Proadrenomedullin NH2-Terminal 20 Peptide Is a Potent Angiogenic Factor, and Its Inhibition Results in Reduction of Tumor Growth

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INTRODUCTION

The formation of new blood vessels from preexisting ones, or angiogenesis, is necessary for normal physiology in processes such as embryogenesis, growth, wound healing, and the endometrial cycle. However, when the balance between proangiogenic and antiangiogenic factors gets distorted, the resulting lack or excess of blood vessels can lead to numerous pathological conditions (1). Angiogenesis is a multistep process that requires at least endothelial cell proliferation, production of extracellular proteases, migration of endothelial cells, tube formation, development of loops, and recruitment of pericytes and smooth muscle cells for larger vessels (2).

Like normal tissues, tumors require an adequate blood supply to maintain their metabolic needs. As the tumor grows, the cells located at the center of the tissue mass experience hypoxia, a physiologic stimulus for the expression and secretion of numerous proangiogenic factors, which in turn will promote enough blood vessel formation to support additional tumor growth (3). Hence the hypothesis that depriving tumors from their proangiogenic factors would stop tumor progression (4, 5). In the last few years, antiangiogenic therapies have been found to successfully delay tumor growth in animals, and several antiangiogenic factors are currently undergoing clinical trials (6, 7).

The regulatory peptide adrenomedullin is a multifunctional molecule (8) that has been recently characterized as a proangiogenic factor with the help of ex vivo and in vivo animal models (9–12; among others). In addition to inducing angiogenesis, adrenomedullin functions in cancer cells as an autocrine growth factor, enhances thymidine incorporation, reduces apoptosis, and is induced by hypoxia, therefore suggesting that this peptide may be an important tumor cell survival factor and a potential target for antitumor therapy (13).

Despite the growing interest in the angiogenic effects of adrenomedullin and its influence in tumor biology, no attention has been paid to the actions of proadrenomedullin NH2-terminal 20 peptide (PAMP) in this field, and we decided to investigate them. Here, we report that PAMP is a very potent angiogenic factor, able to induce neovascularization in animal models at concentrations 6 orders of magnitude lower than other classic proangiogenic factors such as vascular endothelial growth factor (VEGF) and adrenomedullin. We demonstrate that human microvascular endothelial cells have receptors for PAMP and respond to it by increasing migration and cord formation in Matrigel assays. In addition, PAMP stimulation induces expression of classic angiogenic factors in endothelial cells. We use a fragment of PAMP that specifically inhibits PAMP-induced effects as a tool to define for the first time the important role of PAMP in angiogenesis. This fragment acts as an inhibitor of tumor cell–induced angiogenesis and is able to delay tumor growth in xenograft models of tumor progression.

MATERIALS AND METHODS

Chemicals. Synthetic human adrenomedullin, PAMP, and PAMP(12–20) were purchased from Bachem (King of Prussia, PA). Recombinant human VEGF and basic fibroblast growth factor (bFGF) were obtained from R&D Systems (Minneapolis, MN).

Chick Embryo Aortic Arch Assay. The chick embryo aortic arch assay is an ex vivo angiogenesis assay that was performed as described previously (14, 15). In brief, aortic rings of approximately 0.8 mm in length were prepared from the five aortic arches of 13-day-old chicken embryos (CBT Farms, Chestertown, MD), and the soft connective tissue of the adventitia layer was carefully removed with tweezers. Each aortic ring was placed in the center of a well in a 48-well plate and covered with 10 μL of Matrigel (BD Biosciences, San Jose, CA). After the Matrigel solidified, 300 μL of growth factor–free human endothelial-SFM basal growth medium (Invitrogen, Carlsbad, CA) containing the proper concentration of the test substances were added to each well. The plates were kept in a humid incubator at 37°C in 5% CO2 for 24 to 36 hours. Microvessels sprouting from each aortic ring were photographed in an inverted microscope, and the area covered by the newly formed capillaries was estimated as reported previously (14).

Directed In vivo Angiogenesis Assay. Analysis and quantitation of angiogenesis was done using directed in vivo angiogenesis assay as described previously (10, 16). In brief, 10-mm-long surgical-grade silicone tubes with only one end open (angioreactors) were filled with 20 μL of Matrigel alone or mixed with adrenomedullin, bFGF, VEGF, PAMP, and/or PAMP(12–20) at the indicated concentrations. Human lung cancer cell lines (see below) were also premixed with Matrigel alone or in combination with PAMP(12–20) at 10,000 cells per angioreactor. After the Matrigel solidified, the angioreactors were implanted into the dorsal flanks of athymic nude mice (NCI colonies). After 11 days, the mice received i.v. injections of 25 mg/mL FITC-dextran (100 μL/mouse; Sigma, St. Louis, MO) 20 minutes before removing angioreactors. Photographs of the implants were taken for visual examination of angiogenic response. Quantitation of neovascularization in the angioreactors was determined as the amount of fluorescence trapped in the implants and was measured in a HP Spectrophotometer (Perkin-Elmer, Boston, MA). This protocol was approved by the internal NIH animal committee.

The human cancer cell lines used, A549 and H1299, were obtained from the American Tissue Culture Collection (Manassas, VA) and fed with RPMI 1640 containing 10% fetal bovine serum (Invitrogen). Before they were used in...
animals, both cell lines were tested for a panel of human and murine pathogens and found to be pathogen-free.

**Ca**<sup>2+</sup> Measurements. Human dermal microvascular endothelial cells were cultured in 96-well plates at 1.0 × 10<sup>4</sup> cells per well. The cells were loaded for 60 minutes at room temperature with the fluorescent dye FLIPR (Molecular Devices, Sunnyvale, CA) and then transferred to the FlexStation II (Molecular Devices) for analysis. The test compounds were prepared in another plate at 5 × concentration and added to the proper wells by the robotic arm of the FlexStation II. Fluorescence was measured every 5 seconds in each well and recorded. ATP (1 nmol/L; Sigma) was used as a Ca<sup>2+</sup> influx agonist (17).

**Proliferation Assay.** The same microvascular endothelial cells were seeded in 96-well plates at a density of 2.0 × 10<sup>4</sup> cells per well in serum-free medium containing different concentrations of the test peptides. After 3 days in culture, the number of viable cells per well was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as reported previously (10). Results are represented as the percentage of growth over the untreated control.

**Migration Assay.** Cell motility was measured as described previously (10). Test peptides were placed at various concentrations at the bottom of a ChemoTx chamber (NeuroProbe, Inc., Gaithersburg, MD). The intermediate membrane was coated with 10 μg/mL fibronectin, and in the upper chamber, 5.0 × 10<sup>4</sup> human endothelial cells were added. After a 4-hour incubation at 37°C, the membrane was fixed and stained (Protocol Hema3; Biochemical Sciences, Inc., Bridgeport, NJ). The cells trapped in the porous membrane were photographed through a ×25 microscope objective, and the number of cells per photographic field was counted.

**Cord Formation Assay.** Human endothelial cells were seeded at 2.0 × 10<sup>4</sup> cells per well over a solid layer of Matrigel covering the bottom of a 24-well plate in the presence or absence of the test peptides, as described previously (18). After an overnight incubation, the tubular structures were photographed, and the number of knots per photographic field were counted as a measure of lattice complexity.

**Real-Time Polymerase Chain Reaction Quantification of Gene Expression.** Human endothelial cells were cultured in T-75 flasks until they reached a density of approximately 2.5 × 10<sup>6</sup> cells per flask. Cells were treated with 10 nmol/L PAMP in serum-free medium for 24 hours. Total RNA was extracted using the RNeasy Mini kit from Qiagen (Valencia, CA) and reverse transcribed using the SuperScript First-Strand Synthesis system (Invitrogen). Quantification of gene expression was performed by real-time PCR as described previously (19). The PCR reaction was run in an Opticon cycler (MJ Research, Waltham, MA) using Sybr Green PCR master mix (Applied Biosystems, Foster City, CA). Thermocycling was performed in a final volume of 25 μL containing 2 μL of cDNA (1:10 dilution) and 400 nmol/L of primers (see below). All targets were amplified in triplicate in the same run as the house-keeping gene, using the following cycle scheme: After initial denaturation of the samples at 95°C for 2 minutes, 46 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 30 seconds were performed. Fluorescence was measured in every cycle, and mRNA levels were normalized by the 18S reverse, ATT CCT AGC TGC GGT ATC CAG G.

To further characterize this intriguing observation, we used an in vivo assay to more precisely quantitate the angiogenic properties of PAMP. This approach is called directed in vivo angiogenesis assay (10, 16). In this assay, small silicone capsules carrying the test substances are implanted under the skin of nude mice. After 11 days, intratumoral injection, according to their group. Group 1 (control) received 100 μL of PBS; group 2 received 100 μL of 10 mmol/L PAMP(12-20) in PBS; and group 3 received 100 μL of 1 mmol/L PAMP(12-20) in PBS. When the tumor burden became unbearable (larger than 2000 mm<sup>3</sup>), the mice were sacrificed.

**RESULTS**

**Angiogenic Potential of Proadrenomedullin NH<sub>2</sub>-Terminal 20 Peptide in Ex vivo and In vivo Assays.** There are many ways of testing angiogenesis (15), and we chose a chick embryo aortic arch assay for a preliminary assessment of the angiogenic properties of PAMP. Angiogenic factors such as adrenomedullin and VEGF induced a statistically significant increase of the number of sprouting blood vessels over untreated controls at concentrations of 100 nmol/L and higher (results not shown). PAMP induced comparable growth of newly formed blood vessels at concentrations as low as 1 nmol/L (Fig. 1D). At this concentration (1 nmol/L), neither adrenomedullin (Fig. 1B) nor VEGF (Fig. 1C) caused any significant proliferation over the control (Fig. 1A). The sprouting new vessels covered a surface of 2,579,500 ± 378,300 mm<sup>2</sup> after treatment with PAMP, which is about 10 times the surface covered by the primitive capillaries formed in the untreated control (261,500 ± 60,800 mm<sup>2</sup>; P < 0.001). This initial observation suggests that PAMP may be a more potent proangiogenic factor than previously described molecules.

Fig. 1. Comparative angiogenic potential of adrenomedullin, VEGF, and PAMP in the chick embryo aortic ring assay. Aortic rings were embedded in Matrigel and exposed to serum-free medium containing either PBS as a negative control (A) or 1 nmol/L of the peptides adrenomedullin (B), VEGF (C), or PAMP (D). Only in the case of PAMP is the crown of sprouting new vessels significantly larger than the control. Representative examples of four repeats. Bar = 0.5 mm.
the mice received injections of a specific amount of FITC-dextran, and the volume of blood circulating through the implant is quantified by measuring the fluorescence in the capsule. In addition, the new blood vessels growing into the silicone tube can be seen directly by transparency (Fig. 2, A–F). PAMP elicited a measurable angiogenic response at concentrations as low as 1 fmol/L (Fig. 2C), which was clearly distinct from the negative control (Fig. 2A). The angiogenic response elicited by PAMP was dose-dependent (Fig. 2, C–G). When compared with adrenomedullin and VEGF responses at equimolar concentrations, a clearly significant difference was observed over a wide concentration range. In this animal model, adrenomedullin and VEGF induce angiogenesis at nanomolar concentrations, whereas PAMP was already active in the femtomolar range (Fig. 2G).

**P40redrenomedullin NH2-Terminal 20 Peptide Reduces Ca2+ Flux in Endothelial Cells.** Although the adrenomedullin receptor has been well characterized at the molecular level (21), the structure of the PAMP receptor is not yet available; but it has been shown that exposure of adrenal medulla cells to PAMP results in a decrease of carbachol-induced Ca2+ influx (22). To demonstrate whether a similar biological response takes place in endothelial cells, we stimulated them with 1 mmol/L ATP to induce a Ca2+ influx (Fig. 3, squares) as reported previously (17). This response was greatly reduced (>50%) by the presence of 10 nmol/L PAMP in the medium (Fig. 3, diamonds). The peptide fragment PAMP(12-20) has been shown to have opposite actions to full-length PAMP (23), suggesting its specific effect as a PAMP antagonist. To demonstrate the specificity of the PAMP-induced inhibition in Ca2+ influx, we added an excess of PAMP(12-20) and were able to recover the initial response (Fig. 3, circles). Taken together, these data show that there is a functional PAMP receptor in the membrane of the endothelial cells, and therefore this peptide may activate directly the angiogenic response described above.

**Physiologic Effects of P40redrenomedullin NH2-Terminal 20 Peptide on Endothelial Cells.** For angiogenesis to occur, endothelial cells have to proliferate, migrate into new locations, and organize themselves into solid cords that eventually will develop into hollow tubes (2). These processes are promoted by proangiogenic substances, and all proangiogenic molecules must elicit at least one of these physiologic actions. To investigate which of these phenomena are induced by PAMP, we exposed human dermal microvascular endothelial cells to increasing concentrations of PAMP, adrenomedullin, and VEGF and compared their effects on growth (Fig. 4A), migration (Fig. 4B), and cord formation (Fig. 4C).

Endothelial cell growth analysis showed that adrenomedullin and VEGF significantly increase proliferation over the control at a concentration of 10−8 mol/L (P < 0.001 for both). However, PAMP did not significantly enhance cell growth at the concentrations tested (Fig. 4A).

We tested the migratory potential of endothelial cells over a range of peptide concentrations. Adrenomedullin did not modify cell migration significantly on the concentration range tested, whereas both VEGF and PAMP produced a 4-fold increase in migration at a concentration of 10−11 mol/L when compared with untreated controls (P < 0.001 for both). As previously reported for VEGF (24), both VEGF and PAMP showed a peak of migration stimulation at 10−11 mol/L. The shape of the peak was different for both peptides: more steep for PAMP and more gradual for VEGF (Fig. 4).

We also tested the ability of adrenomedullin, VEGF, and PAMP to induce cord formation in a Matrigel assay. All three factors were able to induce statistically higher cord formation in a dose-dependent manner when compared with untreated controls. VEGF was the most efficient mediator, followed by adrenomedullin, whereas PAMP had a modest effect on cord formation (Fig. 4).

We also studied the effects of exogenously added PAMP on the gene expression for other proangiogenic molecules (Fig. 4D). Real-time PCR experiments showed that PAMP modestly induces the
untreated cells. The represents the ratio between the gene of interest in the treated cells and the contents in the mRNA of angiogenic molecules were measured by real-time PCR. Each point indicates no change over untreated conditions.

The complexity of the cord network was estimated by the number of knots per microscopic field. Each point represents the mean and SD of four independent measurements.

The addition of 10 nmol/L PAMP(12-20) did not show any significant difference with the PBS-treated group (results not shown). In contrast, the mice that received 1 μmol/L PAMP(12-20) showed a slower rate of tumor growth (Fig. 6, diamonds). Statistical differences in tumor size between the groups were observed after 9 days of treatment and continued for the rest of the experiment.

We also investigated whether injection of full-length PAMP had any impact in xenograft tumor growth. At the concentrations tested (1 and 100 nmol/L PAMP), no difference was observed between tumors treated with peptide or vehicle (results not shown).

**DISCUSSION**

Here, we have demonstrated that the adrenomedullin gene-related peptide PAMP is a potent angiogenic factor that is active at concentr-

expression of its own gene (adrenomedullin/PAMP), about 50% over basal levels. PAMP was also capable of elevating the expression of VEGF, bFGF, and PDGF C by 80, 300, and 300%, respectively. Conversely, no significant modification in the expression of PDGF A or PDGF B was observed (Fig. 4D). The addition of 10 nmol/L adrenomedullin or VEGF to endothelial cells had no effect on the expression of the adrenomedullin/PAMP gene (results not shown).

A Proadrenomedullin NH2-Terminal 20 Peptide Antagonist Inhibits Angiogenesis In vivo. To further evaluate the role of PAMP in angiogenesis, we evaluated its influence in tumor growth, using PAMP(12-20) as a PAMP-receptor antagonist. First, we studied the competition between synthetic full-length PAMP at 1 nmol/L concentration and increasing doses of PAMP(12-20) in the directed in vivo angiogenesis assay. We observed a dose-dependent inhibition of the angiogenic response elicited by PAMP (Fig. 5A). A 100-fold excess of the peptide fragment (100 nmol/L) inhibited angiogenesis to the basal levels ($P < 0.05$, comparing tumor cells plus PAMP(12-20) with Matrigel only control), indicating that PAMP signaling is somehow necessary for initiating angiogenesis.

For further evaluation of in vivo effects of PAMP, we designed a xenograft experiment. The human lung cancer cell line A549 was injected under the skin of 30 athymic nude mice, and after 2 weeks, all animals developed palpable tumor masses at the injection site. These mice were divided into three groups, and each set received a different treatment three times a week. The control group was treated with the vehicle (PBS), and the tumor mass kept increasing until the mice had to be sacrificed 18 days after treatment began (Fig. 6, squares). The group of animals whose tumors received injections of 10 nmol/L PAMP(12-20) did not show any significant difference with the PBS-treated group (results not shown). In contrast, the mice that received 1 μmol/L PAMP(12-20) showed a slower rate of tumor growth (Fig. 6, diamonds). Statistical differences in tumor size between the groups were observed after 9 days of treatment and continued for the rest of the experiment.

Because tumor cells produce many angiogenic factors (25), we evaluated the contribution of PAMP to the total angiogenic response. Two human non-small cell lung cancer cell lines (A549 and H1299) were embedded in Matrigel and placed in the directed in vivo angiogenesis assay. Both cell lines induced a significant angiogenic response (Fig. 5B) that was completely blocked by 100 nmol/L PAMP(12-20) ($P > 0.05$, comparing tumor cells plus PAMP(12-20) with Matrigel only control), indicating that PAMP signaling is somehow necessary for initiating angiogenesis.
results have been reported for adrenomedullin in breast cancer cells. Additional peptide would not modify the angiogenic response. Similar saturates the receptors present in the endothelial cell surface, thus any speculate that the tumor cells may be producing enough PAMP to experiment when we added full-length peptide. Alternatively, we can dases may also explain the lack of effect observed in the xenograft assay may provide a secluded environment protecting PAMP from proximity of the target cells (endothelial cells), and after effecting its (29). The silicone implant used in the directed in vivo angiogenesis assay may be composed of an interdependent chain of events that when one of those individual steps is blocked, the whole process has to stop. The profound effects of PAMP(12-20) on human lung tumor cells in the directed in vivo angiogenesis assay and the xenograft experiments is consistent with this proposed hypothesis. Our finding that PAMP induces the expression of several proangiogenic genes in endothelial cells is also in agreement with that hypothesis. This would suggest that PAMP has both a direct effect on endothelial cells by binding to its receptor and lowering Ca2+ flux and an indirect effect by inducing production of other angiogenic promoters.

In summary, our experimental findings have revealed PAMP to be an extremely potent angiogenic factor with activity several orders of magnitude higher than the previously established “gold standard,” VEGF. We have shown that the peptide fragment PAMP(12-20) functions as an antagonist that can suppress in vivo angiogenesis induced by the intact ligand or by tumor cell–derived peptide. Finally, PAMP(12-20) treatment partially blocked the in vivo growth of human tumors as assessed in a nude mouse xenograft model. In light of significant recent clinical benefits of antiangiogenic therapies based on VEGF blockade (7), PAMP antagonists provide a conceptually attractive tool to further explore this strategy.

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