Anti-angiogenic Cues from Vascular Basement Membrane Collagen

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ABSTRACT

Vascular basement membrane is an important structural component of blood vessels and has been shown to interact with and modulate vascular endothelial behavior during angiogenesis. During the inductive phase of tumor angiogenesis, this membrane undergoes many degradative and structural changes and reorganizes to a native state around newly formed capillaries in the resolution phase. Such matrix changes are potentially associated with molecular modifications that include expression of matrix gene products coupled with conformational changes, which expose cryptic protein modules for interaction with the vascular endothelium. We speculate that these interactions provide important endogenous angiogenic and anti-angiogenic cues. In this report, we identify an important anti-angiogenic vascular basement membrane-associated protein, the 26-kDa NC1 domain of the α6 chain of type IV collagen, termed arresten. Arresten was isolated from human placenta and produced as a recombinant molecule in Escherichia coli and 293 embryonic kidney cells. We demonstrate that arresten functions as an anti-angiogenic molecule by inhibiting endothelial cell proliferation, migration, tube formation, and Matrigel neo-vascularization. Arresten inhibits the growth of two human xenograft tumors in nude mice and the development of tumor metastases. Additionally, we show that the anti-angiogenic activity of arresten is potentially mediated via mechanisms involving cell surface proteoglycans and the αβ integrin on endothelial cells. Collectively, our results suggest that arresten is a potent inhibitor of angiogenesis with a potential for therapeutic use.

INTRODUCTION

The development of new blood vessels from preexisting ones is generally referred to as angiogenesis (1). In the adult, new blood vessels arise via angiogenesis, a process critical for normal physiological events such as wound repair, the menstrual cycle, and endometrium remodeling (2). In the last three decades, considerable research has been conducted documenting that tumor growth and metastasis require angiogenesis (3). This process is pivotal to the survival and subsequent growth of solid tumors beyond a few cubic millimeters in size (4). Vascular basement membrane constitutes an insoluble structural wall of newly formed capillaries and undergoes several changes during tumor-induced angiogenesis (5). Initially, the membrane is degraded and disassembled but is finally reorganized to a native state around a newly formed capillary (5). Such vascular matrix changes during angiogenesis are associated with the expression of matrix proteins that can interact with vascular endothelium and provide endogenous angiogenic and anti-angiogenic signals (5). Basement membranes are composed of macromolecules such as type IV collagen, laminin, HSPGs, fibronectin, and entactin (6). Type IV collagen is composed of six genetically distinct gene products, namely, α1-α6 (7). The α1 and α2 isofoms are ubiquitously present in human basement membranes (8). The other four isoforms exhibit restricted distributions (9). Type IV collagen promotes cell adhesion, migration, differentiation, and growth (8). It is thought to play a crucial role in endothelial cell proliferation and behavior during the angiogenic process (5). Several studies have shown the anti-angiogenic properties associated with inhibitors of collagen metabolism, supporting the notion that basement membrane collagen synthesis and deposition are crucial for blood vessel formation and survival (10). Additionally, the COOH-terminal globular NC1 domain of type IV collagen is speculated to play an important role in the assembly of type IV collagen suprastructure, basement membrane organization, and modulation of cell behavior (11, 12). Recently, the NC1 domain of the α6 chain of type IV collagen (constatin) was identified as an angiogenesis inhibitor (13). In the present study, we demonstrate the pivotal role of arresten, the NC1 domain of the α6 chain of type IV collagen, in modulating the function of capillary endothelial cells and blood vessel formation using in vitro and in vivo models of angiogenesis and tumor growth.

MATERIALS AND METHODS

Recombinant Production of Arresten in Escherichia coli.

The sequence encoding arresten was amplified by PCR from the α6 NC1 (IV) cDNA vector (14) using a forward primer (5′-CGGGATCCCTCTGTTGATCACGGCTTC-3′) and a reverse primer (5′-CCCAAGCTTTGTTCTTCTCATACAGAC-3′). The resulting cDNA fragment was digested with BamHI and Hind III and ligated into predigested pET22b (+) (Novagen, Madison, WI). This placed arresten downstream of and in frame with the pelB leader sequence, allowing for periplasmic localization and expression of soluble protein. Additional vector sequence was added to the protein encoding amino acids MDGINS. The 3′ end of the sequence was ligated in frame with the polyhistidine tag sequence. Additional vector sequence between the 3′ end of the cDNA and the his tag encoded the amino acids KLAALA. Positive clones were sequenced on both strands. Plasmid constructs encoding arresten were first transformed into E. coli HMS174 (Novagen) and then transformed into BL21 for expression (Novagen). Overnight bacterial culture was used to inoculate a 500-ml culture in Luria-Bertani medium. This culture was grown for ~4 h until the cells reached an A600 of 0.6. Then, protein expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 1–2 mM. After a 2-h induction, cells were harvested by centrifugation at 5,000 × g and lysed by resuspension in 6 M guanidine, 0.1 M NaH2PO4, and 0.01 M Tris-HCl (pH 8.0). Resuspended cells were sonicated briefly, and centrifuged at 12,000 × g for 30 min. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked ‘advertisement’ in accordance with 18 U.S.C. Section 1724 solely to indicate this fact.
min. The supernatant fraction was passed over a 5-mL Ni-nitrilotriacetic acid-agarose column (Qiagen, Chatsworth, CA) four to six times at a speed of 2 mL/min. Nonspecifically bound protein was removed by washing with both 10 and 25 mM imidazole in 8 mM urea, 0.1 M NaH₂PO₄, and 0.01 M Tris-HCl (pH 8.0). Arresten protein was eluted from the column with increasing concentrations of imidazole (50, 125, and 250 mM) in 8 mM urea, 0.1 M NaH₂PO₄, and 0.01 M Tris-HCl (pH 8.0). The eluted protein was diazylized twice against PBS at 4°C. A minor portion of the total protein precipitated during dialysis. Dialyzed protein was collected and centrifuged at ~3,500 × g and separated into pellet and supernatant fractions. Protein concentration in each fraction was determined by the bicinchoninic acid assay (Pierce, Rockford, IL) and quantitative SDS-PAGE analysis. The fraction of total protein in the pellet was ~22%, with the remaining 78% recovered as a soluble protein. The total yield of protein was approximately 100 mg. Recombinant Production of Endostatin in Yeast. Mouse endostatin was produced in *Picchia pastoris* and purified as described previously (15).

Expression of Arresten in 293 Embryonic Kidney Cells. We used the pDS plasmid containing α₁(IV)NC1 (14) to PCR amplify arresten in a way that it would add a leader signal sequence in frame into the pcDNA 3.1 (Invitrogen, Carlsbad, CA) eukaryotic expression vector. The leader sequence from the 5’ end of the full-length α₁(IV) chain was cloned 5’ to the NCI domain to enable protein secretion into the culture medium. The arresten-containing recombinant vectors were sequenced using flanking primers. Error-free cDNA clones were further purified and used for in vitro translation studies to confirm protein expression (data not shown). The arresten-containing plasmid and control plasmid were used to transfect 293 cells using the calcium chloride method. Transfected clones were selected by Geneticin (Life Technologies, Inc., Gaithersburg, MD) antibiotic treatment. The cells were passed for 3 weeks in the presence of the antibiotic until no cell death was evident. Clones were expanded into T-225 flasks and grown until confluent. Then, the supernatant was subjected to affinity chromatography using arresten-specific antibodies (14). Arresten antibody was generated to a purified protein as described previously (14). This antibody recognized only the α₁ NCI domain (14). A major peak was identified, containing a monomer of ~30 kDa that was immunoreactive with arresten antibodies.

Isolation of Native Arresten. Native arresten from human placenta was isolated using bacterial collagenase, anion exchange chromatography, gel filtration chromatography, HPLC, and affinity chromatography (6, 14). Type IV collagen monomers isolated from human placenta were HPLC purified using a C-18 hydrophobic column.

Inhibition of Endothelial Cell Proliferation. CPAE cells were grown to confluence in DMEM with 10% FCS and kept contact inhibited for 48 h. Human renal cell carcinoma cells (786-0; data not shown), PC-3 cells (human prostate adenocarcinoma), HPECs, and A-498 (renal carcinoma) cells (data not shown) were used as controls in this experiment. Cells were harvested by trypsinization (Life Technologies) at 37°C for 5 min. A suspension of 12,500 cells in DMEM with 1% FCS was added to each well of a 24-well plate coated with 0.5% FCS and 3 ng/ml bFGF (R&D Systems, Inc., Minneapolis, MN). All wells were removed, and the wells were washed with PBS. Cells were extracted with 1 M NaOH and added to a scintillation vial containing 4 mL of ScintiVerse II (Fisher Scientific, Springfield, NJ) solution. Thymidine incorporation was measured using a scintillation counter. All groups represent triplicate samples.

Cell Cycle Analysis. Cell cycle analysis was performed as reported previously (16). Briefly, CPAE cells were grown to confluence in DMEM containing 10% FBS and growth arrested by contact inhibition for 48 h. A suspension of 500,000 cells was seeded in each well of a six-well plate in DMEM containing 1% FBS and 5 mg/ml VEGF. Different doses of arresten were added, and the cells were harvested 18 h after treatment. Cells were fixed in ice-cold 95% ethanol and rehydrated 3 h later at room temperature for 30 min in rehydration buffer (2% FBS and 0.1% Tween 20 in PBS). Next, the cells were centrifuged at 1,200 rpm for 10 min and resuspended in 0.5 ml of rehydration buffer. RNase was added at 5 μg/ml and allowed to incubate for 1 h at 37°C, followed by staining with propidium iodide at 5 μg/ml. The data were analyzed using a Becton Dickinson (San Jose, CA) FACStar plus flow cytometer. The percentage of cells in S phase was calculated using ModFit software.

Endothelial Tube Assay. Matrigel (Collaborative Biomolecules, Bedford, MA) was added (320 μl) to each well of a 24-well plate and allowed to polymerize (17). A suspension of 25,000 mouse aortic endothelial cells in EGM-2 (Clonetics, Inc., Walkersfield, MD) medium without antibiotic was passed into each well coated with Matrigel. The cells were treated with arresten, BSA, sterile PBS, or 75% domain in increasing concentrations. All assays were performed in triplicate. Cells were incubated for 24–48 h at 37°C and viewed using an Olympus Optical (Tokyo, Japan) CK2 microscope (3.3 ocular, 10x objective). The cells were then photographed using 400 DK-coated TMAX film (Eastman Kodak, Rochester, NY). Cells were stained with Diff-Quik fixative (Sigma Chemical Co., St. Louis, MO) and photographed again (17). Ten fields were viewed, and tubes were counted and averaged.

Matrigel Assay. Matrigel was thawed overnight at 4°C. Before injection into C57BL/6 mice it was mixed with 20 units/ml heparin (Pierce), 150 ng/ml bFGF (R&D Systems), and either 1 μg/ml arresten or 10 μg/ml endostatin. Control groups received no angiogenic inhibitor. The Matrigel mixture was injected s.c. using a 21-gauge needle. After 14 days, mice were sacrificed, and the Matrigel plugs were removed. Matrigel plugs were fixed in 4% paraformaldehyde (in PBS) for 4 h at room temperature and then switched to PBS for 24 h. The plugs were embedded in paraffin, sectioned, and H&E stained. Sections were examined by light microscopy, and the number of blood vessels from 10 high-power fields was counted and averaged.

Inhibition of Tumor Metastases. C57BL/6 mice were i.v. injected with 1 million MC38/MUC1 cells. Controls (five mice) received sterile PBS, and the experimental group (six mice) received 4 mg/kg arresten every other day for 26 days. Pulmonary tumor nodules were counted for each mouse in both groups and averaged after 26 days of treatment. Two deaths were recorded in each group.

In Vivo Tumor Studies. Human renal cell carcinoma cells (786-0) were maintained in DMEM with 10% FCS until confluent. The cells were harvested, and 2 million were injected into 7- to 9-week-old athymic nude mice. The tumors were allowed to grow to ~700 or 100 mm³. Arresten was injected i.p. daily at a dosage of 10 or 20 mg/kg. Control groups received either BSA or the PBS vehicle daily. Human prostate adenocarcinoma cells (PC-3) were maintained in F12K medium with 10% FCS until confluent. The cells were harvested, and 5 million were injected into 7- to 9-week-old male athymic nude mice. The tumors grew to ~60 or 200 mm³. The mice were injected daily with 10 or 4 mg/kg arresten or 20 mg/kg endostatin. Control groups received daily injections of PBS. In both experiments tumor volume was measured using the standard formula length × width² × 0.52 (18). Each group contained five or six mice.

Immunohistochemistry. Mice were sacrificed after 10–20 days of arresten treatment. Tumors were excised and fixed in 4% paraformaldehyde. Tissues were paraffin embedded, and 3-μm sections were cut and mounted on glass slides. Sections were deparaffinized, rehydrated, and treated with 300 mg/ml protease XXIV (Sigma) at 37°C for 5 min. Digestion was stopped with 100% ethanol, and sections were air dried and blocked with 10% rabbit serum. Then, slides were incubated at 4°C overnight with a 1:50 dilution of rat anti-mouse CD-31 monoclonal antibody (PharMingen, San Diego, CA), followed by a 1:200 dilutions of anti-PCNA antibody (Signet Laboratories, Inc., Dedham, MA). Detection was carried out according to the manufacturer’s recommendations using the USA horseradish peroxidase system (UltraVision). Finally, the slides were counterstained with hematoxylin. Staining for fibronectin and type IV collagen was performed using polyclonal anti-fibronectin (Sigma) at a dilution of 1:500 and anti-type IV collagen (ICN, Costa Mesa, CA) at a dilution of 1:100. The Vectastain Elite ABC kit (Vector...
LABORATORIES, Burlingame, CA) was used for detection according to the manufacturer’s recommendations.

Scatchard Analysis. Scatchard analysis was performed as described previously (19). Briefly, CPAE cells were plated on a 96-well plate (10,000 cells per well) in DMEM with 10% FCS and grown to confluency. The cells were then washed with ice-cold PBS and incubated with 180 pmol of [125I]arresten with and without increasing concentrations of unlabeled arresten ranging from 150 pmol to 100 nmol comprising a total of 27 data points. The cells were incubated with this mixture for 2 h at 4°C. Then, the cells were washed with ice-cold PBS and extracted with 1 N NaOH, and radioactivity was measured in a scintillation counter.

ELISA for HSPG. Direct ELISA was performed as described previously (9). HSPG (100 ng, Sigma) was coated on a 96-well plate in triplicate in a 2-fold molar excess of binding proteins arresten, bFGF, and BSA. Binding was established with antibodies to bFGF, arresten, and BSA. The ELISA was developed with an alkaline phosphatase secondary antibody and read in a plate reader at absorbance of 405 nm.

Cell Adhesion Assay. Ninety-six-well plates were coated with human arresten or human type IV collagen (Collaborative Biomolecules, Bedford, MA) at a concentration of 10 μg/ml or human vitronectin. The cells were grown to confluence. The cells were added to each well, and the plate was incubated for 45 min at 37°C with 5% CO2. Unattached cells were removed by washing with serum-free medium, and attached cells were counted.

RESULTS

Human arresten was produced in E. coli using a bacterial expression plasmid, pET-22b (capable of periplasmic transport, thus resulting in a soluble protein) as a fusion protein with a COOH-terminal 6-histidine tag. The E. coli-expressed protein was isolated predominantly as a soluble protein, and SDS-PAGE analysis revealed a monomeric band at 29 kDa. The additional 3 kDa arise from polylinker and histidine tag sequences and were immunodetected by both arresten and 6-histidine tag antibodies (Fig. 1, a and b). Human arresten was also produced as a secreted soluble protein in 293...
embryonic kidney cells using the pcDNA 3.1 eukaryotic vector. This recombinant protein (without any purification or detection tags) was isolated using affinity chromatography, and a pure monomeric form was detected in the major peak by SDS-PAGE and immunoblot analyses (Fig. 1, c and d). In addition, human arresten was isolated from human placenta by gel filtration, HPLC, and affinity chromatography techniques; a 26-kDa molecule was detected by SDS-PAGE and immunoblot analyses (Fig. 1, e and f).

In assays of endothelial cell proliferation, a dose-dependent inhibition of bFGF-stimulated endothelial cells was detected, with an ED50 value of 0.25 μg/ml (Fig. 2a) using E. coli-produced soluble protein. These results support earlier observations that α1 and α2 type IV collagen isolated from the Engelbreth-Holm-Swarm mouse sarcoma tumor may be inhibitory to capillary endothelial cells (5). No significant effect was observed on the proliferation of renal carcinoma cells (786-O; data not shown), prostate cancer cells (PC-3) or HPECs, even at arresten doses of up to 50 μg/ml (Fig. 2, c and d). In contrast, endostatin inhibited CPAE cell proliferation with an ED50 value of 0.75 μg/ml, 3-fold higher than arresten, and did not inhibit A-498 cancer cells (data not shown; Ref. 15). Cell cycle analysis was also performed using FACSscan technology to assess the antiproliferative properties of arresten in the presence of VEGF. We observed a decrease in the number of CPAE cells in S-phase in the presence of arresten. These results correlate with thymidine incorporation proliferation assays described above (Fig. 2b).

When mouse aortic endothelial cells are cultured on Matrigel, a solid gel of mouse basement membrane proteins, they rapidly align and form hollow tube-like structures (20). Arresten, produced in 293 cells, selectively inhibited endothelial tube formation in a dose-dependent manner (Fig. 2, f and h). Similar results were also obtained using E. coli-produced arresten (data not shown). The 7S domain of

Fig. 2. Inhibition of endothelial cell proliferation. CPAE (a and e) cells and control nonendothelial cells, PC-3 cells (c) and HPECs (d), were treated with concentrations of arresten or endostatin ranging from 0.01 to 50 μg/ml. All wells received 1 μCi of [3H]thymidine at the time of treatment. Thymidine incorporation was measured using a scintillation counter. All groups represent triplicate samples. b, cell cycle analysis. Growth-arrested CPAE cells were treated with concentrations of arresten ranging from 0.1 to 20 μg/ml. The cells were stimulated with 5 ng/ml VEGF, trypsinized, and harvested after 18 h. The VEGF (-) value is the percentage of cells in S-phase at the beginning of the experiment. f–h, endothelial tube assay with mouse aortic endothelial cells. Ten fields were viewed, and tubes were counted and averaged (f). Well-formed tubes can be observed in g treated with 7S domain control (magnification, ×100). Arresten-treated (0.8 μg/ml) mouse aortic endothelial cells (magnification, ×100) are shown in h.
type IV collagen (NH₂-terminal noncollagenous domain) had no effect on endothelial tube formation (Fig. 2g). Maximum inhibition with arresten was attained between 0.8 and 1 µg/ml (Fig. 2f).

To test the in vivo effect of arresten on the formation of new capillaries, we performed a Matrigel plug assay in mice (21). Matrigel was placed in the presence of bFGF, with or without increasing concentrations of arresten. A 50% reduction in the number of blood vessels was observed at 1 µg/ml arresten and 10 µg/ml endostatin (Fig. 3a). Collectively, these results suggest that arresten affects the formation of new blood vessels by inhibiting more than one step in the angiogenic process.

To assess the effect of arresten on metastasis, 1 × 10⁶ MC38/MUC1 cancer cells were administered by tail vein to C57BL/6 mice (22). Treatment with 5 mg/kg arresten (i.p.) was initiated the following day and continued every other day for 26 days. The results show a significant reduction of pulmonary nodules in arresten-treated mice compared with the control group (Fig. 3b).

Next, we tested the effect of arresten on established primary tumors in mice. Arresten, E. coli produced, inhibited the growth of large (Fig. 3c) and small (Fig. 3d) renal cell carcinoma tumors. In experiments performed with PC-3 human prostate tumors in mice, arresten at 10 mg/kg inhibited tumor growth similar to endostatin at 20 mg/kg (Fig. 3e). A similar degree of inhibition was observed with arresten administered at 4 mg/kg, and this inhibition continued for 12 days after arresten treatment was stopped (Fig. 3f). After 12 days, the tumors escaped the effect of arresten and began growing at the same rate as the controls (data not shown). A CD-31 staining pattern of treated (Fig. 3b) versus control (Fig. 3g) mice is shown. Blood vessels in 15 high-magnification fields were counted and averaged. This number was divided by the volume of the tumor and averaged (18.7 ± 6.2 control versus 10.5 ± 7.2 treated; Fig. 3f). Finally, tumor sections were stained for PCNA, fibronectin, and type IV collagen. We found no difference in tumor cell proliferation or in type IV collagen and fibronectin content surrounding tumor cells in the treated and untreated mice, again demonstrating the endothelial cell specificity of arresten (Fig. 3, j–l, representative arresten-treated sections).
To gain further insight into the anti-angiogenic mechanism of action of arresten, we studied its binding to endothelial cells. Labeled human placenta arresten was incubated with CPAE cells, and a Scatchard analysis was performed (19). Our data revealed two different binding sites (Fig. 4a). The high-affinity, low-capacity binding site has a $K_d$ value of $8.5 \times 10^{-11}$ m and a maximum number of binding sites of $3 \times 10^6$ sites per cell. The other low-affinity, high-capacity binding site has a $K_d$ value of $4.6 \times 10^{-8}$ m and a maximum number of binding sites of $6 \times 10^7$ sites per cell. It has been shown that HSPG binds the $\alpha_1$ NC1 domain of type IV collagen (23). Also, recent studies have speculated that $\alpha_1\beta_1$ and $\alpha_6\beta_1$ integrins bind to type IV collagen isolated from the Engelbreth-Holm-Swarm mouse sarcoma tumor (24).

We assessed the capacity of arresten to mediate endothelial cell binding via $\alpha_1\beta_1$ and $\alpha_6\beta_1$ integrins. Our results show that functionally blocking $\alpha_1$ and $\beta_1$ integrin subunit antibodies significantly diminish the binding of HUVECs to arresten-coated culture wells (Fig. 4c). We found an inhibition of endothelial cell attachment to arresten-coated plates of 60% with $\alpha_1$ antibody and 70% with $\beta_1$ integrin antibody. The control $\alpha_6$ integrin antibody showed no binding inhibition to arresten. The $\alpha_6\beta_1$ antibody did not inhibit endothelial cell binding to arresten but increased binding (Fig. 4c). On the other hand, with type IV collagen-coated plates, we observed an inhibition of 30% with $\alpha_1$, 40% with $\beta_1$, and 15% with $\alpha_6\beta_1$ neutralizing antibodies (Fig. 4d). Again, the $\alpha_6$ neutralizing antibody had no effect on binding. We speculate that the difference in cell adhesion between arresten and type IV collagen-coated plates in the presence of $\alpha_1$ and $\beta_1$ integrin antibodies is due to additional integrin binding sites on the entire type IV collagen molecule in comparison with arresten, which may contain a single integrin binding site (Fig. 4, c and d). To demonstrate the efficiency of the $\alpha_6\beta_1$ neutralizing antibody, we performed a control adhesion experiment with its ligand, vitronectin (Fig. 4e). The neutralizing $\alpha_6\beta_1$ and $\alpha_6$ antibodies were able to inhibit endothelial cell binding to vitronectin by 60 and 90%, respectively.

HSPG binding to arresten was assessed by ELISA. ELISA plates were coated with HSPG and incubated with arresten, bFGF, or BSA. Our results show that HSPG binds both arresten and bFGF as reported earlier (Ref. 23; Fig. 4b). Taken together in conjunction with earlier reports (23), these results suggest that arresten may be binding HSPG on the cell surface (Fig. 4, a and b).

DISCUSSION

We propose that the molecular mechanism associated with the tumor-suppressing activity of arresten as well as the specific inhibition of endothelial cell proliferation and migration by arresten may be mediated by the $\alpha_1\beta_1$ integrin. These results suggest that binding of arresten to $\alpha_1\beta_1$ may down-regulate VEGF-induced proliferation and migration of endothelial cells, as suggested previously by VEGF-induced expression of $\alpha_1\beta_1$ integrin on endothelial cells (25).

In support of our findings, it has been shown that $\alpha_1$ integrin neutralizing antibodies can suppress angiogenesis in vivo (24). Among the collagen integrins, $\alpha_1\beta_1$ activates the Ras-Shc-mitogen-activated protein kinase pathway, promoting cell proliferation (26). Our studies suggest that arresten may be antagonizing this effect in endothelial cells. In addition, Pozzi et al. (27) recently described decreased angiogenesis in tumor-bearing $\alpha_1$ integrin-deficient mice.

Whether arresten functions by suppressing the activity of VEGF and/or bFGF directly remains to be elucidated. Future comparative studies with other recently discovered inhibitors such as restin, troponin 1, kringle 5, pigment epithelium-derived factor, and vasostatin will also be very insightful in establishing the unique anti-angiogenic property of arresten (28–30).

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