Aminopeptidase A is a functional target in angiogenic blood vessels

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Summary

We show that a membrane-associated protease, aminopeptidase A (APA), is upregulated and enzymatically active in blood vessels of human tumors. To gain mechanistic insight, we evaluated angiogenesis in APA null mice. We found that, although these mice develop normally, they fail to mount the expected angiogenic response to hypoxia or growth factors. We then isolated peptide inhibitors of APA from a peptide library and show that they specifically bind to and inhibit APA, suppress migration and proliferation of endothelial cells, inhibit angiogenesis, and home to tumor blood vessels. Finally, we successfully treated tumor-bearing mice with APA binding peptides or anti-APA blocking monoclonal antibodies. These data show that APA is a regulator of blood vessel formation, and can serve as a functional vascular target.

Introduction

The realization that angiogenic vasculature is a target for intervention in cancer has long led to an interest in endothelial cell receptors associated with tumor blood vessels and their corresponding ligands. However, so far, only a few such targets have been identified.

We developed a selection system in which circulating ligands that home to specific vascular beds in vivo are isolated from a phage display random peptide library; this approach led to the identification of vascular receptors allowing for systemic targeting of blood vessels in mice and more recently in humans (Arap et al., 2002; Pasqualini and Ruoslahti, 1996). Some of the targets in tumor blood vessels turned out to be membrane-bound proteinases such as matrix metalloproteinases (Koivunen et al., 1999) or aminopeptidases (Pasqualini et al., 2000) that are upregulated in tumor blood vessels. While the biological relevance of the selective expression of proteinases within the angiogenic tumor blood vessel is not entirely understood, one can exploit such a feature to gain control of angiogenesis.

Aminopeptidase A (glutamyl-aminopeptidase, EC 3.4.11.7, APA) is a homodimeric type II membrane-spanning cell surface

SIGNIFICANCE

Angiogenesis is a complex multistep process that can occur in tumors or in response to various stimuli. Many lines of evidence indicate that tumor growth and angiogenesis depend on proteolytic activity. Angiogenic blood vessels express biochemical markers that are differentially expressed and whose functional importance has just begun to be uncovered. While only a few markers associated with angiogenic blood vessels have thus far been reported, it is remarkable that several are cell membrane-associated proteinases. By using genetic elimination and biochemical inhibition, our results demonstrate a novel mechanistic role for APA in pathological angiogenesis. Thus, specific APA inhibitors—such as peptidomimetics or anti-APA antibodies—may prove useful for translational applications targeting tumor vasculature.

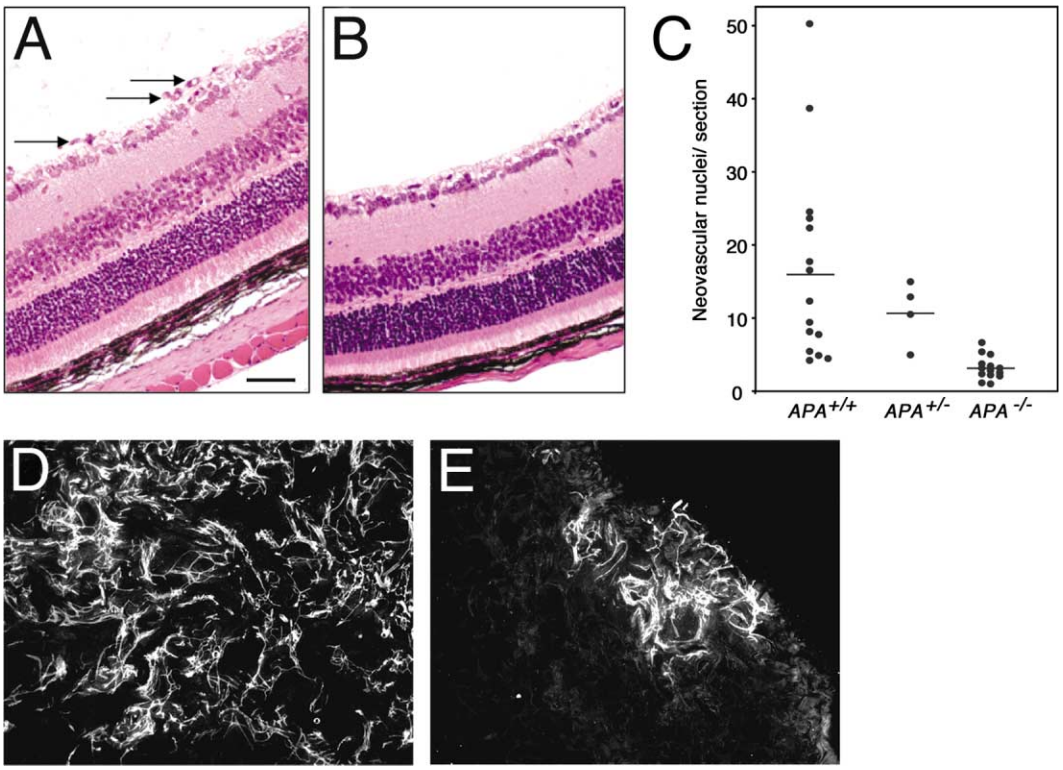

Figure 1. APA-deficient mice have an impaired angiogenesis phenotype

$APA^{-/-}$, $APA^{+/-}$, and $APA^{+/+}$ mouse pups were exposed to 75% oxygen (from P7 to P12) and eyes were enucleated on P19. Paraffin sections were stained with H&E.

A and B: A large number of new blood vessels are seen protruding from the retina into the vitreous space in wild-type ($APA^{+/+}$) eyes (**A**) (arrows point to endothelial cell nuclei), while no neovasculature is present in $APA^{-/-}$ eyes (**B**). Scale bar: 50 μm.

C: The number of neovascular nuclei protruding into the vitreous space was quantified by evaluating several serial sections of multiple eyes. Each circle represents the mean of at least ten eye sections. A horizontal bar represents the mean of the group.

D and E: Gelfoam sponges impregnated with VEGF, bFGF, and TGF-α were implanted in the subcutaneous tissue of $APA^{-/-}$ and $APA^{+/+}$ mice. Sponges were removed 14 days later and sections were stained with anti-CD31 antibody to visualize the endothelial cells. A large number of endothelial cells (2.55 ± 0.08 area units) are seen protruding to the sponges in $APA^{+/+}$ mice (**D**) while the number of new blood vessels is smaller (0.73 ± 0.01 area units, $p < 0.001$) in the sponge implanted in $APA^{-/-}$ mice (**E**). Scale bar: 250 μm.

protein with zinc metallopeptidase activity that hydrolyzes N-terminal glutamyl or aspartyl residues from oligopeptide substrates (Nanus et al., 1993; Wu et al., 1990). Surprisingly, despite widespread tissue distribution (Li et al., 1993), APA null mice develop normally (Lin et al., 1998). Since upregulation of APA has been reported in perivascular cells (termed "pericytes") of tumor blood vessels (Alliot et al., 1999; Schlingemann et al., 1996), we set out to identify and analyze the effects of targeted ligands on the possible role of this enzyme in angiogenesis.

To evaluate whether an APA ligand-receptor system would allow functional targeting of tumor blood vessels, we designed experiments to assess both partners of the protein-protein interaction. We show that (1) APA is strongly upregulated in angiogenic tumor blood vessels but barely detectable in normal blood vessels, (2) the enzymatic activity of APA colocalizes to its expression pattern in human tumors, and finally (3) APA null mice have an impaired angiogenesis phenotype that has not been previously characterized. To gain insight into the functional role of APA in angiogenesis, we used a phage display library to select APA binding peptides and to isolate a novel inhibitory ligand. We show that the consensus motif CPRECESIC (1) specifically binds to APA and inhibits its enzymatic activity, (2) suppresses migration and proliferation of endothelial cells, (3) inhibits in vitro and in vivo angiogenesis, (4) homes to tumor vasculature in vivo, and (5) inhibits tumor growth in vivo. These genetic and biochemical data show that APA is a functional target in tumor blood vessels.

Results

$APA^{-/-}$ mice show impaired angiogenesis

We have evaluated the angiogenic response of $APA^{-/-}$ mice in two models of angiogenesis. First, we used a mouse model of O_2-induced retinopathy (Smith et al., 1994). APA wild-type ($APA^{+/+}$), APA heterozygotes ($APA^{+/-}$), and APA null ($APA^{-/-}$) mice were exposed to 75% O_2 from postnatal day 7 (P7) to P12, and then returned to room air. Retinas were analyzed at P17, when neovascularization had supervened on the vitreal surface. Angiogenesis was quantified by counting endothelial cell nuclei protruding into the vitreous space (Lahdenranta et al., 2001; Smith et al., 1994). Induction of retinal angiogenesis (16.1 ± 3.3 endothelial cell nuclei/section) was seen in wild-type mice on P17 (Figure 1A), but significantly fewer endothelial cell nuclei (3.0 ± 0.4 endothelial cell nuclei/section, t test, $p <$

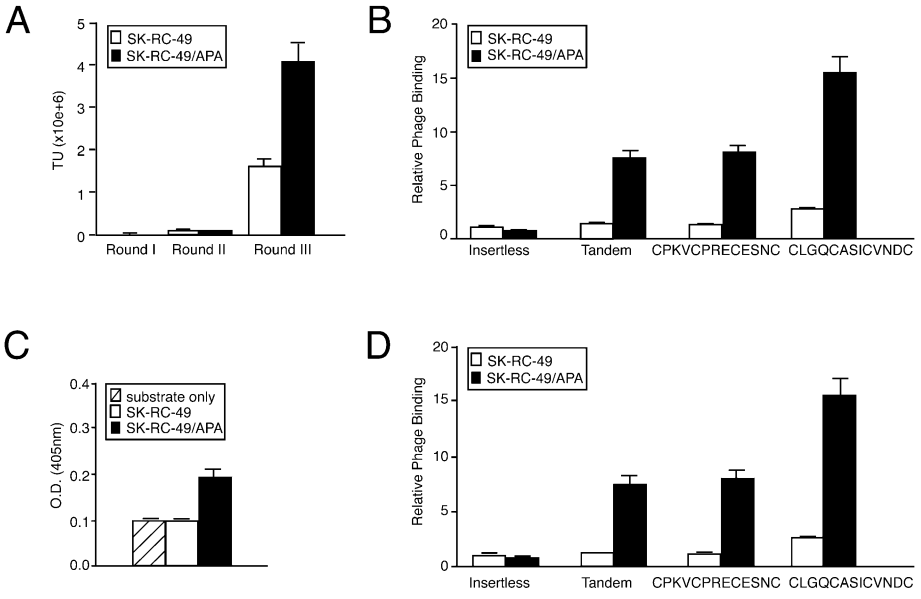

Figure 2. Selection and identification of APA binding peptides

A: A phage peptide library was preabsorbed on APA negative SK-RC-49 cell line and incubated with stably APA-transfected SK-RC-49 cells. Cell-bound phage were recovered by infection and used for further rounds of panning on SK-RC-49/APA cells. By the third round of selection, we observed strong enrichment in phage binding to APA-transfected SK-RC-49 cells. Shown are mean ± standard error of the means (SEM) from triplicate samples.

B: Binding of the selected phage clones to SK-RC-49 /APA cells was compared to binding to SK-RC-49 cells. Insertless fd-tet phage was used as a negative control. The designation tandem refers to the peptide sequence CYNLCIRECESIC GADGACWTWCADGCSRSC (see text). Phage binding to the cells is shown relative to the negative control phage binding to the parental SK-RC-49 cells (value was set to be 1). Shown are mean ± SEM from triplicate samples.

C: APA was immobilized on RC38-coated microwells from SK-RC-49/APA cell lysates, and APA enzyme activity was evaluated with the specific substrate α-glutamyl-p-nitroanilide by measuring absorbance. RC38-immobilized SK-RC-49 cell lysate showed no activity above background from the substrate alone. Shown are mean ± SEM from triplicate samples.

D: Binding of the selected phage clones to RC38-immunocaptured APA was compared to the binding to RC38-immunocaptured SK-RC-49 cell lysate. The individual wells were then incubated with equal amounts of each individual phage. Insertless fd-tet phage was used as a negative control for the phage binding. Phage binding to immunocaptured APA is shown relative to the negative control phage binding to immunocaptured SK-RC-49 cell lysate (value was set to be 1). Shown are mean ± SEM from triplicate samples.

0.001) were found in retinas from *APA*$^{-/-}$ mice (Figure 1B). An intermediate decrease in retinal angiogenesis was observed in the *APA*$^{+/-}$ mice (10.1 ± 2.2 endothelial cell nuclei/section), suggesting dose dependence (Figure 1C). No endothelial cell nuclei protruding into the vitreous space were found in mice exposed only to room air regardless of their genotype or time of analysis.

As a second model, we adopted a quantitative in vivo angiogenesis assay utilizing implanted gelfoam sponges saturated with angiogenic growth factors VEGF, bFGF, and TGF-α (McCarty et al., 2002). To compare angiogenesis induction from subcutaneous tissue, sponges were implanted into the flanks of wild-type (*APA*$^{+/+}$) or APA null mice (*APA*$^{-/-}$). After two weeks, the sponges were harvested and stained with anti-CD31 antibodies to detect the endothelial cells of newly formed blood vessels permeating the sponge. Strong induction of CD31-positive capillary structure formation was observed in the growth factor-absorbed sponges implanted into wild-type mice (Figure 1D), whereas the number of CD31-positive capillary structures was much smaller in *APA*$^{-/-}$ mice and the only limited capillary formation observed was observed at the outer surface of the sponge (Figure 1E).

These results suggest a deficient angiogenic response in *APA*$^{-/-}$ mice to relative hypoxia or to angiogenic growth factors as compared with their otherwise isogenic *APA*$^{+/+}$ counterparts.

Selection and identification of APA binding peptide motifs

To identify peptides capable of binding to APA, we screened APA-transfected cells with a phage display library (Smith and Scott, 1993). We stably transfected SK-RC-49 renal carcinoma cells with a vector expressing full-length APA cDNA. We verified that APA was functional in transfected cells by an enzyme activity assay specific for APA. Parental SK-RC-49 cells showed neither APA expression nor activity. An increase in phage binding to SK-RC-49/APA cells relative to SK-RC-49 cells was observed in the third round of selection (Figure 2A). DNA sequencing revealed an enrichment of the sequence CYNLCIRECESIC-GADGA-CWTWCADGCSRSC containing tandem repeats of the general library sequence $CX_3CX_3CX_3C$ on each side of the pIII peptide linker GADGA sequence. 50% of randomly selected phage clones displayed such tandem repeat after the second round and 100% displayed it after the third round (Table 1).

Validation of APA-specific phage ligand binding

Phage displaying enriched peptide motifs were tested individually for APA binding. Phage displaying the inserts CPKVCPRECE SNC, CYNLCIRECESICGADGACWTWCADGCSRSC, or CLGQ CASICVNDC preferentially bound to SK-RC-49/APA cells relative to SK-RC-49 cells; control insertless phage (fd-tet) showed no binding preference (Figure 2B). We synthesized pep-

Table 1. APA binding peptide sequences

Peptide sequences	Round 2 (%)	Round 3 (%)
CYNLCI**RECESIC**GADGACWTWCADGCSRSC	50	100
CPKVCP**RECES**NC	5	–
CLGQCA**SIC**VNDC	5	–
CGTG**CAVECE**VVC	5	–
CAVACWADCQLGC	5	–
CSGLCTVQ**CLEGC**	5	–
CSMM**CLEGC**DDWC	5	–
Other	20	–

tides containing APA binding motifs (consensus sequence CPRECESIC; Table 1). Binding of phage displaying CPKVCPRE CESNC or CYNLCIRECESICGADGACWTWCADGCSRSC peptides to SK-RC-49/APA cells was specific because it was inhibited by the synthetic peptide; several negative control peptides had no inhibitory effect. Binding of CLGQCASICVNDC phage to SK-RC-49/APA cells was not affected by the synthetic peptide, suggesting that the SIC sequence in the consensus motif is not critical for binding. We also evaluated whether the selected peptides would bind to the isolated APA protein. We immunocaptured APA from SK-RC-49/APA lysate with the RC38 antibody and showed that the immunocaptured APA remained enzymatically active (Figure 2C). We used phage displaying CPKVCPRECESNC, CLGQCASICVNDC, or CYNLCIRECESIC GADGACWTWCADGCSRSC in binding assays to the immunocaptured APA. We found up to 12-fold enrichment of phage binding to APA compared to immunocaptured cell lysates from the SK-RC-49 cells; control insertless phage (fd-tet) showed no binding preference (Figure 2D).

These data support the specific binding of the selected peptides to isolated functional APA enzyme and to cell membrane-bound APA in vitro.

CPRECESIC is an inhibitor of APA enzymatic activity

To evaluate the effect of synthetic CPRECESIC peptide on APA enzyme activity, we incubated APA-transfected cells with the APA-specific substrate α-glutamyl-p-nitroanilide with increasing concentrations of APA binding or control peptides. The APA binding peptide inhibited APA enzyme activity (colorimetric assay), while the control peptide had no effect (Figure 3A). We calculated the IC_{50} of the peptide for APA enzyme inhibition to be $\sim$800 μM. The inhibition was specific to APA because the peptide did not affect the enzymatic activity of aminopeptidase N, a related protein with homology to APA (Nanus et al., 1993; Wu et al., 1990).

CPRECESIC suppresses endothelial cell function

Because of the enzymatic inhibitory activity of APA binding peptides, we reasoned that they might affect endothelial cell function. We correlated the effect of APA inhibition by the CPRECESIC peptide on the migration and proliferation of endothelial cells. We used human dermal microvascular endothelial cells (HMEC) in which the APA expression was verified by immunostaining with RC38 and the APA activity by its specific enzyme assay. CPRECESIC peptide suppressed the migration of HMECs in the Boyden chamber assay (Figure 3B). Suppression in cell migration was dose-dependent and commensurate with inhibition of APA enzymatic activity. Up to 60% suppression in cell migration was observed at 1 mM of peptide, but significant inhibition was detected at concentrations as low as 100 μM (Figure 3B). Similarly, proliferation of HMECs was suppressed by CPRECESIC ($\sim$40% at 1 mM) in a dose-dependent manner (Figure 3C). Inhibition of cell proliferation was also observed at 0.3 mM, but lower peptide concentrations had no significant effect. A negative control peptide affected neither cell migration nor proliferation.

These data indicate that an APA binding peptide functions as a specific inhibitor of enzymatic activity and that inhibition of APA enzymatic activity is associated with suppression of migration and proliferation in human endothelial cells in vitro.

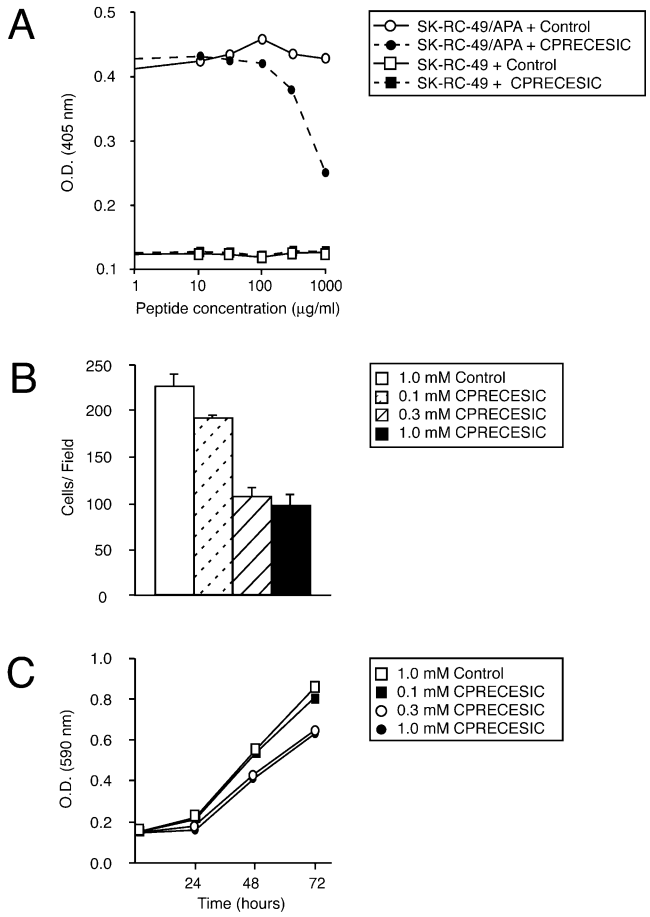

Figure 3. CPRECESIC is a specific inhibitor of APA activity and blocks endothelial cell migration and proliferation

A: APA enzymatic activity in SK-RC-49/APA cells was measured in the presence of increasing concentrations of either targeting or control peptide. SK-RC-49 cells served as a negative control.

B: HMECs were stimulated with complete M199 culture medium. Migration assay was performed in a Boyden microchemotaxis chamber. Cells were allowed to migrate through an 8 μm pore filter for 5 hr at 37°C in the presence of increasing concentrations of CPRECESIC or control peptides. Cells migrating through the membrane were stained and five high-power fields for each microwell were counted. Shown are means ± SEM from triplicate wells.

C: HMECs were stimulated with complete M199 culture medium in the presence of increasing concentrations of APA binding or control peptides. Cell proliferation was measured at 24, 48, and 72 hr by a colorimetric assay based on crystal violet staining.

CPRECESIC inhibits capillary tube formation and angiogenesis

To determine whether inhibition of APA enzyme activity with CPRECESIC peptide would affect angiogenesis, we tested the effect of peptide in vitro and in vivo. First, we evaluated the ability of HMECs to differentiate into capillary-like structures characteristic of angiogenesis in the presence of CPRECESIC on a gel of reconstituted basement membrane matrix (Matrigel). The formation of cord/tube-like structures was progressively impaired by increasing concentrations of CPRECESIC peptide, relative to the network formation in controls without peptide (Figure 4A, upper left panel) or with an unrelated control peptide at 1 mM concentration (Figure 4A, upper right panel). The num-

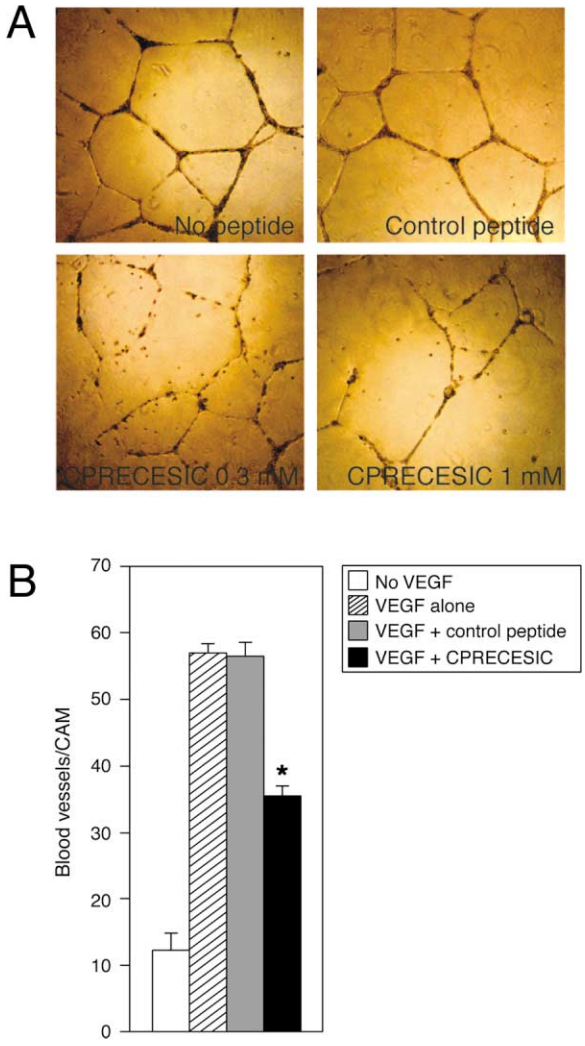

Figure 4. Inhibition of angiogenesis by CPRECESIC

A: HMECs were plated on Matrigel in the presence of increasing concentrations of APA binding or control peptides and photographed after 24 hr (magnification: 40×).

B: Eight-day-old chicken egg CAMs were implanted with PBS- or VEGF-adsorbed sponges. CPRECESIC or control peptides were tested at 1 mM concentration in the VEGF-adsorbed sponge. At day 12, CAMs were evaluated and the number of neovessels quantified. There were significantly fewer blood vessels in the CAMs treated with APA binding peptides (*t test, $p < 0.01$). Shown are means ± SEM from triplicate samples.

ber and length of capillary-like branching structures was reduced at 0.3 mM CPRECESIC peptide (Figure 4A, lower left panel), whereas cell interconnections and the capillary network organization were severely lost at a peptide concentration of 1 mM (Figure 4A, lower right panel).

Next, we tested CPRECESIC activity in a CAM assay. In this in vivo model, neovascularization is stimulated with VEGF adsorbed on to a gelatin sponge placed on the CAM. CAMs were stimulated on embryonic day 8 (E8) with sponges containing VEGF only, VEGF plus CPRECESIC, or VEGF plus control peptides. CAMs were examined on E12 and neovascularization was quantified by counting the number of emerging capillaries (Figure 4B). Significant induction of neovascularization (57.0 ± 1.41 blood vessels) was found when a sponge with VEGF was placed on the CAM compared to a sponge with no growth factors (12.0 ± 2.8 blood vessels; t test, $p < 0.01$). CPRECESIC peptide (1 mM) inhibited VEGF-induced CAM neovascularization by 40% (35.5 ± 1.4 blood vessels; t test, $p < 0.01$). Equimolar concentrations of a negative control peptide (56.5 ± 2.12 blood vessels; t test, $p < 0.01$, compared to the active CPECE SIC peptide) or lower CPRECESIC peptide concentrations (0.1 mM and 0.3 mM) did not affect the number or branching of the growing blood vessels (data not shown).

These data show that the CPRECESIC motif inhibits blood vessel formation in standard models of angiogenesis.

APA expressed in blood vessels of human tumors is active

The extensive proliferation of blood vessels in malignant gliomas suggests an early role of angiogenesis in brain tumors (Wesseling et al., 1995). To investigate the expression of APA in brain tumor vasculature, we compared the localization of APA expression by immunostaining to the localization of APA activity by enzyme histochemistry in a panel ($n = 14$) of primary and metastatic human brain tumors and in the incidental normal tissue adjacent to the tumors. APA enzyme activity in tissue sections colocalized with APA expression detected by an RC38 antibody. The staining pattern was consistent with APA localization to perivascular cells in the tumor microvasculature (Figures 5A and 5B, glioblastoma multiforme; 5C and 5D, squamous cell carcinoma of the lung metastatic to the brain). Staining and enzymatic activity were apparent in sites of glomerular vascular proliferation in malignant gliomas and metastatic carcinomas. In contrast, APA expression and activity were barely detectable in capillaries in normal brain parenchyma (data not shown). By double staining with RC38 and with PAL-E antibody, a general endothelial marker (Schlingemann et al., 1985), APA expression in colon adenocarcinoma metastatic to the brain appears to be restricted to perivascular cells (Figure 5E). We also showed by immunostaining that blood vessels in human nonmalignant granulation tissue express APA (Figure 5F). Finally, we localized APA staining to perivascular cells by immuno-electron microscopy (Figure 5G).

These results show that the APA present in blood vessels during the progression of malignant tumors is enzymatically active and therefore may contribute to the angiogenic process associated with human brain tumors.

APA binding phage targets tumor vasculature

We next evaluated the ability of APA binding peptides to home to tumor blood vessels in vivo. Phage were administered intravenously to nude mice bearing human breast carcinoma MDA-MB-435-derived xenografts. Phage homing was quantified by recovery from tissue homogenates by bacterial infection. CPKVCPRECESNC phage were enriched in tumors compared to control organs; in contrast, negative control phage (without a peptide insert) did not enrich in tumors (Figure 6A). Homing of CPKVCPRECESNC phage to tumor vasculature was confirmed by immunohistochemical staining of phage on tissue sections. Strong phage staining was observed in tumor blood vessels—but not in normal brain blood vessels—of mice that received CPKVCPRECESNC phage; in contrast, negative control phage without insert did not home to tumor blood vessels

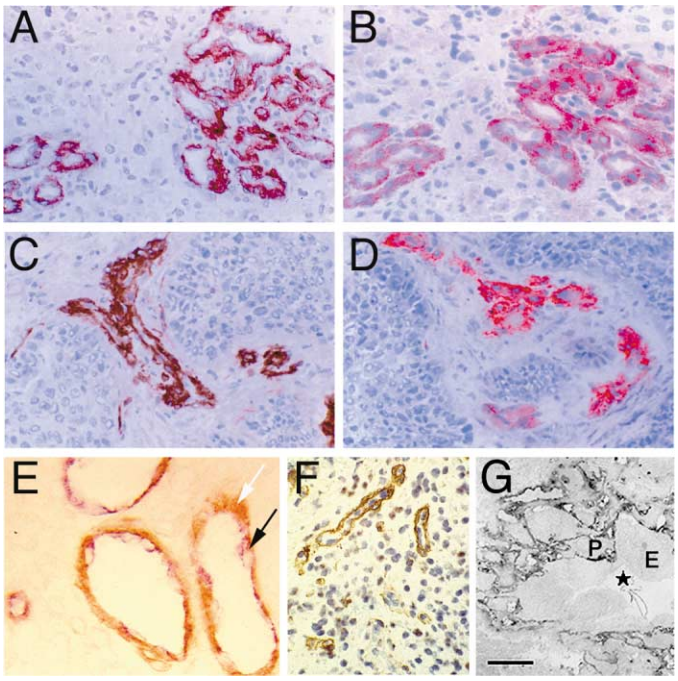

Figure 5. APA expression and activity in human brain tumor blood vessels

A–E: RC38 immunohistochemistry (**A** and **C**) and APA enzyme activity (**B** and **D**) of an area of glomeruloid vascular proliferation in glioblastoma multiforme (**A** and **B**) and in brain metastases from squamous cell carcinoma of the lung (**C** and **D**). Note the similar distribution of reaction products in both primary (**A** and **B**) and metastatic tumors (**C** and **D**), and the absence of staining of endothelial cells lining the vascular lumina, findings consistent with a localization of APA in the perivascular cells of these microvessels. Scale bar: 50 μm.

E: Double staining of human adenocarcinoma metastasis to the brain with anti-APA antibody (RC38) and the antiendothelial cell antibody (PAL-E). RC38 was detected by using DAB as the substrate producing brown staining of perivascular cells (white arrow), and PAL-E was detected by using 4-chloro-1-naphtol as the substrate, producing blue-gray staining of endothelial cells (black arrow). Note the absence of the RC38 staining in PAL-E positive cells. Scale bar: 50 μm.

F: RC38 immunohistochemistry of an area of human granulation tissue. Scale bar: 50 μm.

G: RC38 immuno-electron microscopy of an area of glomeruloid vascular proliferation in human glioblastoma multiforme. Perivascular cells (P) are positive for RC38 staining, whereas endothelial cells (E) are negative. Asterisk indicates the lumen. Scale bar: 5 μm.

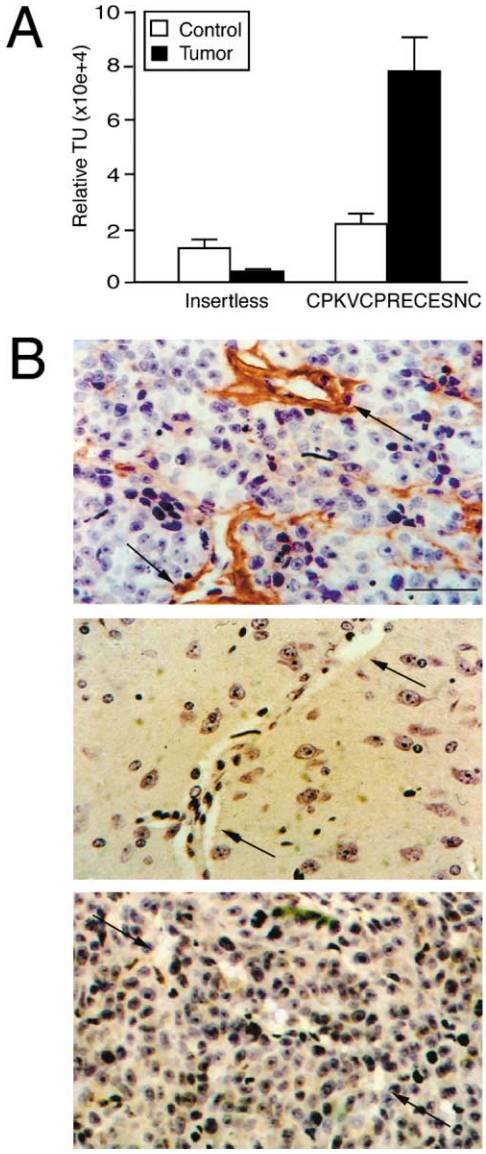

Figure 6. CPKVCPRECESNC-displaying phage targets tumor vasculature

A: The ability of the APA binding phage to home to tumors was evaluated after intravenous administration into mice bearing human MDA-MB-435 breast carcinoma-derived tumor xenografts. Phage were recovered from tumor and control tissues after perfusion. Shown are mean ± SEM of TU from triplicate platings. Brain was used as a control tissue.

B: Phage displaying the peptide CPKVCPRECESNC or insertless negative control phage were injected intravenously into MDA-MB-435-derived tumor-bearing mice. An anti-phage antibody was used for staining. Arrows indicate blood vessels. The upper panel shows the tumor from the mouse that received the CPKVCPRECESNC phage; the middle panel shows the brain from the same mouse; the lower panel shows the tumor from the mouse that received the insertless control phage. Scale bar: 70 μm.

(Figure 6B). These results show that APA binding phage target tumor blood vessels in vivo.

APA binding peptides or anti-APA antibodies can suppress tumor growth

To evaluate whether APA inhibitory ligands (APA binding peptides or anti-APA blocking antibodies) suppress tumor growth in vivo, we used EF43-*fgf4*-derived tumors, an established mouse mammary carcinoma model (Deroanne et al., 1997, Hajitou et al., 2001). We have chosen this model because APA expression is undetectable in EF43-*fgf4* cells but strongly induced in the tumor blood vessels associated with EF43-*fgf4*-derived tumors (Figure 7A) and homing of APA binding phage to tumor vasculature was experimentally validated (data not shown). Tumor-bearing mice received vehicle, CPRECESIC, or control peptides, and tumors were monitored (Figure 7B). We observed differences in tumor growth as early as 5 days after treatment initiation, and when the experiment was terminated at the end of two weeks, CPRECESIC-treated mice had significantly smaller tumors (1,366 ± 190 mm^3) relative to tumor-bearing control mice that received control peptide (2,626 ± 335 mm^3; $p = 0.006$); tumors in mice treated by control peptide behaved similarly to those tumors in mice receiving vehicle alone (2,295 ±

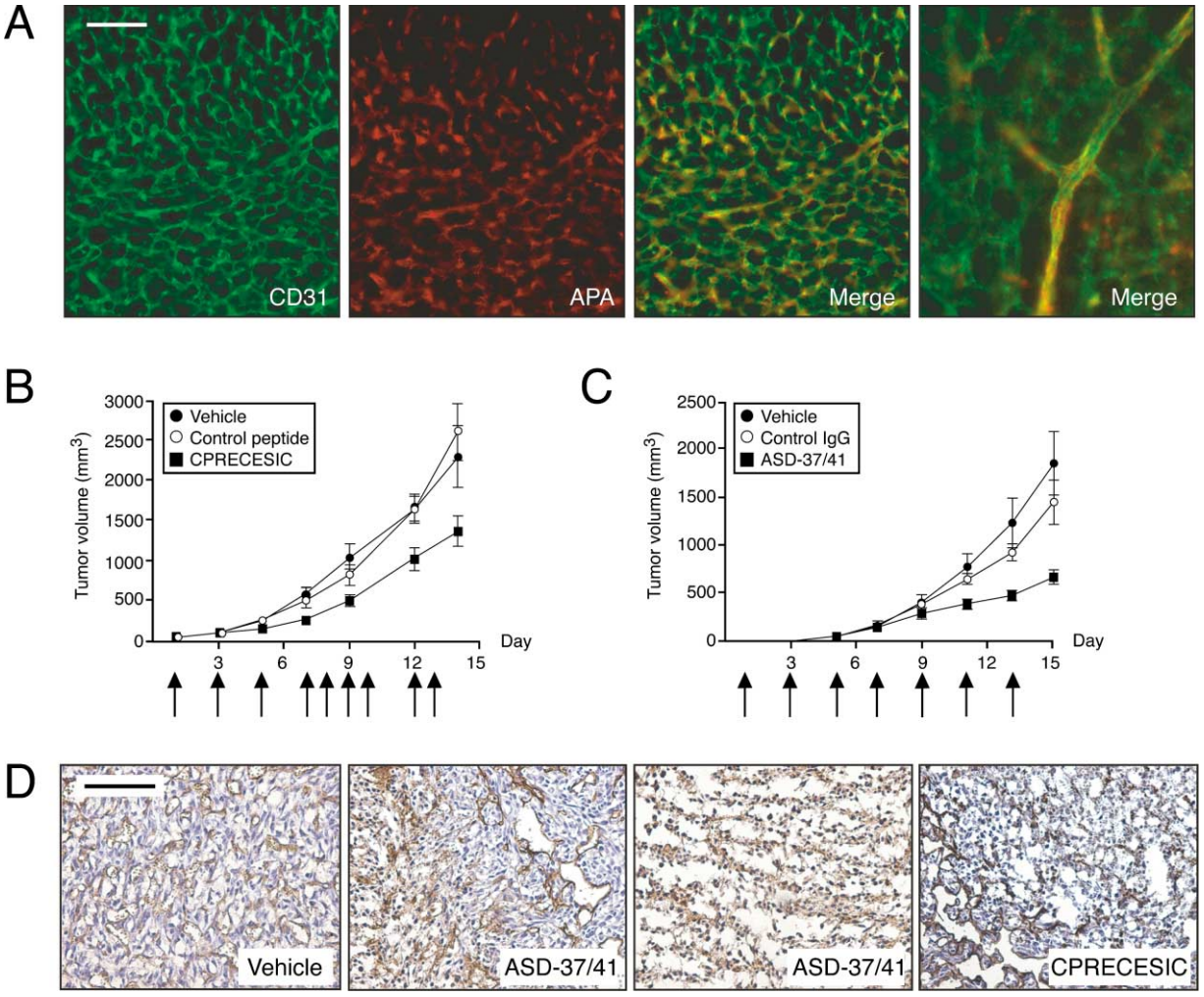

Figure 7. Treatment of tumor-bearing mice with APA binding peptides or anti-APA blocking monoclonal antibodies can suppress tumor growth

A: Immunofluorescence staining of EF43-*fgf4*-derived tumors shows APA expression on tumor blood vessels. Frozen tumor sections were stained for CD31 and APA. CD31 was detected with a FITC-conjugated secondary antibody (green), and APA was detected with a Cy3-conjugated secondary antibody (red); merged images indicate that APA expression is vascular and not detectable on EF43-*fgf4* cells. Scale bar: 125 μm (or 250 μm, far right panel).

B: Balb/c mice bearing EF43-*fgf4*-derived tumors were divided in 3 size-matched cohorts (n = 7 mice per group) that received vehicle (DMEM), CPRECESIC peptide, or control (CARAC) peptide. Peptides were administered at 250 μg/mouse/dose (indicated by arrows). Shown are mean tumor volumes ± SEM.

C: Balb/c mice bearing EF43-*fgf4*-derived tumors were divided in 3 size-matched cohorts (n = 7 mice per group) that received vehicle (DMEM), ASD-37/41 antibodies, or isotype control (rat IgG). Antibodies were administered at 200 μg/mouse/dose (indicated by arrows). Shown are mean tumor volumes ± SEM.

D: After treatments (**B** and **C**) were terminated at two weeks, tumor sections were immunostained for CD31 and counterstained by hematoxylin. Representative histopathological panels are shown as indicated. Relatively to controls (far left panel), ASD-37/41-treated tumors (middle panels) contained codominant areas of disrupted vascular structure next to islands of viable tissue (middle left panel) as well as large areas of tumor destruction with extensive cell death (middle right panel); inhibitory peptides also had an evident effect on treated tumors (far right panel). Scale bar: 100 μm.

381 mm^3; $p = 0.53$), indicating that the control peptide had no measurable effect. Of note, another synthetic APA binding peptide (sequence CPRECESN) also showed efficacy in vivo (data not shown).

However, because the APA-inhibitory peptides may require a relatively high molar range (~250 nanomoles/mouse/dose) to be effective, we also evaluated the effects of anti-APA antibodies on tumor growth. We used an established regimen of two anti-APA monoclonal antibodies (ASD-37/41) with synergistic APA inhibitory properties (Assmann et al., 1992, Mentzel et al., 1999) on the same animal model. Tumor-bearing mice received vehicle, ASD-37/41, or isotype control IgG, and tumors were monitored (Figure 7C). Again, we noticed clear differences in tumor growth. By the end of two weeks when the experiment was terminated, ASD-37/41-treated mice had significantly smaller tumor volumes (663 ± 77 mm^3) relative to tumor-bearing control mice that received isotype negative control IgG (1,442 ± 231 mm^3, $p = 0.013$); tumors in mice treated by control IgG behaved similarly to those tumors in mice receiving vehicle only (1,845 ± 313 mm^3, $p = 0.38$), indicating that control IgG had no detectable effect on tumor growth. Finally, to analyze post-treatment effects of APA-inhibitory antibodies in tumors and their angiogenic blood vessels, we examined tissue sections of all groups by CD31 immunostaining (Figure 7D). In contrast

to the recognized (Deroanne et al., 1997; Hajitou et al., 2001) extensive blood vessel network typically observed in EF43-*fgf4*-derived tumors, ASD-37/41-treated tumors exhibited a mixture of viable tissue (toward the outer rim of the tumors) and neighboring regions of disrupted vascular structure along with large areas of widespread cell death and destruction of the tumor architecture; inhibitory peptides had similar but less pronounced effect on treated tumors (Figure 7D).

Taken together, these results indicate that APA-inhibitory ligands can specifically target angiogenic vasculature and suppress tumor growth in vivo.

Discussion

Several approaches indicate that the vascular endothelium of angiogenic blood vessels expresses surface markers that are accessible from the circulation in tumors but are undetectable, inactive, or inaccessible in normal blood vessels. Such cell markers include growth factor receptors, cell adhesion molecules, proteoglycans, and proteases. Membrane-associated proteases such as gelatinases and aminopeptidases have been found to be molecular targets in tumor blood vessels (Koivunen et al., 1999; Pasqualini et al., 2000).

Here we set out to determine the role of APA as a functional target for inhibition of angiogenesis. We predicted that genetic elimination of APA or biochemical inhibition of its enzymatic activity would lead to insights into mechanisms of abnormal blood vessel formation. We also reasoned that identification of a specific APA ligand might yield a desirable targeting tool, given the high expression and activity of this protease in the newly formed blood vessels of human tumors.

Knockout $APA^{-/-}$ mice develop normally (Lin et al., 1998), but our study shows a severely impaired angiogenic response to oxygen-induced retinopathy in a mouse model of retinopathy of prematurity (Smith et al., 1994). They also failed, compared to $APA^{+/+}$ mice, to sprout new blood vessels into subcutaneously implanted angiogenic factor-containing sponges (McCarty et al., 2002). The fact that $APA^{-/-}$ mice develop normally with no gross phenotypic abnormalities suggests that while APA may participate in embryonic angiogenesis, it is not an essential participant. The molecular events associated with de novo formation of blood vessels ("vasculogenesis") during early development may be redundant or compensated by other proteases. Our data indicate a functional role for APA in the formation of new blood vessels from a preformed vascular bed ("angiogenesis"). These data strengthen the candidacy of APA as a specific vascular target to inhibit abnormal angiogenesis associated with tumor formation and retinal neovascularization.

In addition to probing the two mouse models in $APA^{-/-}$ mice, we screened a combinatorial peptide library to gain insights into the role of APA activity in angiogenesis. Given the molecular diversity of the vasculature (St. Croix et al., 2000; Arap et al. 2002), we reasoned that this approach would yield probes to study APA function in endothelium-derived cells, and possibly ligands to target tumor blood vessels in vivo. We selected APA-biding phage displaying the sequences CYNLCIRECESICGAD GACWTWCADGCSRSC, CPKVCPRECESNC, and CLGQCAS ICVNDC. The first two phage clones bound specifically to APA and their binding was specifically inhibited by the cognate synthetic CPRECESIC motif. Binding of the CLGQCASICVNDC phage to APA was not inhibited by CPRECESIC peptide, possibly due to the short sequence shared with the other isolated peptides leading to a low-affinity interaction; this result suggests that the sequence CPRECES alone is sufficient to promote inhibition. Functionally, the peptide CPRECES is the dominant sequence of the motif, since another APA binding peptide (sequence CPRECESN) was effective in vitro and also yielded therapeutic effects in vivo (data not shown). Because a search of human databases for sequences homologous to the selected peptides did not yield matches consistent with potential APA ligands, the biological substrate responsible for APA inhibition by the consensus motif CPRECESIC may remain unknown until the three-dimensional structure of the interaction of ligand peptides and the APA active site has been solved.

To define the functional basis for the role of APA in blood vessel formation, we used in vitro and in vivo angiogenesis assays. Because our evaluation of APA enzyme activity in freshly isolated endothelial cells led to the observation of high enzyme activity in HMECs and in some tumor-derived endothelial cells (our unpublished data), we used HMECs in our in vitro angiogenesis assays. We found that the synthetic CPRECESIC peptide inhibited VEGF-induced migration and proliferation of HMECs. CPRECESIC peptide also inhibits cord/tube formation of microvascular endothelial cells in a Matrigel assay and angiogenesis in VEGF-stimulated CAMs. These results show (1) that the consensus motif CPRECESIC is an inhibitor of APA enzymatic activity and a suppressor of migration and proliferation of endothelial cells and (2) that inhibition of APA enzymatic activity leads to a dose-dependent decrease in cord/tube formation during angiogenesis.

APA appears to be one of the many molecules that have been shown to play different roles, according to the organ and time period examined. A broad spectrum of tissues expresses APA (Li et al., 1993), but its only well understood role is the conversion of angiotensin II to angiotensin III in the renin-angiotensin system (Jackson, 2001). In vivo, angiotensin II remains the sole characterized substrate for APA. While angiotensins are usually investigated in relation to mechanisms of arterial hypertension, several studies suggest a role for angiotensins in blood vessel formation (Le Noble et al., 1996; Monton et al., 1998; Nadal et al., 2002; Walsh et al., 1997). Furthermore, inhibition of angiotensin-I-converting enzyme suppresses angiogenesis (Volpert et al., 1996; Yoshiji et al., 2001). While APA is strongly expressed by angiogenic microvessels (Schlingemann et al., 1996), we have confirmed that it is undetectable or barely detectable in normal vasculature. The established involvement of angiotensins in angiogenesis referenced above and the overexpression of APA in angiogenic blood vessels suggest a mechanism for APA in neovascularization. As predicted by our hypothesis that APA is a functional target for molecular agents that can inhibit angiogenesis, we observed a decreased neovascularization in $APA^{-/-}$ mice following induction of angiogenesis by hypoxia and by growth factors and reduced tumor growth in mice treated with APA inhibitors. Our data are reminiscent of those showing a stimulatory effect of angiotensins on endothelial cell migration (Kifor and Dzau, 1987) and proliferation (Monton et al., 1998). Also, angiotensin receptors are expressed in the CAM, suggesting that angiotensins might play a role in angiogenesis and capillary branching (Le Noble et al., 1996). Our results, however, could also reflect a more general effect of APA on angiogenic mechanisms such as a role in degradation of extracellular matrix by processing other unknown substrates

(Sang, 1998). Natural and synthetic inhibitors of other metalloproteases inhibit not only in vivo angiogenesis (Egeblad and Werb, 2002; Koivunen et al., 1999; Pasqualini et al., 2000; Sang, 1998), but also migration and proliferation of microvascular cells in vitro (Murphy et al., 1993; Zempo et al., 1996). Also, knockout mice in which other metalloproteases have been eliminated (such as MMP-2 or -9) show a defect in angiogenesis (reviewed in Egeblad and Werb, 2002).

In addition to mouse models, we evaluated whether abnormal angiogenesis associated with certain human diseases is related to APA in the vasculature. We examined expression and activity of APA in malignant gliomas and metastatic carcinomas to the brain. We showed not only that APA is present in angiogenic blood vessels of human malignant gliomas, but also that the protein overexpressed in the perivascular cells is enzymatically active. In contrast, the presence of the enzyme and its corresponding proteolytic activity were undetectable in the blood vessels and adjacent normal brain. Pericytes, which were once seen merely as the contractile microvessel equivalent of smooth muscle cells surrounding larger blood vessels, also play an active role in neovascularization and maturation, remodeling, and maintenance of the vascular system (Morikawa et al., 2002; Schlingemann et al., 1991). Similarly to endothelial cells, perivascular cells also exhibit molecular, functional, and structural heterogeneity (Morikawa et al., 2002), of which APA overexpression in tumor blood vessels is one example. APA may play a significant role in several functions of perivascular cells, such as secretion of growth factors, modulation of the extracellular matrix, and regulation of vascular permeability.

The overexpression of APA in activated blood vessels has been associated with perivascular cells in human tumors here and elsewhere (Schlingemann et al., 1996), but the exact cellular location of active APA in the vascular endothelium of blood vessels during tumorigenesis is still not entirely clear. A further level of complexity arises because tumor cells per se express APA in several human cancers (Fujimura et al., 2000; Geng et al., 1998; Ino et al., 2000), making it difficult to differentiate the origin of APA enzymatic activity. In this study, we show that APA expression and activity are strongly increased in tumor blood vessels during late stages of human malignant glioma progression. We have also observed that endothelial cells derived from mature macrovessels like human umbilical vein endothelial cells express little or no APA, while HMECs, extracted from small, immature blood vessels, show much higher APA expression (unpublished data). One might speculate that this process mimics an angiogenic switch (Hanahan and Folkman, 1996) in which endothelial cells are recruited to form new capillaries for extensive tumor growth. Once tumor blood vessels mature to the stage at which they are covered with perivascular cells, APA expression is reduced in the endothelial cells. In turn, activated perivascular cells covering more mature tumor blood vessels express high levels of APA. Due to its specific expression and accessibility to tumor blood vessels, APA in the cell membranes of activated endothelial cells or perivascular cells can be systemically targeted, which makes it a suitable candidate receptor for targeted imaging or therapy. Openings between defective endothelial cells of tumor blood vessels (Hashizume et al., 2000) enable access to the perivascular cell layer of the vasculature; other cell surface receptors of activated perivascular cells (such as the proteoglycan NG2) have been targeted by in vivo phage display (Burg et al., 1999). Combinatorial strategies targeting both the endothelial and perivascular cell compartments are likely to provide improved efficacy for antiangiogenic therapies in multiple stages of tumorigenesis (Bergers et al., 2003).

Given that the selected APA binding phage home to tumor vasculature, and the consensus motif deduced from APA binding peptides specifically inhibits APA enzymatic activity, we evaluated whether APA binding peptides can be used as targeted inhibitory carriers. Although APA is also expressed in many cell types in normal tissues (Alliot et al., 1999; Li et al., 1993), our results show that APA is exposed, active, and available for binding to circulating ligands from the luminal side of the vascular endothelium because APA binding phage selectively target tumor vasculature. Based on the phage recovery from tissues and phage immunohistochemistry, we showed that the CPKVCPRECESNC phage targets angiogenic blood vessels in human breast carcinoma-derived tumor xenografts.

Finally, after documenting the expression of the target in the tumor vasculature, in a mouse model of mammary carcinoma (Deroanne et al., 1997, Hajitou et al., 2001), we successfully targeted this tumor with the same APA binding phage and have chosen this model for therapy experiments. We showed that APA-inhibitory ligands—such as the APA binding peptides described here or anti-APA monoclonal antibodies (Assmann et al. 1992)—specifically target angiogenic vasculature and can suppress tumor growth in vivo in this experimental system. We determined the IC_{50} of the ASD-37 monoclonal antibody for the APA enzyme activity and found it to be $\sim$60 nM (data not shown). The apparently superior inhibitory ability of anti-APA monoclonal antibodies is commensurate with, and one likely explanation for, the more evident histopathological findings observed after treatment with such antibodies relative to our APA binding peptides. Considering these promising preclinical results, and the fact that antibody-related toxicity occurred only when mice received over 20-fold the doses that yielded the antitumor activity observed here (Assmann et al. 1992, Mentzel et al., 1999), anti-APA inhibitory antibodies should be at least considered as candidates for drug development and, possibly, translation into clinical applications.

In sum, our results reveal a new role for APA and its targeted ligands in angiogenesis. The interaction between APA and its ligands has a functional role in abnormal angiogenesis. We show that APA binding peptides inhibit its proteolytic activity and affect endothelial cell functions and angiogenesis. The ligand motif described here may therefore serve as a peptidomimetic drug lead against angiogenic mechanisms. Taken together, this study shows that APA is a specific vascular target to be critically evaluated in translational clinical trials against pathologic angiogenesis.

Experimental procedures

Reagents

VEGF-A_{165}, bFGF, and TGF-α were from R&D Systems (Minneapolis, MN) and anti-CD31 antibody from BD-Pharmingen (San Diego, CA). RC38 (Schlingemann et al., 1996), ASD-37, ASD-41 (Assmann et al., 1992), and PAL-E antibody (Schlingemann et al., 1985) have been described. Synthetic peptides were purchased from AnaSpec (San Jose, CA). Unless otherwise indicated, an unrelated synthetic peptide (sequence GACVRLSACGA) was used as a negative control. A phage display random peptide library displaying the insert $CX_3CX_3CX_3C$ (C, cysteine; X, any amino acid residue) was constructed as described (Smith and Scott, 1993).

Cell culture

Renal carcinoma cells SK-RC-49 (Ebert et al., 1990) were stably transfected with a mammalian expression vector encoding the full-length APA cDNA (Geng et al., 1998) and HMECs stably transfected with large T (Ades et al., 1992). EF43-*fgf4* cells have been described (Deroanne et al., 1997, Hajitou et al., 2001).

Animals

Institutional animal care and utilization committees approved animal experiments. This study adhered to the Association for Research in Vision and Ophthalmology (ARVO) guidelines. The $APA^{-/-}$ strain of inbred C57BL/6 mice has been described (Lin et al., 1998). Nude mice and Balb/c mice were obtained from Jackson Laboratory (Bar Harbor, Maine).

Mouse model of retinal neovascularization

P7 mouse pups were exposed to 75% oxygen for 5 days. Mice were returned to room air (20.8% O_2) on P12 (Smith et al., 1994). For histological analysis, mice were sacrificed on P19, eyes were enucleated, fixed, serially sectioned, and stained with hematoxylin and eosin (H&E). Endothelial cell nuclei on the vitreous side of the internal limiting membrane were counted. At least 10 H&E-stained sections were evaluated per eye, and the average number of nuclei was counted from 4–16 eyes for each condition.

In vivo angiogenesis assay

We used an angiogenesis assay in which gelfoam sponges (Pharmacia & Upjohn, Kalamazoo, MI) impregnated with VEGF, bFGF, and TGF-α (200 ng/ml each) are implanted in vivo as described (McCarty et al., 2002). Sponges were implanted into the subcutaneous tissue of homozygous $APA^{+/+}$ or $APA^{-/-}$ mice. After 14 days, anesthetized mice were perfused with 4% paraformaldehyde (PAF). Sponges were fixed with 4% PAF, frozen, and sectioned at 50 μm. Sections were stained with an anti-CD31 antibody and a Cy^3-conjugated secondary antibody. Quantification of CD31+ cells was determined (McCarty et al., 2002).

Cell surface panning

We preadsorbed 10^{10} transducing units (TU) of a $CX_3CX_3CX_3C$ (C, cysteine; X, any amino acid residue) phage display random library on SK-RC-49 parental cells. Next, the precleared $CX_3CX_3CX_3C$ phage library ($\sim 10^{10}$ TU) was added to 10^6 detached APA-transfected SK-RC-49 cells in binding medium (20 mM HEPES, 2% FCS in DMEM). Cell panning was performed at 4°C to minimize postbinding events such as receptor-mediated internalization (Giordano et al., 2001). Cells were washed with binding medium and cell bound phage were recovered and amplified by infection of K91Kan *E. coli*. Serial dilutions were plated on Luria-Bertani (LB) agar plates with tetracycline and kanamycin. The number of TU was determined by bacterial colony counting.

APA immunocapture and enzymatic assay

Cells lysates were prepared in ice-cold 100 mM N-octyl-β-glucopyranoside in PBS. Microtiter wells were coated with 2 μg of RC38 and blocked with 3% BSA. Cell lysates (1 mg of protein/ml) were incubated on RC38-coated wells overnight at 4°C. Wells were washed with 0.1% Tween-20 in PBS. The assay for APA enzymatic activity with α-L-glutamyl-p-nitroanilide has been described (Lin et al., 1998). Activity was determined by measuring absorbance at 405 nm.

Binding assays on APA or on APA-transfected cells

Binding assays were performed on cells with 10^9 TU phage input. Increasing concentrations of synthetic CPRECESIC or control peptides were used to evaluate competitive inhibition of phage binding. APA was immunocaptured on microtiter wells with RC38 and incubated with 10^9 TU of phage in 3% BSA in PBS. Wells were washed with 1% BSA, 0.01% Tween-20 in PBS, and phage were recovered by infection of K91kan *E. coli*. Serial dilutions were plated on LB agar plates containing tetracycline and kanamycin. The number of TU was determined by bacterial colony counting.

Endothelial cell growth assay

HMECs were seeded ($\sim$10,000 cells/well) and allowed to attach for 24 hr in complete M199 medium (Sigma; St. Louis, MO). The indicated concentrations of CPRECESIC or 1 mM negative control peptide were added to the cells in complete M199 medium. HMECs were then serially fixed in 2.5% glutaraldehyde, stained with 0.1% crystal violet in 20% methanol, and solubilized in 10% acetic acid. Cell growth was evaluated by measuring absorbance at 590 nm. A calibration curve with linear correlation between absorbance and cell number (10^3–10^5 HMECs) was used for the assay.

Chemotaxic assay

Cell migration assays were performed in a 48-well Boyden chamber (NeuroProbe; Gaithersburg, MD) as described (Bussolino et al., 1995). Indicated concentrations of CPRECESIC peptide or negative control peptide (1 mM) were placed in the lower chamber compartment in complete M199 medium. Subconfluent HMECs were harvested and 1×10^5 cells were seeded in the upper chamber compartment in complete M199 medium. Cells were allowed to migrate for 5 hr at 37°C in a humidified 5% CO_2 atmosphere. Filter was removed and cells on the upper side were scraped off. Migrated cells were fixed and Giemsa stained.

Three-dimensional cell culture

Matrigel (BD Biosciences; Bedford, MA) was added to 48-well tissue culture plates and allowed to solidify at 37°C. HMECs ($\sim 10^4$ cells) were added to the wells and allowed to adhere to the Matrigel for 30 min at 37°C. Cell medium was replaced containing the indicated concentrations of CPRECESIC or a negative control peptide (both at 1 mM) in complete medium. Plates were examined and photographed after 16 hr.

Chick embryo chorioallantoic membrane assay

Fertilized chicken eggs were incubated in constant humidity at 37°C for 3 days. 2–3 ml of albumin was removed through a square opening to detach the developing CAM. The opening was sealed with a glass plate and the eggs were incubated for 5 days. 1 mm^3 gelfoam sponges were adsorbed with 20 ng of VEGF, or 20 ng of VEGF plus 1 mM CPRECESIC or negative control peptide, and implanted on top of the growing CAMs. CAMs were examined daily until day 12, photographed in ovo with a stereomicroscope and emerging capillaries were counted (Ribatti et al., 1997).

In situ enzyme histochemistry

Biopsy materials were snap frozen and serially sectioned at 5 μm. Acetone-fixed tissue sections were incubated at 37°C with 1.6 mM of the APA-specific substrate N-Glutamyl-4-methoxy-3-naphtylamide (Bachem; Bubendorf, Switzerland) and 1.1 mM Fast Blue B salt in 0.1 M Tris (pH 7.0) containing 2.5 mM $CaCl_2$. Tissue sections were fixed, stained with H&E, and examined with a light microscope.

Immunoperoxidase, immunofluorescence, and immuno-electron microscopy

Frozen sections of colon adenocarcinoma metastases to the brain were immunoperoxidase-stained with RC38 antibody by using diaminobenzidine (DAB) as substrate (brown staining product). Tissue sections were washed with 0.1 M Glycine-HCL (pH 3.0) to block peroxidase activity and then stained in a second round of immunoperoxidase staining with PAL-E antibody by using 4-chloro-1-naphtol as substrate (blue-gray staining product). 60 μm frozen sections of EF43-*fgf4* tumors were double stained with ASD-37 and anti-CD31 antibodies. ASD-37 staining was detected by Cy3-conjugated secondary antibody and CD31 staining was detected by FITC-conjugated secondary antibody.

Immuno-electron microscopy was performed for biopsy materials as described by Schlingemann et al. (1996).

Tumor homing in vivo and therapy experiments

Nude mice bearing tumor xenografts derived from MDA-MB-435 human breast cancer cells were generated as described (Arap et al., 1998). 10^9 TU of CPKVCPRECSNC-displaying phage or fd-tet phage were injected intravenously to anesthetized mice. Phage were allowed to circulate for 5 min and the animals were perfused through the left ventricle of the heart with DMEM. The tumor and control organs were dissected and equal amounts of tissue were homogenized. Homogenates were washed with ice-cold DMEM containing a protease inhibitor cocktail (Sigma; St. Louis, MO) and 0.1% BSA. Tissue-bound phage were recovered as described above the cell panning. Tissue samples from the injected animals were fixed, sectioned at 5 μm, and stained with an anti-M13 antibody.

For experimental therapy, Balb/c mice bearing EF43-*fgf4*-derived tumors were established and tumor volumes determined as described (Deroanne et al., 1997; Hajitou et al., 2001). Treatments in tumor-bearing mice were started 5 (peptides) to 7 days (antibodies) after cell inoculation (10^5 cells/mouse). Reagents were administered by ip and sc routes.

Statistical analysis

Student's t tests were used for statistical analysis.

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