rabbit HPRG induced caspase-3 activity at levels similar to HKa, a representative experiment is shown in Fig. 7. Thus, induction of endothelial cell apoptosis may be one of the mechanisms underlying the antiangiogenic activity of HPRG.

HPRG Treatment Leads to Decreased Tumor Cell Number in the Modified Matrigel Plug Assay. Many antiangiogenic compounds also inhibit tumor growth (22–25). We tested the H/P domain for its ability to reduce tumor cell number in a modification of the Matrigel plug assay. Two million cells of the mouse lung carcinoma cell line 3LL or the rat prostate cancer line MLL transfected with GFP were mixed with Matrigel with or without the H/P domain and then injected into the flank of a mouse, according to the protocol described for the Matrigel plug assay. A mass of tumor cells (0.3–0.6 g) was observed in the control plugs after 7 days (Fig. 8). Although the H/P domain did not have any direct cytotoxic effect on 3LL, MLL, or HeLa cells in culture (data not shown), inclusion of the H/P domain in Matrigel plugs containing either 3LL or a GFP-transfected MLL tumor cell line significantly reduced the number of tumor cells (Fig. 8 and Table 1). The total number of tumor cells in the plug was estimated by plug weight (3LL) or by total GFP fluorescence and plug weight (GFP-MLL) with good agreement between the two values. The total hemoglobin content of the plugs was also decreased in the presence of the H/P domain (Table 1). The activity of an inhibitor of angiogenesis with known antitumor activity, endostatin, was also compared with the H/P domain in the Matrigel/GFP-MLL and Matrigel/3LL models (Table 1). The levels of reduction in tumor cell number and total hemoglobin content for the H/P domain were similar to those for endostatin at the same molar concentration.

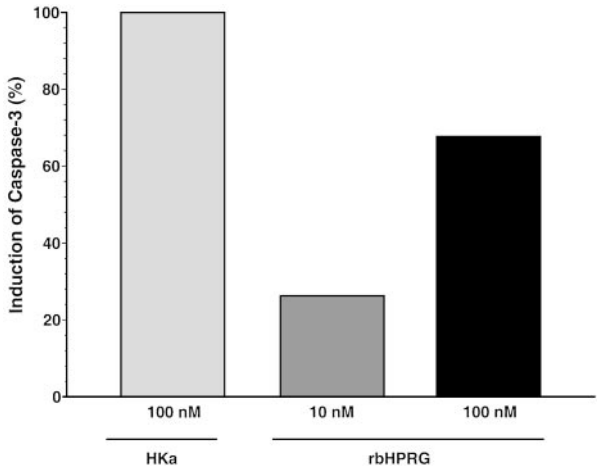

Fig. 7. rbHPRG activates caspase-3 in FGF-2-stimulated HUVECs. HUVECs, were serum starved overnight (2% FBS), and the next day, 500,000 cells were plated in medium containing 2% serum, 10 ng/ml FGF-2, 10 μM $ZnCl_2$, and rbHPRG (10 or 100 nM) or HKa (100 nM) was added. After 6 h, adherent and nonadherent cells were harvested, pelleted down, and resuspended in 100 μl of lysis buffer. Caspase-3 activity was measured using EnzCheck Caspase-3 assay kit 2 from Molecular Probes according to the manufacturer's instructions.

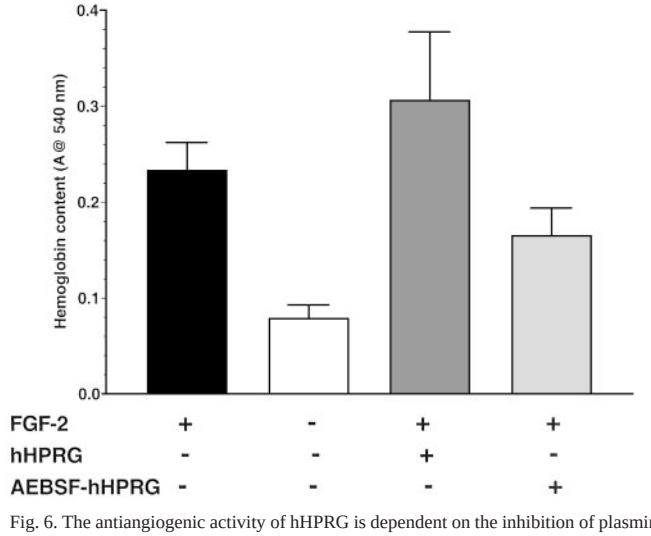

Fig. 6. The antiangiogenic activity of hHPRG is dependent on the inhibition of plasmin contamination. Matrigel plugs containing buffer alone (negative control; □), 400 ng/ml FGF-2 plus 50 μg/ml heparin (FGF-2 control; ■), 250 nM hHPRG (▩), or 250 nM AEBSF-treated (▤) hHPRG were implanted in mice. After 5 days, the plugs were removed, and hemoglobin levels were determined by the Drabkin method. *Bars*, SD.

DISCUSSION

There is increasing evidence that angiogenic pathways are regulated through the activity of endogenous plasma and extracellular matrix proteins (26) that are activated to inhibit angiogenesis by specific proteolytic cleavage. Several such proteins have been identified, including angiostatin (from plasminogen; Ref. 27), endostatin (from type XVIII collagen; Ref. 28), HKa (from HK; Ref. 17), tumstatin (from type IV collagen; Ref. 29), and antithrombin III (30). In this study, we have identified a novel antiangiogenic protein, HPRG, and have further localized its antiangiogenic activity to a single region, the H/P domain. HPRG stimulates endothelial cell apoptosis similarly to other antiangiogenic proteins (17, 31, 32) and is dependent on zinc for its activity, making it mechanistically similar to HKa and endostatin (17, 33). We have also demonstrated that the addition of the H/P domain to a Matrigel plug containing tumor cells implanted in mice leads to a reduction in the total hemoglobin content and in the number of tumor cells. It is very likely that the reduction in the number of tumor cells in the treated plugs is attributable to inhibition of angiogenesis by the H/P domain because (*a*) rbHPRG is not directly cytotoxic to tumor cells *in vitro*, and (*b*) strong evidence has been presented that rbHPRG has antiangiogenic effects directly on endothelial cells. However, other possible indirect effects of the H/P domain on tumor cells cannot be completely ruled out. Human HPRG has recently been shown to be mildly proangiogenic and also to abrogate the antiangiogenic activity of TSP-1 (15). We have demonstrated (Fig. 6) that bound plasmin(ogen) appears responsible for the proangiogenic activity of human HPRG, at least in the Matrigel plug model. Simantov *et al.* (15) also suggested that the region of HPRG that was implicated in binding to TSP-1 is different from the antiangiogenic H/P domain. Thus, it is possible that HPRG may contain both pro- and antiangiogenic activities that can be revealed independently of each other. On the other hand, the possible proangiogenic

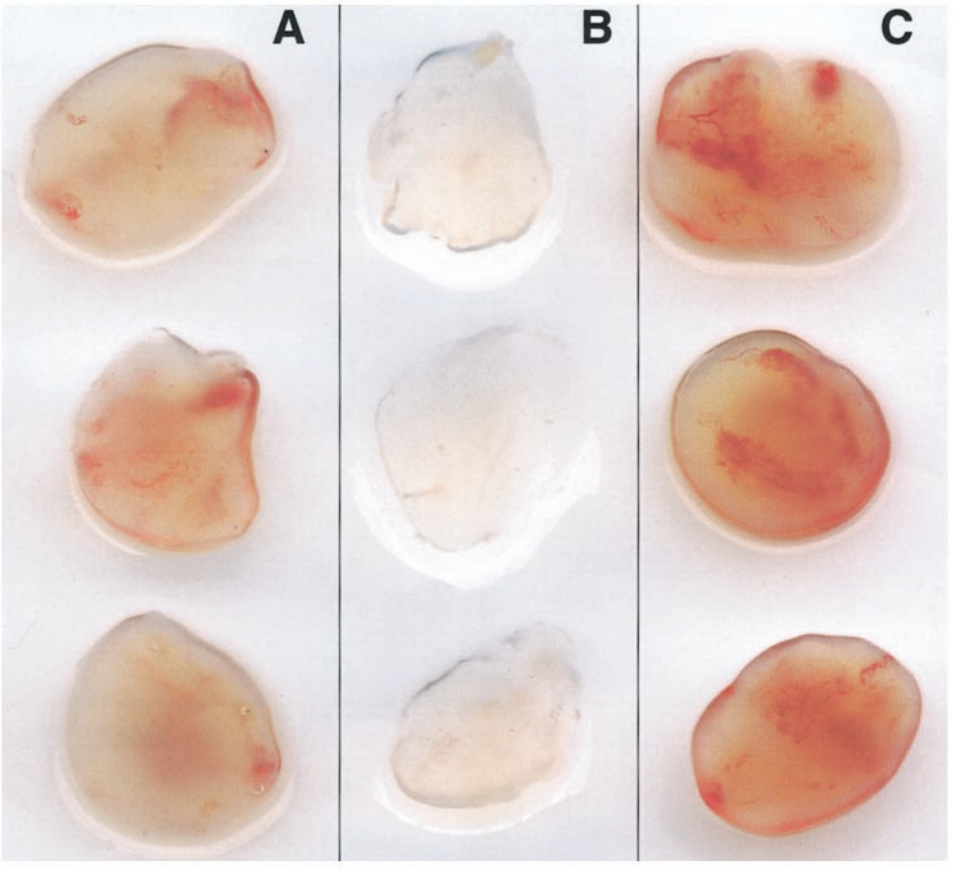

Fig. 8. The H/P domain inhibits hemoglobin content and tumor cell growth of 3LL cells. Two million cells of the mouse lung carcinoma cell line 3LL were mixed with Matrigel plus 1.8 μM H/P domain and then injected into the flank of a mouse according to the protocol described for the Matrigel plug assay. After 7 days, the animals were sacrificed, and the plugs were removed, weighed, and scanned at high resolution. *Panel A*, 1.8 μM H/P domain with 2×10^6 3LL cells; *Panel B*, saline buffer; *Panel C*, 2×10^6 3LL cells. A robust angiogenic response can be seen in the Matrigel plugs containing 3LL cells *versus* control.

contribution of plasmin(ogen) bound to hHPRG in the reported inhibition of TSP-1 antiangiogenic activity deserves further scrutiny.

Although our data demonstrate the potent inhibition of angiogenesis by the H/P domain, the contribution of the proangiogenic plasminogen bound to the COOH-terminal lysine of human HPRG raises issues about the physiological function of HPRG. Plasmin(ogen) contamination of human HPRG stimulated angiogenesis in the Matrigel plug assay, which is consistent with other reports describing the dependence of angiogenesis on plasmin catalytic activity (13, 14). However, plasmin(ogen) can also have antiangiogenic activity as the precursor for the antiangiogenic molecule angiostatin (34), which makes the assessment of the true contribution of plasmin to angiogenesis difficult. It is possible that release of plasminogen bound to HPRG may be required for the full antiangiogenic activity of HPRG to be revealed, and this could occur near the surface of a cell. For example, there are abundant binding sites for plasminogen on the surface of endothelial cells (35) that could compete with plasminogen bound to HPRG. Alternatively, as with many other antiangiogenic molecules (17, 26–30), a proteolytic event may be required to reveal the antiangiogenic potential of the H/P domain.

Several antiangiogenic proteins or fragments of proteins are presently in clinical trials. However, there are inherent limitations in using proteins or fragments of proteins as pharmaceuticals. Because of the typically poor pharmacological behavior of proteins, very large and repeated doses are generally needed to obtain a biological response, and the cost of large-scale production of these proteins may make commercialization cost-prohibitive. Thus, an alternative approach would be to develop protein fragments or peptides of the smallest size possible that retain the potency of the parent molecule. The H/P domains of both human and rabbit HPRG, as well as from other species (18, 36), are highly homologous and are arranged as repeats of five-amino acid consensus sequences. Studies are presently under way to identify the consensus sequence responsible for the antiangiogenic activity of the parent protein as well as the receptor for the H/P domain.

HPRG, by virtue of the high His content of its H/P domain, has been found to behave like a pH sensor (7). Binding of HPRG to matrix and endothelial cells occurs in conditions of low pH through interactions with glycosaminoglycans (7) because of the protonation of the abundant His residues of the H/P domain under those conditions. Thus, HPRG may be induced to bind to endothelial cells by a slight

Table 1 *Inhibition of tumor cell growth and hemoglobin levels by the H/P domain*

Matrigel plugs containing 2×10^6 tumor cells (3LL or GFP-MLL) and the H/P domain (1.8 μM) or endostatin (1.8 μM) were injected into the flank of a mouse according to the protocol described for the Matrigel plug assay. The tumor weight or GFP total fluorescence, and hemoglobin content (Drabkin's method) for treated and untreated tumor cell containing plugs are shown as the mean of the percentage of inhibition ±SD. *n* represents the number of plugs.

	Inhibition (%)							
	3LL				GFP-MLL			
Agent	Weight	*n*	Hemoglobin	*n*	Fluorescence	*n*	Hemoglobin	*n*
H/P domain	41.9 ± 7.4	9	53.8 ± 17.5	15	55.5 ± 11.1	3	50.7 ± 17.0	3
Endostatin	66.0 ± 12.8	3	56.4 ± 23.5	3	40.6 ± 25.3	3	24.1 ± 19.8	3

drop in the local pH (0.25–0.5) sufficient to protonate the His residues. Hypoxia, one the most powerful triggers of angiogenesis, can induce a decrease of up to one unit relative to the physiological pH of extracellular fluid or plasma (37), which may promote binding of HPRG to endothelial cells. Zinc has been shown to bind with high affinity to HPRG and to shift its pH dependence for binding to heparin toward physiological pH values (7). Likewise, we have also shown that the endothelial antiproliferative activity of HPRG under physiological pH is dependent on Zn^{2+}. Many tumors are prothrombotic, and in this setting, zinc concentrations can increase 30–60-fold after activated platelets release their zinc-rich α-granules (38). Therefore, the H/P domain of HPRG may selectively bind to endothelial cells under conditions of local acidosis, such as those of hypoxia, abundant zinc, or both. As such, the H/P domain may be a hypoxic vasculature targeting agent.

This apparent selectivity for a hypoxic environment may be relevant to therapy in light of recent findings by Yu *et al.* (39). They demonstrated that tumors in which the *p53* gene has been inactivated are more resistant to antiangiogenic therapy because they have a lower rate of hypoxia-induced apoptosis. In the above-mentioned study, the tumors were treated with a combination therapy of DC101, an antibody against VEGF receptor-2, and metronomic vinblastine chemotherapy. In the study by Yu *et al.* (39), p53-deficient tumor cells were able to eventually overcome the antiangiogenic effects of DC101 because they could survive under hypoxic conditions. However, if one could combine a conventional antiangiogenic agent with an antiangiogenic agent that functions in a hypoxic environment, the end result could be global inhibition of angiogenesis, leading to anoxic conditions and tumor cell death. The H/P domain by itself or conjugated to a cytotoxic agent may be an example of such an agent. We are presently testing the hypothesis that the H/P domain is capable of inhibiting angiogenesis in hypoxic tumors.

In summary, HPRG is a novel, potent antiangiogenic and antitumor molecule that induces apoptosis in activated endothelial cells, with the activity localized to its H/P domain. This domain, which is composed of several related pentapeptide repeats, may be amenable to development as a pharmaceutical agent. The H/P domain may inhibit angiogenesis under both normoxic and hypoxic conditions and could thus overcome some of the problems of induced resistance in tumors that adapt to survive under hypoxic conditions.

REFERENCES

1. Morgan, W. T. Histidine-rich glycoprotein. In: T. Creighton (ed.), Wiley Encyclopedia of Molecular Medicine, pp. 1644–1647. New York: John Wiley & Sons, Inc., 2002.
2. Brown, K. J., and Parish, C. R. Histidine-rich glycoprotein and platelet factor 4 mask heparan sulfate proteoglycans recognized by acidic and basic fibroblast growth factor. Biochemistry, *33:* 13918–13927, 1994.
3. Borza, D. B., and Morgan, W. T. Acceleration of plasminogen activation by tissue plasminogen activator on surface-bound histidine-proline-rich glycoprotein. J. Biol. Chem., *272:* 5718–5726, 1997.
4. Leung, L. L. Interaction of histidine-rich glycoprotein with fibrinogen and fibrin. J. Clin. Investig., *77:* 1305–1311, 1986.
5. Leung, L. L., Nachman, R. L., and Harpel, P. C. Complex formation of platelet thrombospondin with histidine-rich glycoprotein. J. Clin. Investig., *73:* 5–12, 1984.
6. Morgan, W. T. The histidine-rich glycoprotein of serum has a domain rich in histidine, proline, and glycine that binds heme and metals. Biochemistry, *24:* 1496–1501, 1985.
7. Borza, D. B., and Morgan, W. T. Histidine-proline-rich glycoprotein as a plasma pH sensor. Modulation of its interaction with glycosaminoglycans by pH and metals. J. Biol. Chem., *273:* 5493–5499, 1998.
8. Lamb-Wharton, R. J., and Morgan, W. T. Induction of T-lymphocyte adhesion by histidine-proline-rich glycoprotein and concanavalin A. Cell Immunol., *152:* 544–555, 1993.
9. Olsen, H. M., Parish, C. R., and Altin, J. G. Histidine-rich glycoprotein binding to T-cell lines and its effect on T-cell substratum adhesion is strongly potentiated by zinc. Immunology, *88:* 198–206, 1996.
10. Chang, N. S., Leu, R. W., Rummage, J. A., Anderson, J. K., and Mole, J. E. Regulation of macrophage Fc receptor expression and phagocytosis by histidine-rich glycoprotein. Immunology, *77:* 532–538, 1992.
11. Leung, L. Histidine-rich glycoprotein: an abundant plasma protein in search of a function. J. Lab. Clin. Med., *121:* 630–631, 1993.
12. Lijnen, H. R., Hoylaerts, M., and Collen, D. Isolation and characterization of a human plasma protein with affinity for the lysine binding sites in plasminogen. Role in the regulation of fibrinolysis and identification as histidine-rich glycoprotein. J. Biol. Chem., *255:* 10214–10222, 1980.
13. Pepper, M. S. Role of the matrix metalloproteinase and plasminogen activator-plasmin systems in angiogenesis. Arterioscler. Thromb. Vasc. Biol., *21:* 1104–1117, 2001.
14. Brodsky, S., Chen, J., Lee, A., Akassoglou, K., Norman, J., and Goligorsky, M. S. Plasmin-dependent and -independent effects of plasminogen activators and inhibitor-1 on ex vivo angiogenesis. Am. J. Physiol. Heart Circ. Physiol., *281:* H1784–H1792, 2001.
15. Simantov, R., Febbraio, M., Crombie, R., Asch, A. S., Nachman, R. L., and Silverstein, R. L. Histidine-rich glycoprotein inhibits the antiangiogenic effect of thrombospondin-1. J. Clin. Investig., *107:* 45–52, 2001.
16. Schmaier, A. H., Rojkjaer, R., and Shariat-Madar, Z. Activation of the plasma kallikrein/kinin system on cells: a revised hypothesis. Thromb. Haemost., *82:* 226–233, 1999.
17. Zhang, J. C., Claffey, K., Sakthivel, R., Darzynkiewicz, Z., Shaw, D. E., Leal, J., Wang, Y. C., Lu, F. M., and McCrae, K. R. Two-chain high molecular weight kininogen induces endothelial cell apoptosis and inhibits angiogenesis: partial activity within domain 5. FASEB J., *14:* 2589–2600, 2000.
18. Borza, D. B., Tatum, F. M., and Morgan, W. T. Domain structure and conformation of histidine-proline-rich glycoprotein. Biochemistry, *35:* 1925–1934, 1996.
19. Passaniti, A., Taylor, R. M., Pili, R., Guo, Y., Long, P. V., Haney, J. A., Pauly, R. R., Grant, D. S., and Martin, G. R. A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin, and fibroblast growth factor. Lab. Investig., *67:* 519–528, 1992.
20. Kubota, Y., Kleinman, H. K., Martin, G. R., and Lawley, T. J. Role of laminin and basement membrane in the morphological differentiation of human endothelial cells into capillary-like structures. J. Cell Biol., *107:* 1589–1598, 1988.
21. Brooks, P. C., Montgomery, A. M., and Cheresh, D. A. Use of the 10-day-old chick embryo model for studying angiogenesis. Methods Mol. Biol., *129:* 257–269, 1999.
22. Liekens, S., De Clercq, E., and Neyts, J. Angiogenesis: regulators and clinical applications. Biochem. Pharmacol., *61:* 253–270, 2001.
23. Folkman, J. Tumor angiogenesis: therapeutic implications. N. Engl. J. Med., *285:* 1182–1186, 1971.
24. Hanahan, D., and Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell, *86:*353–364, 1996.
25. Carmeliet, P., and Jain, R. K. Angiogenesis in cancer and other diseases. Nature (Lond.), *407:* 249–257, 2000.
26. Browder, T., Folkman, J., and Pirie-Shepherd, S. The hemostatic system as a regulator of angiogenesis. J. Biol. Chem., *275:* 1521–1524, 2000.
27. O'Reilly, M. S., Holmgren, L., Shing, Y., Chen, C., Rosenthal, R. A., Moses, M., Lane, W. S., Cao, Y., Sage, E. H., and Folkman, J. Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. Cell, *79:* 315–328, 1994.
28. O'Reilly, M. S., Boehm, T., Shing, Y., Fukai, N., Vasios, G., Lane, W. S., Flynn, E., Birkhead, J. R., Olsen, B. R., and Folkman, J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell, *88:* 277–285, 1997.
29. Maeshima, Y., Colorado, P. C., and Kalluri, R. Two RGD-independent $\alpha v\beta 3$ integrin binding sites on tumstatin regulate distinct anti-tumor properties. J. Biol. Chem., *275:* 23745–23750, 2000.
30. O'Reilly, M. S., Pirie-Shepherd, S., Lane, W. S., and Folkman, J. Antiangiogenic activity of the cleaved conformation of the serpin antithrombin. Science (Wash. DC), *285:* 1926–1928, 1999.
31. Dhanabal, M., Ramchandran, R., Waterman, M. J., Lu, H., Knebelmann, B., Segal, M., and Sukhatme, V. P. Endostatin induces endothelial cell apoptosis. J. Biol. Chem., *274:* 11721–11726, 1999.
32. Jimenez, B., Volpert, O. V., Crawford, S. E., Febbraio, M., Silverstein, R. L., and Bouck, N. Signals leading to apoptosis-dependent inhibition of neovascularization by thrombospondin-1. Nat. Med., *6:* 41–48, 2000.
33. Boehm, T., O'Reilly, M. S., Keough, K., Shiloach, J., Shapiro, R., and Folkman, J. Zinc-binding of endostatin is essential for its antiangiogenic activity. Biochem. Biophys. Res. Commun., *252:* 190–194, 1998.
34. Dong, Z., Kumar, R., Yang, X., and Fidler, I. J. Macrophage-derived metalloelastase is responsible for the generation of angiostatin in Lewis lung carcinoma. Cell, *88:* 801–810, 1997.
35. Hajjar, K. A., and Acharya, S. S. Annexin II and regulation of cell surface fibrinolysis. Ann. NY. Acad. Sci., *902:* 265–271, 2000.
36. Hulett, M. D., and Parish, C. R. Murine histidine-rich glycoprotein: cloning, characterization and cellular origin. Immunol. Cell Biol., *78:* 280–287, 2000.
37. LaManna, J. C. Hypoxia/ischemia and the pH paradox. Adv. Exp. Med. Biol., *388:* 283–292, 1996.
38. Gorodetsky, R., Mou, X., Blankenfeld, A., and Marx, G. Platelet multielemental composition, lability, and subcellular localization. Am. J. Hematol., *42:* 278–283, 1993.
39. Yu, J. L., Rak, J. W., Coomber, B. L., Hicklin, D. J., and Kerbel, R. S. Effect of p53 status on tumor response to antiangiogenic therapy. Science (Wash. DC) *295:* 1526–1528, 2002.