Parstatin, the Cleaved Peptide on Proteinase-Activated Receptor 1 Activation, Is a Potent Inhibitor of Angiogenesis

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ABSTRACT

The proteolytic activation by thrombin of the proteinase-activated receptor 1 unveils the tethered peptide ligand and cleaves a 41-amino acid peptide. In this report, we show that this peptide, which we have designated as “parstatin,” is a potent inhibitor of angiogenesis. Synthesized parstatin suppressed both the basic angiogenesis and that stimulated by basic fibroblast growth factor and vascular endothelial growth factor in the chick embryo model in vivo and in the rat aortic ring assay. Parstatin also abrogated endothelial cell migration and capillary-like network formation on the Matrigel and fibrin angiogenesis models in vitro. Treatment of endothelial cells with parstatin resulted in inhibition of cell growth by inhibiting the phosphorylation of extracellular signal-regulated kinases in a specific and reversible fashion and by promoting cell cycle arrest and apoptosis through a mechanism involving activation of caspases. We have shown that parstatin acts as a cell-penetrating peptide, exerting its biological effects intracellularly. The uptake into cells and the inhibitory activity were dependent on parstatin hydrophobic region. These results support the notion that parstatin may represent an important negative regulator of angiogenesis with possible therapeutic applications.

Proteinase-activated receptors (PARs) comprise a novel family of G protein-coupled receptors, which are activated by proteolytic cleavage of their N-terminal extracellular domain (Ossowskaya and Bunnett, 2004). PAR1 is the first member of this family to be cloned in which proteolytic cleavage at the Arg41/Ser42 bond releases a 41-amino acid peptide and unveils a tethered peptide ligand with the recognition sequence SFLLRN, which acts as an agonist (Vu et al., 1991). PAR1 is primarily activated with very high affinity by thrombin, but it has been shown that other proteases, such as activated protein C (Riewald et al., 2002) and matrix metalloprotease (MMP)-1 (Boire et al., 2005), are able to activate this receptor under certain conditions and induce downstream signal events.

Thrombin has been shown to be a key positive regulator of normal and abnormal angiogenesis (Tsopanoglou and Maragoudakis, 2004). Thrombin’s angiogenic activity is mainly dependent on signaling via PAR1. Thrombin, through PAR1 signaling, interacts with and stimulates a variety of vascular cells and regulates the release, expression, and activation of the majority of angiogenesis mediators. For example, in human platelets, it has been demonstrated that PAR1 activation regulates, in opposing fashion, the release of vascular endothelial growth factor (VEGF) and endostatin, which is an endogenous inhibitor of angiogenesis (Ma et al., 2005). In endothelial cells, it has also been shown that the activation of PAR1 by thrombin resulted in marked up-regulation of growth-regulated oncogene-a, which in turn mediated the thrombin-induced increase of vascular regulatory proteins (MMP-1, MMP-2), growth factors (VEGF, angiopoietin-2), and angiogenic receptors (VEGF receptor 2) (Caunt et al., 2006). In addition, signaling through PAR1 has been pro-

ABBREVIATIONS: PAR, protease-activated receptor; MMP, matrix metalloprotease; VEGF, vascular endothelial growth factor; FITC, fluorescein isothiocyanate; HUVEC, human umbilical vein endothelial cell; CAM, chorioallantoic membrane; bFGF, basic fibroblast growth factor; FBS, fetal bovine serum; BSA, bovine serum albumin; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; HB, heparin binding; EGFr, epidermal growth factor; ERK, extracellular signal-regulated kinase; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; DAPI, 4,6-diamidino-2-phenylindole; MAPK, mitogen-activated protein kinase; parst, parstatin; Z, N-benzylxycarbonyl; FMK, fluoromethyl ketone.
posed to influence a wide range of pathophysiological responses, including platelet activation, intimal hyperplasia, inflammation, and tumor growth and metastasis (Tsopanoglou and Maragoudakis, 2007). In this context, PAR1 presents a novel target for developing new therapeutic agents for treating these conditions and angiogenesis-related diseases (Zania et al., 2006).

Despite the wealth of information relating to the role of PAR1 in physiology, disease states, and angiogenesis, a potential biological role of the PAR1-cleaved peptide remains unknown. In the present study, we showed that the PAR1-cleaved peptide inhibits angiogenesis in the chick embryo angiogenesis model in vivo and in angiogenesis models in vitro. We also show that this peptide abrogates endothelial cell growth by induction of apoptosis. These effects are associated with the ability of the PAR1-cleaved peptide to penetrate the cell membrane and act intracellularly. We coined the term “parstatin” for this biologically active fragment of PAR1.

**Materials and Methods**

**Peptides and Cell Culture.** Synthesized peptides were purified (purity level, >95%) by high-performance liquid chromatography and characterized by mass spectrometry. Their amino acid sequences and sources were as shown in Table 1. Human parstatin corresponds to the 1- to 41-amino acid-cleaved N-terminal fragment of human PAR1. Mouse parstatin corresponds to the 1- to 41-amino acid-cleaved N-terminal fragment of mouse PAR1. Scrambled human parstatin contains a random rearrangement of the amino acid sequence of human parstatin. Human short parstatin corresponds to amino acids 24 to 41 of human parstatin. Modulated human parstatin contains scrambled the 24 to 41 amino acids of human parstatin. Human parstatin and short and modulated parstatins were conjugated with fluorescein isothiocyanate (FITC). Human umbilical vein endothelial cells (HUVECs) were obtained, cultured, and used as described previously (Tsopanoglou and Maragoudakis, 1999).

**Chick Chorioallantoic Membrane Model.** The in vivo chick chorioallantoic membrane (CAM) angiogenesis model was used as described previously (Pyriochou et al., 2006). In brief, on incubation day 9 of fertilized chicken eggs, an O-ring (1 cm²) was placed on the surface of the CAM, and the vehicle or the indicated substances were placed inside this restricted area. After 48 h, CAMs were fixed in saline-buffered formalin, photographed, and analyzed using Scion Image software (Scion Image Release Beta 4.0.2 software; Scion Corporation, Frederick, MD).

**Rat Aorta Model of Angiogenesis.** All animal procedures were performed with approval from the Veterans Administration Puget Sound Health Care System Institutional Animal Care and Use Committee and according to National Institutes of Health guidelines. Thoracic aortas were dissected from CO₂- euthanized 5- to 10-week-old Fischer 344 male rats (Harlan, Indianapolis, IN). Freshly cut aortic rings were embedded in collagen gels and cultured in 16-mm wells (four-well NUNC dishes; Nalge Nunc International, Rochester, NY), each containing 0.5 ml of serum-free endothelial basal medium (Lonz WalkerSvilles, Inc., Walkersville, MD), as described previously (Zhu and Nicosia, 2002). Where indicated, VEGF (human recombinant; R&D Systems, Minneapolis, MN) or basic fibroblast growth factor (human recombinant bFGF; R&D Systems) were added to medium. The angiogenic response of aortic cultures was measured in live cultures by counting the number of neovessels over time, according to published criteria.

**Tube Formation Angiogenesis Models.** The Matrigel tube formation assay was used as described previously (Zania et al., 2006). In brief, Matrigel (BD Biosciences Pharmingen, San Diego, CA) was used to coat 24-well plates (0.25 ml/well). After polymerization of Matrigel at 37°C for 1 h, indicated concentrations of peptides were added in 0.5 ml of serum-free medium. HUVECs (40,000 cells/well) were then added suspended in 0.5 ml of medium containing fetal bovine serum (FBS), making 5% final concentration in FBS. After 18 h of incubation, the cells were fixed and stained, and the tube-like structures formed were quantitated.

The ability of endothelial cells to form three-dimensional structures was also assessed using a fibrin gel in vitro angiogenesis assay kit (Chemicon International, Temecula, CA). Fibrin gels were formed in 48-well plates by mixing fibrinogen and thrombin solutions according to the manufacturer’s instructions. HUVECs (40,000 cells/well) were then added and cultured in medium containing 2% FBS for 18 h. After the addition of a second layer of fibrin gel, endothelial cells were cultured in serum-free medium containing 0.5% bovine serum albumin (BSA) and the combination of VEGF/bFGF (human recombinant; Chemicon International) for 24 h. Where indicated, parstatin or other peptides were added. The capillary-like network was photographed and measured.

**Cell Migration Assay.** HUVEC migration was assessed by the modified Boyden’s chamber assay in Transwell cell culture chambers (Corning Life Sciences, Acton, MA). Polycarbonate filters with 8-μm pores were used to separate the upper and lower chambers. Cells were added to the upper compartment at a density of 10,000 cells/100 μl in serum-free medium containing 0.5% BSA and incubated for 6 h. Directional migration (chemotaxis) to the lower chamber was induced by the addition of medium containing 5% FBS to the lower chamber. Where indicated, parstatin or other peptides were added to the lower chamber. Cells on the filters were fixed and stained. Nonmigrated cells (cells on the upper surface) were removed by wiping with cotton swabs. The cells on the lower surface were counted manually with a microscope in six predetermined fields.

**Cell Proliferation Assay.** Cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma-Aldrich, St. Louis, MO) assay. In brief, endothelial cells (10,000/well) were seeded in 24-well tissue culture plates and incubated with growth medium for 24 h. Cells were then treated with the vehicle or the

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**TABLE 1**  
Parstatin peptides

<table>
<thead>
<tr>
<th>Peptides and Sources</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>Human parstatin (Peptide Specialty Laboratories GmbH, Heidelberg, Germany; EZBiolab Inc., Westfield, IN; Bio-Synthesis, Inc., Lewisville, TX)</td>
<td>NH₂-MGPRLLLVLAAACFLSCLGPPLLSTRARRPESKATNATLDPDR-OH</td>
</tr>
<tr>
<td>Human parstatin-FITC (Bio-Synthesis, Inc.)</td>
<td>FITC-Ahx-MGPRLLLVLAAACFLSCLGPPLLSTRARRPESKATNATLDPDR-OH</td>
</tr>
<tr>
<td>Mouse parstatin (Peptide Specialty Laboratories GmbH)</td>
<td>NH₂-MGPRLLLVLAAACFLSCLGPPLLSTRARRPESKATNATLDPDR-OH</td>
</tr>
<tr>
<td>Scrambled human parstatin (Peptide Specialty Laboratories GmbH)</td>
<td>NH₂-LRTNASLVLPTTKRASSGTRAADPPRLMCLRPLARRCG-OH</td>
</tr>
<tr>
<td>Short human parstatin (Peptide Specialty Laboratories GmbH)</td>
<td>NH₂-TRARRPESKATNATLDPDR-OH</td>
</tr>
<tr>
<td>Short human parstatin-FITC (EZBiolab Inc.)</td>
<td>NH₂-TRARRPESKATNATLDPDR-FITC</td>
</tr>
<tr>
<td>Modulated human parstatin (EZBiolab Inc.)</td>
<td>NH₂-MGPRLLLVLAAACFLSCLGPPLLSTRARRPESKATNATLDPDR-OH</td>
</tr>
<tr>
<td>Modulated human parstatin-FITC (EZBiolab Inc.)</td>
<td>FITC-Ahx-MGPRLLLVLAAACFLSCLGPPLLSTRARRPESKATNATLDPDR-OH</td>
</tr>
</tbody>
</table>
indicated peptides in medium containing 5% FBS for 1 to 3 days. After 24, 48, or 72 h, MTT solution (5 mg/ml) was added to each well and incubated for 3 h at 37°C. Absorbance at 450 nm was recorded by using a 96-well plate reader.

**[3H]Thymidine Incorporation Assay.**HUVECs were incubated until 60 to 80% confluence in 24-well plates. Cells were then treated with indicated peptides in serum-free medium containing 0.5% BSA or VEGF or bFGF or epidermal growth factor (human recombinant EGF; Promega, Madison, WI) or heparin binding EGF [human recombinant heparin binding (HB)-EGF; R&D Systems] or medium containing 5% FBS for 18 h. In some experiments, HUVECs were seeded sparsely into the well and incubated until confluence. After washes, confluent cells were then treated with parstatin for 18 h in medium containing 5% FBS. All cells were pulsed with 0.5 Ci/ml [3H]thymidine (ICN Biomedicals Inc., Irvine, CA) for an additional 6 h. Radioactivity was determined in a liquid scintillation counter.

**Western Blot Analysis.**HUVECs were cultured in 35-mm tissue culture plates. After reaching 80% confluence, cells were growth-factor-starved and subsequently stimulated for 10 min with vehicle or the agents indicated. In combination experiments, cells were pretreated with parstatin or other peptides for 10 to 60 min. Cell lysates were resolved in 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were incubated with primary antibodies against phospho-p42/44 mitogen-activated protein kinases (p-Erk1/2; New England Biolabs, Ipswich, MA) and p42/44 Erk1/2 (t-Erk1/2; New England Biolabs).

For analysis of poly(ADP-ribose) polymerase (PARP), endothelial cells were treated with the indicated agents or their combinations for 24 h. Attached and suspended cells were lysed with radioimmuno-precipitation assay lysis buffer. Cell lysates were processed as described above using the following primary antibodies: mouse anti-PARP monoclonal (BD Biosciences, San Jose, CA) that detects both the 116-kDa intact and 85-kDa cleaved forms of PARP, anti-α-tubulin monoclonal (Sigma-Aldrich).

**Cell Cycle Analysis.**HUVECs were treated in the absence or in the presence of parstatin for 24 h in serum-free medium containing either 0.5% BSA or bFGF. Attached cells were collected by trypsinization, pooled with suspended cells, washed, and fixed. Fixed cells were then stained with propidium iodide (50 μg/ml; Sigma-Aldrich) for 20 min at 4°C in the dark. Flow cytometry was performed on a FACs flow cytometer (EPICS XL-MCL; Beckman Coulter, Fullerton, CA). The propidium iodide-stained cell population in sub-G0/G1, G1, S, and G2/M phases were represented by distinct and quantified peaks in the fluorescence histograms obtained using the WinMDI logical program.

**Assessment of Apoptosis by Flow Cytometry Analysis.**We used the annexin V-FITC assay kit (BD Biosciences Pharmingen). Endothelial cells were treated in the absence or in the presence of parstatin for 24 h in serum-free medium containing 0.5% BSA or bFGF. Attached cells were collected by trypsinization, pooled with suspended cells, washed, and fixed. Fixed cells were then stained with parstatin (50 μg/ml; Sigma-Aldrich) for 20 min at 4°C in the dark. Flow cytometry was performed on a FACs flow cytometer (EPICS XL-MCL; Beckman Coulter, Fullerton, CA). The propidium iodide-stained cell population in sub-G0/G1, G1, S, and G2/M phases were represented by distinct and quantified peaks in the fluorescence histograms obtained using the WinMDI logical program.

**Caspase-3 Activity Assay.** The activity of caspase-3 in treated endothelial cells was measured with an assay kit (Promega). The colorimetric substrate, Ac-DEVD-p-nitroanilide, which cleaved by caspase-3 to release yellow p-nitroanilide, was measured by absorbance at 405 nm. HUVECs, at approximately 80% confluence, were treated in the absence or in the presence of the indicated peptides for 24 h in serum-free medium containing either 0.5% BSA or bFGF. Suspended and adherent cells were collected and lysed. Caspase-3 activity was measured by absorbance at 405 nm.

**Cell Loading and Assessment of Peptide Uptake into Cells.**HUVECs in the exponential growth phase were exposed to various concentrations of indicated FITC-labeled peptides in serum-free medium containing 0.5% BSA. After incubation at the indicated times, cells were washed extensively and incubated for 10 min with trypsin to remove the cell surface-bound peptides. Suspended cells were then analyzed on a FACS flow cytometer.

For imaging, endothelial cells were incubated with the indicated FITC-labeled peptides, and their distribution was monitored with fluorescence microscopy. 4,6-Diamidino-2-phenylindole (DAPI; Sigma-Aldrich) was used to stain nuclei of all cells. Cell fluorescence was imaged on a Nikon Eclipse TE2000-U microscope (Nikon, Tokyo, Japan). FITC and DAPI were excited using 490 and 360 nm, respectively. The emission signals were sorted out using 514 and 460 filters for the FITC and DAPI, respectively.

**Data Analysis.** Data are expressed as means ± S.E. of the indicated number of observations. Statistical comparisons between groups were performed using analysis of variance or Student’s t test as appropriate. Differences were considered significant when p < 0.05.

**Results**

**Parstatin Is a Potent Inhibitor of Angiogenesis.** The application of human parstatin on CAM of chick embryo in vivo angiogenesis model resulted in a significant inhibition of the basal level of angiogenesis (Fig. 1A). The antiangiogenic effect of parstatin was dose-dependent and more pronounced when angiogenesis was stimulated by bFGF or VEGF. Mouse parstatin also inhibited vessel formation in the CAM model but to a lesser extent compared with human parstatin (Fig. 1A). The application of short and scrambled parstatins, at a concentration similar to that of human or mouse parstatins (10 nmol), did not result in any significant effect on angiogenesis (Fig. 1A). These data demonstrate that parstatin peptides are effective across species (i.e., human and mouse parstatin on chicken) and that there are sequence- and dose-specific effects of both human and mouse parstatin peptides on vascular growth in the CAM model.

In rat aortic ring assay, parstatin inhibited microvessel formation in a dose-dependent manner, with complete inhibition at a concentration of 10 μM (Fig. 1B). This inhibitory effect was evident either in basal conditions or in VEGF- or bFGF-induced angiogenesis. Similarly to the CAM assay, the ability of parstatin to function across species is evident. Human parstatin effectively inhibited angiogenesis in rat tissue in a dose-dependent manner.

**Parstatin Inhibits Capillary Tube-Like Formation by Endothelial Cells.** The formation of capillary-like structures by endothelial cells is a multistep process involving cell adhesion, migration, and differentiation and can be enhanced in vitro by use of Matrigel or fibrin clots to coat plastic culture dishes. When human parstatin was tested on the Matrigel model, it exhibited a significant inhibitory effect on the rate and extent of tube formation (Fig. 2A). At concentrations ranging from 0.3 to 10 μM, parstatin treatment resulted in a dose-dependent inhibition of tube formation by endothelial cells cultured in medium containing 5% serum. Similar results were evident in fibrin in the in vitro angiogenesis model, where endothelial cells were cultured in a sandwich mode between two fibrin gels and formed three-dimensional capillary-like tubes (Fig. 2B). The total capillary tube length induced by VEGF and bFGF was significantly
reduced by parstatin. Control scrambled parstatin and short parstatin did not affect the ability of endothelial cells to form capillary-like networks in both models (Fig. 2, A and B). Exposure of endothelial cells to mouse parstatin resulted in a less pronounced, but still significant, inhibitory effect (Fig. 2, A and B). In agreement with these findings, parstatin also attenuated cell migration through a microporous membrane in response to serum (Fig. 2C). Endothelial cells readily migrate toward 5% FBS. However, when human parstatin was added to the medium, the number of migrated cells was significantly reduced in a concentration-dependent manner.

**Parstatin Inhibits Growth of Endothelial Cells.** To investigate the effect of parstatin on endothelial cell proliferation, HUVECs were grown in medium containing 5% FBS for 72 h. On each of 3 consecutive days, cell growth was determined indirectly by MTT assay. As shown in Fig. 3A, endothelial cell number doubled every 18 to 26 h over the 72-h period. In the presence of parstatin, the rate of endothelial cell growth was significantly decreased. HUVEC proliferation was essentially blocked by parstatin at a concentration of 10 μM. This inhibitory effect of parstatin was dose-dependent, with an IC_{50} of approximately 3 μM. Similar results were obtained when cell growth was stimulated by VEGF or bFGF, with an IC_{50} of 1 μM (data not shown). Mouse parstatin was less effective inhibiting cell proliferation, with an IC_{50} of approximately 20 μM, whereas scrambled parstatin and short parstatin were without effect (data not shown).

Likewise, parstatin reduced DNA synthesis in HUVECs in a dose-dependent manner, with the inhibitory effect on bFGF- or VEGF-stimulated DNA synthesis to be more pronounced than that measured in the presence of serum (Fig. 3B). These data suggest that parstatin has a more potent effect on stimulated dividing cells than quiescent cells. When
DNA synthesis inhibition was also evident after short exposure of parstatin on cell culture was not necessary because of cell cycle arrest and apoptosis. As shown in Fig. 4, treatment of endothelial cells with parstatin inhibited Erk1/2 signaling. Pretreatment of endothelial cells with parstatin for 1 h did not have any effect on basal levels of Erk1/2 (data not shown) but strongly inhibited the activation of Erk1/2 stimulated by FBS, bFGF, or VEGF (Fig. 4A). The inhibitory effect was concentration-dependent, and parstatin blocked the bFGF-induced Erk1/2 phosphorylation levels from a concentration of 3 μM (Fig. 4B). This inhibitory effect was also observed at shorter exposure times. At 10-min pretreatment of endothelial cells, the inhibition of Erk1/2 activation was approximately 50% of the maximum, indicating a time-dependent effect of parstatin (Fig. 4C). In addition, the blockage of Erk1/2 phosphorylation by parstatin was found to be reversible (Fig. 4D). HUVECs exposed to human parstatin for 1 h, then washed free of parstatin, and subsequently incubated for a further 1 to 3 h in fresh medium, regaining the ability to respond to bFGF on the stimulation of Erk1/2. Scrambled parstatin did not alter the Erk1/2 activation, and mouse parstatin had a less pronounced effect compared with human parstatin at similar concentrations (Fig. 4C). It is interesting that the growth-inhibitory effect of parstatin was specific for bFGF or VEGF because parstatin did not have any effect on EGF or HB-EGF-induced DNA synthesis (Fig. 3C) and Erk1/2 activation (Fig. 4E).

**Growth Inhibition of Endothelial Cells Is Associated with Induction of Apoptosis.** Flow cytometric cell cycle analysis was performed to determine whether results of cell growth were a reflection of cytostatic or cytotoxic effects because of cell cycle arrest and apoptosis. As shown in Fig. 5A, parstatin increased the sub-G0/G1 cell fraction (subdiploid region on the DNA content histogram), which represents the percentage of apoptotic cells. Parstatin also increased the percentages of endothelial cells in early and late apoptotic stages. In parallel, the percentage of early and late apoptotic cells was measured by the annexin V/propidium iodide-based assay, which is a valuable and very sensitive technique to detect apoptosis (van Heerde et al., 2000). The data presented in Fig. 5B, revealed that parstatin increased the percentages of endothelial cells in the S and G2/M phases. Similar results were obtained when endothelial cells were stimulated by bFGF (Fig. 5A) or VEGF (data not shown). These data provide evidence that parstatin inhibits cell cycle progression triggered by major angiogenic agonists and leads to apoptosis of endothelial cells.

To further support this observation, we analyzed the pro-apoptotic effect of parstatin in endothelial cells with the annexin V/propidium iodide-based assay, which is a valuable and very sensitive technique to detect apoptosis (van Heerde et al., 2000). The data presented in Fig. 5B, revealed that parstatin increased the percentages of endothelial cells in early and late apoptotic stages. In parallel, the percentage of healthy/alive cells was equally decreased. Parstatin promoted cell apoptosis, with the effect being greater in endothelial cells stimulated by bFGF or VEGF (Fig. 5B). However, parstatin was without significant effect on EGF-treated cells.
The proapoptotic effect of parstatin was concentration-dependent and was reversed by caspase inhibitor Z-VAD-FMK (Fig. 5C), indicating that caspase activation was involved in parstatin-mediated apoptotic cell death.

When examining the effect of parstatin on caspase-3 activa-
Fig. 5. Promotion of endothelial cell apoptosis. A, HUVECs were incubated in medium containing either 0.5% BSA or bFGF (5 ng/ml) in the absence or in the presence of human parstatin (parst, 10 μM) for 24 h. Harvested cells were stained with propidium iodide and analyzed with a flow cytometer. Results are expressed as the mean percentage of cell population in sub-G0/G1, G1, S, and G2/M phases of the cell cycle ± S.E. Experiments were run in duplicate and repeated three times. Statistical analysis was performed between indicated groups. *, p < 0.05; **, p < 0.01. B, HUVECs were incubated in medium containing 0.5% BSA or VEGF (10 ng/ml) or bFGF (5 ng/ml) in the presence of vehicle or human parstatin (parst, 10 μM) for 24 h. C, HUVECs were incubated in medium containing 0.5% BSA in the presence of vehicle (C) or indicated concentrations of human parstatin (parst) or caspase inhibitor Z-VAD-FMK (100 μM) or the indicated combination for 24 h. Cells were fixed, stained with annexin V-FITC and PI, and analyzed for healthy cells (annexin V- and PI-negative), early apoptotic cells (annexin V-positive and PI-negative), and late apoptotic or dead cells (annexin V- and PI-positive) by flow cytometry. The corresponding percentages of stained cells are shown in representative dot plots (B) or expressed as mean percentage of cell population ± S.E. (C). Statistical analysis was performed in early apoptotic cell population versus control. *, p < 0.05; **, p < 0.01.
tion in cultured endothelial cells, it increased caspase-3 activity in a concentration-dependent manner (Fig. 6A). As expected, bFGF alone reduced the activity of caspase-3, whereas the presence of parstatin resulted in a dramatic increase in caspase-3 activity. The combination of parstatin with Z-VAD-FMK blocked the stimulatory effect of parstatin, suggesting its specificity (Fig. 6B). In addition, the caspase-promoting activity of parstatin was observed as early as 3 h after the exposure of cell to parstatin (data not shown). Mouse parstatin caused a moderate increase in caspase-3 activity, and scrambled parstatin was without effect (Fig. 6B). Furthermore, parstatin promoted PARP cleavage to its signature 85-kDa fragment in a concentration-dependent manner (Fig. 6C). PARP cleavage by caspases produces 85- and 24-kDa fragments from the full-length 116-kDa protein, which leads to its inactivation and constitutes an early event in apoptosis. Parstatin also increased PARP cleavage in bFGF-stimulated endothelial cells. Together, these results suggest that parstatin promoted apoptosis in growing endothelial cells and provide strong evidence that the cytotoxicity observed was because of caspase activation.

Parstatin Is a Cell-Penetrating Peptide. Parstatin, because of highly hydrophobic properties of its first 23 amino acids, may possess the ability to interact with cell membrane lipid bilayers and to penetrate inside the cells. To investigate whether parstatin exerts its cellular effects as a cell-permeable peptide, human parstatin was conjugated with FITC, and its uptake into the endothelial cells was assessed by flow cytometry. As shown in Fig. 7A, the fraction of FITC-positive cell population exposed to parstatin-FITC for 30 min was increased in a dose-dependent manner. In addition, the uptake kinetics of parstatin into the cells suggested a nonsaturable, nonreceptor-mediated uptake (Fig. 7B). Even at the shortest time of exposure to parstatin (10 μM) studied, 1 min, the FITC-positive cell population was 13.4% and reached the maximal level after 30 min of treatment. For imaging, endothelial cells were incubated with FITC-parstatin, and the distribution was observed with fluorescent microscopy. As shown in Fig. 7C, a positive FITC signal (green) was detected as early as 5 min of cell exposure to parstatin-FITC. At this time point, parstatin signal was exclusively localized in cell membranes. When endothelial cells were exposed to parstatin-FITC for 10 min, the FITC signal was detected in cell membranes and in the cytosol. The exposure of cells for 30 min resulted in signal localization only in the cytosol, preferentially around the nucleus (Fig. 7C). These data suggest that parstatin possesses the ability to interact with cell membranes and enter cells at a rate dependent on the exposure time and the concentration applied. It is interesting that this kinetic profile was in agreement with the initiation of parstatin-mediated biological effects (e.g., the inhibition of bFGF-induced MAPK activation in Fig. 4C).

As a control peptide, we used the short parstatin conjugated with FITC, which lacks the hydrophobic domain of parstatin. When endothelial cells were exposed to short parstatin, no fluorescence was observed (Fig. 7C), and the FITC-positive cell population was minimal and comparable with that of control cells (Fig. 7A). The inability of short parstatin to interact with and penetrate cell membranes provides a plausible explanation for the absence of cellular effects discussed above. However, the exposure of endothelial cells to FITC-labeled modulated parstatin, which is scrambled at residues 24 to 41, resulted in a marked increase of the fraction of FITC-positive like that observed with FITC-parstatin (Fig. 7A). In accordance, the microscope images presenting a similar time distribution of the FITC signal as that observed in parstatin-treated cells (Fig. 7C). These results are in perfect agreement and associated with the ability of modulated parstatin to inhibit bFGF-induced Erk1/2 activation and DNA synthesis (Fig. 7D). The peptide corresponding to residues 1 to 23 was not feasible to be investigated because it formed aggregates because of its highly hydrophobic nature. However, the existence of the scrambled sequence

![Fig. 6. Activation of caspase activity in endothelial cells. A and B, caspase-3 activity was determined in cell extracts of HUVECs cultured in medium containing 0.5% BSA in the presence of vehicle or indicated concentrations of human parstatin (parst) or bFGF (5 ng/ml) or Z-VAD-FMK (Z-VAD, 100 μM) or mouse parstatin (mouse, 10 μM) or scrambled parstatin (scr, 10 μM) or the indicated combination for 24 h. Results are expressed as mean of optical density at 405 nm (OD405) ± S.E. Experiments were run in triplicate and repeated three times. Statistical analysis was performed versus controls or between indicated groups. * p < 0.05; ** p < 0.01. C, HUVECs were cultured in medium containing either 0.5% BSA or bFGF (5 ng/ml) in the presence of vehicle or indicated concentrations of human parstatin (parst) for 24 h. Protein lysates were immunoblotted with anti-PARP monoclonal antibody. Total protein levels were determined by probing membranes with α-tubulin antibody. Representative membrane blot is presented.](https://jpet.aspetjournals.org/article-pdf/10.1124/jpet.117.246320/jpet.117.246320.htm)
in modulated parstatin prevented this phenomenon and renders the peptide active. In addition, when we added a random-sequenced octapeptide in addition to the hydrophobic region, this peptide was also active (data not shown). These results suggest that the inhibitory sequence of parstatin is localized within its hydrophobic domain, and this moiety needs to be framed with additional amino acids to facilitate its solubility and activity.

**Discussion**

In the present study, we have shown that parstatin, a synthetic peptide that corresponds to the cleaved peptide on PAR1 activation, inhibits basal and bFGF- and VEGF-induced endothelial cell proliferation and cell cycle progression and induces endothelial cell apoptosis. These effects can explain the ability of parstatin to suppress angiogenesis both in vivo and in vitro. The only reported functions for PAR1-cleaved peptide (parstatin) come from studies in platelets (Furman et al., 1998; Claytor et al., 2003). According to these studies, parstatin can act as platelet agonist and promote platelet-endothelial cell adhesion (Claytor et al., 2003). However, many of these effects were evident only in washed platelets and in absence of serum. This was attributed to the presence of a potential endogenous inhibitor of parstatin in the plasma. In our experiments, however, parstatin was active in vivo in the chick embryo model and inhibited endothelial cell functions.
either in the absence or in the presence of serum. Experiments in which parstatin was incubated with human serum for several time periods did not result in peptide degradation, suggesting that parstatin is quite stable in physiological fluids (data not shown). It also has been shown that the cleaved peptide of PAR1 induced marked decrease in the platelet surface binding of a panel of GPIb-IX-V-specific monoclonal antibodies because of the redistribution of the platelet sur-

Fig. 7. (continued) C, fluorescence microscopy images. HUVECs were incubated with 10 mM FITC-labeled (green) parstatin (parst-FITC) or modulated parstatin (mod-parst-FITC) or short parstatin (shrt-parst-FITC) for the indicated time intervals. All cells were also stained with the nuclear dye DAPI (blue). Arrows show the FITC signal localization on cell membrane or in cytosol. Experiments were run in triplicate and repeated three times. D, modulated parstatin mimics parstatin in inhibition of MAPK activation and DNA synthesis in endothelial cells. For MAPK activation (left), HUVECs were pretreated with parst (10 \mu M) or short parstatin (shrt-parst, 10 \mu M) or modulated parstatin (mod-parst, 10 \mu M) for 1 h and then stimulated with bFGF (5 ng/ml) for 10 min. Cells were then processed as described in Fig. 4. Representative blots are shown. For DNA synthesis (right), HUVECs were incubated in serum-free medium supplemented with bFGF (5 ng/ml) in the absence or in the presence of parst (10 \mu M) or short parstatin (shrt, 10 \mu M) or modulated parstatin (mod, 10 \mu M) for 18 h. All cells were pulsed with [3H]thymidine for an additional 6 h. All experiments were run in triplicate and were repeated at least three times. Results are expressed as mean ± S.E. of DPM per well and presented as percentage change of control (0%). Statistical analysis was performed between indicated groups. **, p < 0.01.
face GPIb-IX-V complex to the surface-connected canalicular system (Furman et al., 2000). This effect suggests that parstatin negatively regulates the functional roles of GPIbα, including the negative regulation of the GPIbα binding sites for von Willebrand factor and thrombin.

Parstatin possesses a highly hydrophobic domain (MG-PRRLLLVAACSFSLCPLLSAR-) that may represent a putative signal sequence (Vu et al., 1991). Bioinformatic analysis indicates that PAR1 has a functional signal peptide, belonging to the small subgroup of G protein-coupled receptors (5–10%) that possess putative N-terminal signal peptides. Signal peptides have been shown to facilitate export of many proteins across eukaryotic endoplasmic reticulum and are believed to be cleaved off after mediating the endoplasmic reticulum targeting/insertion process (Kochl et al., 2002). However, this may not always be the case. It was reported recently that corticotrophin-releasing factor receptor type 2a did not possess a conventional cleaved signal peptide (Rutz et al., 2006). Instead, it contained a signal peptide that it was uncleaved and formed part of the mature receptor protein. It was also shown that a glycosylated asparagine in the signal sequence played a negative role in signal peptide cleavage. It is of interest that parstatin contains an asparagine (Asn35) within a consensus glycosylation site that may prevent proteolysis of signal sequence. In addition, evidence that parstatin may be released from thrombin-activated platelets has also been reported (Ramachandran et al., 1997; Furman et al., 2000). Using specific antibodies against the PAR1 N-terminal domain, it has been suggested that at least residues 19 to 41 are expressed on the platelet surface. Nevertheless, expression experiments of PAR1 in mammalian cell systems are in progress to address in detail the precise length of the peptide cleaved upon activation of PAR1.

The physiological significance of angiogenesis inhibition by parstatin is a critical point and is currently under investigation. In normal conditions, where most blood vessels remain quiescent, and angiogenesis is limited, the number of PAR1 molecules expressed in the vascular system is limited and probably not enough to provide sufficient concentration levels of parstatin for endothelial cell suppression and induction of apoptosis (Hirano, 2007). However, when angiogenesis is initiated (i.e., at sites of wound healing and repair, inflammation, and tumor growth), endothelial cells and many other recruited cell types are activated, and the expression of PAR1 is up-regulated (Nelken et al., 1992; Even-Ram et al., 1998; Ellis et al., 1999; Darmoul et al., 2003; Kaushal et al., 2006; Leger et al., 2006). In the microenvironment of robust angiogenesis, a plethora of cells, which overexpress PAR1 (i.e., platelet, macrophages, mural cells, tumor cells), are temporally and spatially coordinated. Under these conditions, the local concentration of parstatin may be high enough for negative regulation of the angiogenic process and endothelial cell functions. Furthermore, because angiogenesis seems to be under the control of additional negative regulatory factors (Nyberg et al., 2005), which also represent cleavage fragments of bigger molecules, it is possible that parstatin cooperates and acts in synergy with such locally released angiogenesis inhibitors.

The precise molecular mechanism by which parstatin exhibits its effect in angiogenesis and on endothelial cell functions is under investigation. Several points have been considered in the present study. 1) The inhibition of parstatin on endothelial cell growth was not because of the result of a nonspecific cytotoxic mechanism. Parstatin blocked in a reversible fashion the MAPK phosphorylation in endothelial cells induced by serum, bFGF, and VEGF, but not by EGF or HB-EGF. 2) The inhibitory effect of parstatin was evident after a short time of exposure of endothelial cells. This makes it unlikely that new protein synthesis is involved in this process. 3) The inhibitory effect of parstatin on endothelial cell growth was more evident in fast-growing endothelial cells. Therefore, it is anticipated that parstatin will not have a significant effect on the normally quiescent endothelial cells but rather on the activated cells involved in angiogenesis.

Furthermore, the present study provides evidence that parstatin crosses the plasma membrane to reach its molecular targets. Some signal peptides, because of their highly hydrophobic properties, possess the ability to interact with the cell membrane lipid bilayers and penetrate inside the cells (Lin et al., 1995). This observation was further supported by the fact that short parstatin, which lacks first 23-amino acid hydrophobic domain, was without effect in angiogenesis and endothelial cell functions. This suggests a crucial functional role for the hydrophobic domain of parstatin. The modulated parstatin, which has intact the hydrophobic sequence but scrambled the residues 24 to 41, mimicked the cellular effects of human parstatin. In addition, mouse parstatin, which also exerted significant inhibitory effects, possesses greater identity (78%) with human parstatin in the hydrophobic domain than in the remaining hydrophilic domain (44%). The mechanism by which parstatin crosses the plasma membrane is unknown. Over the past years, two noncompeting mechanisms have been proposed for the cell entry of peptides (Vives et al., 2008). One is direct translocation through the plasma membrane by the peptide-induced reorganization of the membrane following several possible structural alterations. A second mechanism involves a form of endocytosis by which the peptides gets localized into late endosomes from where it may eventually leak out partially toward the cytoplasm. However, we cannot rule out the possibility that parstatin produces its antiangiogenic effect by competing with the proangiogenic growth factors, bFGF and VEGF, for binding to low-affinity heparin sulfate coreceptors and thereby attenuating the ability of growth factors to bind their high-affinity endothelial cell protein receptors and produce their mitogenic effects (Rapraeger et al., 1991; Yayon et al., 1991).

Angiogenesis is a complex biological process where the contribution of numerous regulatory factors probably depends on the microenvironment at the site of tissue and the pathophysiology involved. We propose that thrombin, through its multiple cellular actions related to angiogenesis, may play a pivotol role by orchestrating the actions of many factors in the regulation of angiogenesis. In analogy to the role of thrombin in hemostasis, where it can be both prothrombotic and antithrombotic, the role of thrombin in angiogenesis can be proangiogenic and antiangiogenic. Parstatin, the cryptic peptide generated by thrombin, may represent an important negative regulator of angiogenesis, with possible therapeutic applications.

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References


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