Mutant Anthrax Toxin B Moiety (Protective Antigen) Inhibits Angiogenesis and Tumor Growth

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Abstract
Bacillus anthracis protective antigen (PA), the B subunit of the binary anthrax toxin, binds to the cellular receptors capillary morphogenesis gene 2 protein and tumor endothelial marker 8 with high affinity. Both receptors are expressed on endothelial cells during angiogenesis. We sought to determine whether one could inhibit angiogenesis by interfering with the binding of these receptors to their endogenous ligands. Here, we show that wild-type PA inhibits both vascular endothelial growth factor–induced and basic fibroblast growth factor–induced angiogenesis at moderate but statistically significant levels. Structure-activity studies identified a PA mutant that exhibited markedly enhanced inhibition of angiogenesis and also inhibited tumor growth in vivo. This mutant, PA(SSR), is unable to undergo normal cellular processing and, thus, remains bound to the surface receptor. Further mutation of PA(SSR) so that it does not bind to these cell surface receptors abolished its ability to inhibit angiogenesis. We conclude that high-affinity anthrax toxin receptor (ATR) ligands, such as PA and PA(SSR), are angiogenesis inhibitors and that ATRs are useful targets for antiangiogenic therapy. These results also suggest that endothelial cell-binding proteins from additional pathogens may inhibit angiogenesis and raise the question of the role of such inhibition in pathogenesis.

Introduction
Bacillus anthracis protective antigen (PA) is the 83-kDa B subunit of anthrax toxin. Anthrax toxicity is initiated by the binding of PA to cell surface receptors that include tumor endothelial marker 8 (TEM8) and capillary morphogenesis gene 2 protein (CMG2): refs. 1, 2). Following receptor binding, PA is proteolytically activated by a cell surface–associated furin-like protease (3, 4). Proteolysis removes a steric barrier in PA and prompts oligomerization to a heptameric species (5). PA oligomerization generates binding sites for the A subunits, lethal factor (LF) and edema factor (EF), and triggers endocytosis of the resulting complex (5). EF is a Ca2+, calmodulin-dependent adenylate cyclase, and LF is a mitogen-activated protein kinase kinase–selective protease. Any combination of three A subunit molecules can bind to the PA heptamer. After internalization, the low pH environment of the endocytic compartment triggers a conformational rearrangement in the toxin complex that allows translocation of A subunits into the cytosol where they act on their intracellular substrates (5). The activity of these enzymes is currently believed to be responsible for anthrax pathogenesis.

PA binds to two known cell surface receptor proteins, known as anthrax toxin receptor (ATR) 2 (or CMG2) and ATR1 (or TEM8; refs. 1, 2). The ATRs form their own subfamily of von Willebrand factor (vWF) domains, with 60% amino acid identity between them (2), compared with <25% for other human and mouse genes. Site-directed mutagenesis studies of PA have shown the importance of individual amino acid residues for receptor binding and activity (6–10). X-ray crystal structures are available for the CMG2 vWF domain in complex with individual PA molecules (11) and PA63 heptamer (12). The cocryystals show that CMG2 shares structural homology with other vWF domains (e.g., integrin α subunits) and binds PA in a manner that mimics the binding of integrins to their ligands (11, 13).

The exact biological roles of the anthrax receptors are incompletely understood. However, their likely function involves binding to, and signaling from, the extracellular matrix. CMG2 was initially identified as the product of capillary morphogenesis gene 2, a gene up-regulated in endothelial cells forming tubes in a collagen gel (14). CMG2 is a 54-kDa transmembrane protein whose intracellular domain has multiple signaling motifs (2, 14). CMG2 is expressed on endothelial cells in most tissues, with the exception of the brain, as well as on other cell types, such as monocytes (15, 16). Mutations in the protein result in juvenile hyaline fibromatosis and infantile systemic hyalinosis, underscoring its importance in cell-extracellular matrix interactions (16–18). CMG2 has been shown to bind specifically to extracellular matrix molecules in vitro, including collagen IV and fibronectin, suggesting that these may be natural ligands of the protein (14). The NH2-terminal vWF domain of CMG2 binds to PA in a divalent cation-dependent manner with an affinity as high as 170 pmol/L (19). This domain is highly conserved, with 94% amino acid identity between mouse and human, increasing to 96% in PA-binding residues. PA also binds to TEM8 (ATR1) in a divalent cation-dependent manner with high affinity (100 nmol/L). TEM8 was originally identified as a protein expressed on colon tumor endothelium but not on normal endothelial cells (20). It has since been shown to be expressed on fetal endothelial cells as well as angiogenic tumor endothelium (21). Overexpression of the protein and blocking of collagen-binding sites affect endothelial cell migration but not tube formation or proliferation (22), whereas knockout of the gene with hammerhead ribozymes inhibits both cell migration and tube formation (23). These data suggest that TEM8, like CMG2, may be involved in vascular growth. Like CMG2, TEM8 is a transmembrane protein with an NH2-terminal vWF domain. It is also highly conserved, with 99% amino acid identity between mouse and...
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Materials and Methods

Protein production. PA and PA mutants were produced recombinantly in Escherichia coli BL21 (DE3) Star using the pET22b expression system. Cultures were grown to an $A_{600}$ of 3 to 10 in a 5-L fermentor at 37°C using ECPM1 growth medium supplemented with carbenicillin (50 mg/L; ref. 19). The culture was induced with isopropyl-$\beta$-thiogalactopyranoside (0.8–1 mmol/L) for 3 to 4 h at 27°C to 30°C and cells grew to a final $A_{600}$ of 6 to 20. Periplasmic lysates were generated as described previously (26). The lysate was buffered with 20 mmol/L Tris-Cl (pH 8.5) and purified by Q-Sepharose anion-exchange chromatography (GE Healthcare) eluted by 0.8–1 mmol/L NaCl (pH 8.5) and by 0–250 mmol/L NaCl gradient using buffers A (20 mmol/L Tris-Cl, 1 mmol/L CaCl$_2$ (pH 8.5)) and B (A + 1 mol/L NaCl). Pure fractions (as determined by SDS-PAGE) were concentrated and then purified by SEC (Sephacryl 200) using buffer C (A + 150 mmol/L NaCl). Pure fractions following SEC (as determined by SDS-PAGE) were concentrated and stored at $-80°C$. Final protein concentrations were determined by spectrophotometry ($\varepsilon_{280}$ of 75,670 mol/L$^{-1}$ cm$^{-1}$).

Mouse corneal micropocket assay. The corneal micropocket assay was done as described (27) using pellets containing 90 ng of basic fibroblast growth factor (bFGF) or 160 ng of human carrier-free recombinant vascular endothelial growth factor (VEGF; R&D Systems) in C57BL/6J mice. The treated groups received daily or twice daily i.p injections for 5 (bFGF) or 6 (VEGF) consecutive days of protein in PBS. Treatment was started on the day of pellet implantation; control mice received only vehicle i.p. The area of vascular response was assessed on the 5th (bFGF) or 6th (VEGF) postoperative day using a slit lamp. Typically, 10 eyes per group were measured.

Proliferation assay. Human microvascular endothelial cells (Cambrex) were maintained in EGM-2 (Cambrex) according to the vendor’s instructions and used before passage 7. BCE cells (a kind gift of Dr. Judah Folkman, Children’s Hospital, Harvard Medical School, Boston, MA) were maintained on DMEM supplemented with 10% fetal bovine serum and 2 ng/ml bFGF. On day 0, proliferating cultures of BCE or HMVEC-d cells were seeded at $-19%$ confluence into 96-well plates. After attachment, medium was exchanged for medium containing 1 pmol/L to 1 pmol/L of the indicated protein. Cells were allowed to grow for 7 days and then quantitated using CyQUANT (Invitrogen) according to manufacturer’s directions. The degree of proliferation in culture was measured by comparing wells in each plate fixed in absolute ethanol on day 0 with experimental wells, with fold proliferation calculated by dividing CyQUANT fluorescence in experimental wells by that in day 0 wells. Groups were compared using Student’s t test, with Bonferroni correction where appropriate.

Migration assay. Human microvascular endothelial cells were maintained as above. Polycarbonate Transwell inserts, 6.5 mm diameter with 8.0 μm pores, were coated with fibronectin (BD Biosciences) and type I rat tail collagen (Upstate) or left uncoated. Cells were harvested and resuspended in EBM (Cambrex) containing 0.1% bovine serum albumin (Fisher Chemical). Cells (10,000 per well) were plated onto wells containing medium alone or medium containing the molecule to be tested. These wells were suspended above wells containing 5 to 10 ng/ml recombinant human VEGF (R&D Systems). Cells were allowed to migrate for 4 h. Membranes were rinsed once in PBS and then fixed and processed using Diff-Quick (Dade Diagnostics). Cells on the top of the membrane were removed using cotton-tipped applicators and the membrane was removed from the insert using a scalpel. Membranes were then mounted on slides, and the number

Figure 1. The SSSR mutation increases the antiangiogenic activity of PA. Results of the corneal neovascularization assay (vascularized corneal area in mm$^2 \pm $ 95% confidence interval) showing the inhibition of VEGF-induced neovascularization by PA (A) and the inhibition of VEGF-induced (left) and bFGF-induced (right) corneal neovascularization by the furin cleavage–resistant SSSR mutant of PA (B).

of cells in a microscopic field was counted either manually or with computer assistance using Scion Image. BCE assays were done similarly, except that membranes were preincubated with complete medium to improve cell adherence, and migration was toward complete medium.

**Tube formation assay.** Human microvascular endothelial cells were maintained as above. Before the assay, a 1- to 2-mm layer of Matrigel was plated into the wells of a 12-well cluster. Approximately 10^5 cells were plated on this layer in EGM-2. Plates were examined at 12, 14, 16, 18, and 24 h for differences in network formation. In each experiment, good network formation was observed in untreated control wells.

**Tumor models.** To determine the in vivo antitumor activity of PASSR, C57BL/6J male mice (8–10 weeks old; The Jackson Laboratory) were inoculated s.c. with f^3/C210^6 mouse Lewis lung carcinoma cells. The murine Lewis lung tumor line was maintained by in vivo passages as described (28). Treatment was started after the development of measurable tumor (tumors f<0.1 cm^3). Animals were sorted into groups of five containing approximately equal tumor volume, and the group with slightly larger aggregate tumor size was selected for treatment. Daily i.p. drug injections were done with protein in PBS. Serial caliper measurements of perpendicular diameters were used to calculate tumor volume using the following formula: (shortest diameter)^2 × (longest diameter) × 0.52. Animals were sacrificed after 2 weeks of treatment. All animal studies were conducted according to protocols approved by the Animal Ethics Committee of Children’s Hospital. Animals were anesthetized by isoflurane inhalation before all procedures and observed daily for signs of toxicity. Animals were sacrificed by CO₂ asphyxiation.

**Results**

As described above, both CMG2 and TEM8 are expressed on endothelial cells during angiogenesis. We sought to determine whether PA could inhibit angiogenesis by interfering with the binding of these receptors to their endogenous ligands. The ability of PA and PA mutants to inhibit angiogenesis was evaluated using the standard mouse corneal neovascularization assay developed by our laboratory (27). The corneas of C57BL/6J mice were implanted with slow-release pellets containing 160 ng VEGF. Mice were then treated with 10 mg/kg PA i.p. As shown in Fig. 1A, treatment with wild-type (wt) PA resulted in a small, but statistically significant, decrease in vessel area of ~15%.

To establish whether receptor binding was responsible for the observed effect, several PA mutant proteins were also tested. If PA

**Figure 2.** The effect of dose and schedule on the antiangiogenic activity of PA^SSSR^ Results of the corneal neovascularization assay (vascularized corneal area in mm^2 ± 95% confidence interval) showing the effect of SSSR dose on VEGF-induced corneal neovascularization (A), the effect of SSSR dose on bFGF-induced corneal neovascularization (B), and the effect of daily versus twice daily SSSR doses (C).

**Figure 3.** Receptor-binding mutants affect the antiangiogenic activity of PA^SSSR^. Results of the corneal neovascularization assay (vascularized corneal area in mm^2 ± 95% confidence interval) showing that the N682A, D683A mutant, which is reported to exhibit no receptor binding in vitro, is ineffective at inhibiting bFGF-induced angiogenesis (A). B, the D683N mutant, which binds to CMG2 with 30-fold reduced affinity and fails to bind to TEM8, inhibits corneal neovascularization, albeit the difference is not statistically significant. Note that both mutants were generated on an SSSR background.
binding to the anthrax receptor(s) was responsible for the observed antiangiogenic effect, then increasing the time that PA spent bound to the receptor could be expected to increase the effect. When PA binds to its receptor, it is cleaved, rapidly multimerizes, and is internalized, thus removing it from the cell surface. In an effort to increase the time PA is bound to the cell surface receptors, we tested the ability of various PA mutants to inhibit angiogenesis. PA$^{\text{SSSR}}$ (R164S, K165S, K166S, and R167R) is a PA mutant that is resistant to cleavage by endogenous furin-like proteases (3). Because the absence of a furin cleavage site on PA$^{\text{SSSR}}$ interferes with normal cellular processing and internalization of the PA-receptor complex, this PA mutant is presumed to have a longer extracellular lifetime and receptor residency than wt PA. As shown in Fig. 1B, PA$^{\text{SSSR}}$ was significantly more active than wt PA at inhibiting angiogenesis ($P < 0.05$; Fig. 1B). Mutants PA$^{\text{D512K}}$, PA$^{\text{D512K},\text{K199E}}$, PA$^{\text{R468A}}$, PA$^{\text{R470D}}$ fail to multimerize in solution (9) but have unknown oligomerization capacity at the cell surface. Neither of these mutants showed an appreciable increase in angiogenesis inhibition, suggesting that the in vitro oligomerization defect may not be replicated in vivo (data not shown).

Using the most effective mutant that we had identified, we found that treatment with PA$^{\text{SSSR}}$ inhibited both VEGF- and bFGF-induced angiogenesis in a dose-dependent manner with a maximal inhibition of ~60% (Fig. 2). To test whether the difference in efficacy between wt PA and PA$^{\text{SSSR}}$ could also be ascribed to differences in the circulating half-life of the two compounds rather than receptor binding, we determined whether the effect of PA$^{\text{SSSR}}$ could be increased by increasing the frequency of dosing. However, we observed no difference between daily and twice daily injections (Fig. 2C). These data indicate that the IC$_{50}$ for PA$^{\text{SSSR}}$ is 10 to 20 mg/kg and that the maximum inhibition likely to be obtained by daily dosing is about 60% to 70%. These data also support the idea that receptor binding may be the cause of the observed inhibition of angiogenesis.

To further support ATR binding as the mechanism responsible for the observed effect, PA mutations known to interfere with receptor binding (13, 29) were overlaid onto the PA$^{\text{SSSR}}$ background. Significantly, a PA$^{\text{SSSR}}$ mutant that lacks the ability to bind to either of the ATRs (PA$^{\text{SSSR},\text{N682A},\text{D683A}}$) was unable to inhibit corneal angiogenesis (Fig. 3A). In addition, the PA$^{\text{SSSR},\text{D683N}}$ mutant, which lacks the ability to bind to TEM8 and binds to CMG2 with 30-fold reduced affinity, failed to inhibit corneal angiogenesis in a statistically significant manner (Fig. 3B). Together, these data are strong demonstrations that PA interaction with the receptor is required for antiangiogenic activity in vivo.

Antiangiogenic molecules are expected to also inhibit tumor growth. To test the antitumor effects of PA$^{\text{SSSR}}$, we treated Lewis lung carcinoma tumors with daily doses of 10 mg/kg PA$^{\text{SSSR}}$. Beginning at ~1 week of treatment and extending to the end of the treatment experiment, we observed that tumor growth was inhibited. Final tumor size in the treated group was ~60% that of the control tumors, a highly statistically significant difference ($P < 0.0001$; Fig. 4).

Several mechanisms might explain the antiangiogenic and antitumor effects of PA. Endothelial cell killing, inhibition of endothelial cell proliferation, and inhibition of other endothelial cell function have all been shown to inhibit vascularization. To assess these possibilities, we tested the effects of PA$^{\text{SSSR}}$ and...
endostatin works through affinity (Fig. 5). The in vivo activity of integrins. For example, canstatin works through these fragments seem to act by blocking normal function of endostatin, endorepellin, restin, and vastatin (30). In many cases, angiogenesis inhibitors, such as arresten, canstatin, tumstatin, and esis. For example, fragments of several extracellular matrix proteins from multiple pathogens may inhibit angiogenesis. Previous studies have shown that intact anthrax lethal toxin (PA + LF) has antivascular activity (31, 32) through the LF-mediated killing of endothelial cells, similar to many other vascular killing agents (33). In these previous reports, the observed antivascular effect is due to delivery of LF to the cell via receptor-mediated internalization. However, in this work, we show that blockade of the ATR(s) themselves inhibits angiogenesis in vivo without any requirement for endothelial cell killing. Thus, inhibition of endothelial migration can be obtained without the requirement receptor-mediated toxin internalization and endothelial cell killing. Moreover, because administration of PA alone avoids cell toxicity associated with the presence of LF, we were able to use much higher doses of PA (~ 1,000-fold) than were used in previous studies involving the complete anthrax lethal toxin (31, 32).

In addition to anthrax PA, proteins from other pathogens bind endothelial cell receptors. It will be interesting to ascertain whether some of these also have antiangiogenic effects. Strikingly, whereas nonpathogenic hantaviruses bind to β₁ integrins, pathogenic hantaviruses [whose pathology is similar to that of anthrax (34)] bind to β₂ integrins found on endothelial cells. As is true of anthrax PA, these viruses can inhibit endothelial cell migration (35), suggesting that they may also have the capacity to inhibit angiogenesis. This notion is supported by the observation that these integrins are also the targets of several antiangiogenic agents, including canstatin and tumstatin (30). Thus, endothelial cell-binding proteins from multiple pathogens may inhibit angiogenesis.

In conclusion, we have shown that an antiangiogenic effect can be obtained with PA and PA mutants. Maintaining PA bound to the extracellular surface receptor, through mutations that inhibit internalization, markedly enhances the antiangiogenic and antitumor effects. We are currently pursuing the design of small molecule and peptide mimetics for preclinical development.

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