Simplified Production of a Recombinant Human Angiostatin Derivative That Suppresses Intracerebral Glial Tumor Growth

Patricio I. Meneses, Lauren E. Abrey, Katherine A. Hajjar, S. Humayun Gultekin, Robert M. Duvoisin, Kenneth I. Berns, and Myrna R. Rosenfeld

Division of Neurosurgery [M. R. R.], and Departments of Neurosciences [P. I. M., R. M. D., M. R. R.] and Pediatrics and Medicine [K. A. H.], Weill Medical College of Cornell University, New York, New York 10021; Departments of Neurology [L. E. A.] and Pathology [S. H. G.], Memorial Sloan-Kettering Cancer Center, New York, New York 10021; and Department of Molecular Genetics and Microbiology [K. I. B.], University of Florida College of Medicine, Gainesville, Florida 32610

ABSTRACT

Angiostatin is an endogenous inhibitor of tumor neo-vascularization that inhibits the proliferation of endothelial cells. Production of sufficient quantities of biologically active angiostatin by the enzymatic cleavage of plasminogen has proven difficult in that it has delayed clinical testing. We have cloned, expressed, and purified a recombinant human angiostatin derivative (K1–3) using a mammalian expression system. Through the addition of a secretory signal and polyhistidine sequence tag, K1–3 can be purified from post-culture medium by simple column chromatography. Purified K1–3 protein is apparently folded in an active conformation, as evidenced by its ability to bind to lysine-Sepharose. In vitro, recombinant K1–3 significantly suppressed endothelial cell proliferation in a dose-dependent manner with an IC50 of 50 nM. Using an animal model of intracranial brain tumors in immune-competent rats, systemic administration of purified recombinant K1–3 resulted in up to 85% suppression of tumor growth (P = 0.01). Growth suppression was accompanied by a 32% decrease (P = 0.01) in tumor neo-vascularization.

This study demonstrates a simple method to produce a biologically active recombinant angiostatin derivative. The ability to suppress intracerebral tumor growth after systemic administration suggests that K1–3 is likely to have therapeutic value in the treatment of malignant glial tumors.

INTRODUCTION

Angiostatin has been recently described as an endogenous inhibitor of angiogenesis (1). Angiostatin has been isolated by a laborious and low yield process requiring limited proteolytic digestion of plasminogen purified from urine or serum (2). The difficulties in manufacturing sufficient quantities of biologically active angiostatin have hampered bringing this agent to clinical use (3), and alternative production methods have been sought. Several studies suggest that recombinant angiostatin can be produced by viral or yeast expression systems (4–6). In addition to facilitating production, the use of recombinant angiostatin derivatives avoids the risks of using human plasma, which has the potential for contamination with transmissible agents.

We have made a recombinant human angiostatin molecule consisting of the first three kringle domains (K1–3) of plasminogen (7). Expressed and secreted by a mammalian cell line, recombinant K1–3 protein is easily purified. Recombinant K1–3 maintains angiogenic activity in vitro. Furthermore, we demonstrate that K1–3 is active in suppressing the growth of an intracranial glial tumor after systemic administration.

Angiogenesis plays a role in the growth of many tumors. Because malignant gliomas are among the most highly vascularized of all tumors (8), they are likely to be affected by antiangiogenic agents such as angiostatin. In glial tumors, an increase in microvascular density is associated with higher grade and poorer prognosis (9). Both glial cell lines and glial tumors have been shown to express several growth factors and growth factor receptors that are capable of stimulating endothelial cell proliferation. These include bFGF (10–12). In some glial tumors, the level of bFGF expression correlates with the degree of malignancy and vascularity (13). In vitro, the growth of transformed astrocytes and glial tumor cell lines are suppressed when bFGF expression is inhibited (14, 15) and increased when bFGF is overexpressed or blocked (16, 17). These studies suggest that both autocrine and paracrine angiogenic signaling pathways exist in some glioblastomas. These features make glial neoplasms reasonable targets for antiangiogenic therapy. In contrast to studies that use s.c. glioma xenografts in nude mice to test the therapeutic efficacy of novel agents (18, 19), we tested K1–3 against an intracerebral brain tumor model in immunocompetent rats to more closely approximate the clinical situation.

The abbreviations used are: bFGF, basic fibroblast growth factor; HUVE, human umbilical vein endothelial; HEK, human embryonal kidney; LPS, lipopolysaccharide.
MATERIALS AND METHODS

Cell Lines and Animals. HEK 293 cells were obtained from American Type Culture Collection (Rockville, MD) and were grown in DMEM supplemented with 10% FCS and 5,000 units/ml penicillin/streptomycin. HUVE cells, which were obtained at delivery as described previously (20), were grown in Medium 199 containing 20% pooled human serum, 20 μg/ml endothelial cell growth factor, 2.5 μg/ml fungizone, and 50 units/ml penicillin/streptomycin. The 9L rat glioma cell line was grown in DMEM supplemented with 10% heat-inactivated FCS, 2 μg/ml t-glutamine, and 5,000 units/ml penicillin/streptomycin. Inbred female Fisher 344 rats (200 g) were purchased from Charles River Laboratories (Wilmington, MA). Animal studies were done in accordance with guidelines of the Animal Care and Use Committee of the Memorial Sloan-Kettering Cancer Center.

Plasmid Construction. A cDNA encoding kringle domains 1, 2, and 3 of human plasminogen (Val-79 to Ser-337) was amplified by PCR using human plasminogen cDNA as the template (courtesy of F. J. Castellino, University of Notre Dame, Notre Dame, IN) and oligonucleotide primers (5'-CCCAAGCTTGGTGTATCTCTCAGGG-3' and 5'-GCCCTCTAGAGGGAGGTAGCAGA-3') containing linkers with HindIII and XbaI restriction sites, respectively. The 789-bp fragment was cloned into pSecTagA (Invitrogen, San Diego, CA) downstream from the cytomegalovirus promoter and the murine immunoglobulin κ-chain leader peptide and upstream of the myc epitope and polyhistidine tag. The integrity of the resulting plasmid, pSecK1–3, was confirmed by automated sequencing.

Purification of K1–3 Protein. pSecK1–3 was transfected into HEK 293 cells using the calcium phosphate method (21). Stable transfectants were obtained by selection in Zeocin (250 μg/ml). Stable transfectants were grown in 30 ml of media in T-175-cm² flasks to 80–90% confluence. The media from each of the eight flasks was collected and passed over a Ni²⁺ affinity column as per the manufacturer’s instructions (Promega, Madison, WI). K1–3 protein was eluted with 1 M imidazole, and the fractions with maximum absorbance pooled and extensively dialyzed against 1000 volumes of PBS (pH 7.4) at 4°C for 16 h. K1–3 protein was also purified by passage of media over a dialyzed against 1000 volumes of PBS (pH 7.4) at 4°C for 16 h. The fractions with maximum absorbance pooled and extensively dialyzed against 1000 volumes of PBS (pH 7.4) at 4°C for 16 h.

Western Blot. Purified protein or 15 μl of media from transfected HEK 293 cells were resolved by 12% SDS-PAGE. To determine the purity of the protein preparations, gels were stained with Coomassie Brilliant Blue. For Western blotting, the resolved proteins were transferred to nitrocellulose. After overnight blocking in 5% Carnation nonfat milk, the nitrocellulose was sequentially incubated with mouse anti-myc IgG diluted 1:5000 (Invitrogen; 2 h) and with rabbit antismouse IgG conjugated to alkaline phosphatase diluted 1:10,000 (1 h). The blot was then immersed in an enhanced chemiluminescence solution (Amersham, Arlington, IL) for 60 s and exposed to Kodak XAR5 film (Sigma, St. Louis, MO). Between steps, the nitrocellulose was washed with 0.1% Tween Tris-buffered saline. All incubations were done at room temperature.

Cell Proliferation Assay. HUVE cells were plated on 24 well plates at 30,000 cells/well. After 24 h, purified K1–3 protein was added to the media at increasing concentrations and then incubated for 72 h at 37°C. Cell number was quantified with a cell proliferation assay kit using 4.5 dimethylthiazol-3-carboxymethoxy-phenyl-4-sulfophenyl-tetrazolium (MTS; Promega) or by trypan blue exclusion.

Animal Model. Rats were anesthetized by i.m. injection of chlorpromazine (1.5 mg/kg) and ketamine (100 mg/kg; Fort Dodge Laboratories, Fort Dodge, IN). After making a midline sagittal incision, a burr hole 2 mm in diameter was drilled, and a 3–4-μl suspension containing 5 × 10⁴ or 1 × 10⁵ 9L cells in sterile saline was injected into the right basal ganglia using a 25-gauge Hamilton syringe and a small animal stereotactic frame (Kopf, Tujunga, CA). The coordinates used for injection were 4 mm lateral to the midline at the bregma and 5 mm below the dural surface. Animals were given s.c. injections of 0.5 mg/kg purified K1–3 protein in PBS every 12 h starting 24 h after 9L implantation for a total of 10 treatment days. Control animals were treated with column fractions obtained from media from nontransfected HEK 293 cells.

Tumor Size. Following 10 days of K1–3 treatment, animals were euthanized. The brains were embedded in Optimal Cutting Temperature medium (Miles, Elkhart, IN) and snap frozen in isopentane chilled by liquid nitrogen. Consecutive 10-μm sections were cut through the extent of the tumor determined by examining hematoxylin-stained sections under light microscopy. Tumor volume was calculated by multiplying the area of the tumor by the slice thickness and the number of slices containing the tumor.

Immunohistochemical Analysis and Microvessel Quantification. Frozen sections were fixed in 10% buffered formalin then sequentially incubated with 0.1% hydrogen peroxide to destroy endogenous peroxidase activity, 10% normal horse serum as a blocking agent, mouse antiamouse CD31 (1:1000; Serotec, Raleigh, NC), biotinylated rat adsorbed horse antimouse (1:1000; Vector, Burlingame, CA), and peroxidase-conjugated streptavidin (1:1000; Boehringer, Indianapolis, IN). Diaminobenzidine (0.05%) was used as the chromogenic substrate, and hematoxylin was used as the counterstain. All incubations were performed at room temperature. Quantitation of anti-CD31 staining (microvessels per field) was performed by two investigators who were blinded to the experimental status of the section. After screening the tumors at low power (×40 and ×100), microvessels in the areas with the highest number of discrete microvessels were counted in the ×400 field. A vessel lumen was not required for the identification of a microvessel; CD31-positive single endothelial cells or cell clusters were counted (9, 22). The average vessel counts of all fields studied by both investigators were recorded.

Statistical Methods. Numerical values are expressed as the mean ± SE. Statistical significance was assessed using the
 Student's t test. Ps <0.05 were considered to be statistically significant.

RESULTS

Gene Construction and Expression. The cDNA fragment corresponding to kringles 1–3 of human plasminogen was cloned into pSecTagA to produce pSecK1–3 (Fig. 1). The murine immunoglobulin κ-chain leader peptide was included to serve as the secretory signal, whereas an myc epitope and a polyhistidine chain were included to allow for antibody tagging and protein purification, respectively. Using this construct, stable transfectants of HEK 293 cells were derived by antibiotic selection. Resolution of the protein preparations isolated from the media bathing stable transfectants after Ni²⁺ column purification on SDS-polyacrylamide gels revealed the presence of a single protein band at 32 kDa (Fig. 2A). Western blot analysis using a monoclonal anti-myc epitope antibody confirmed the identity of the protein to be the product of pSecK1–3 (Fig. 2B). A similar protein was not identified in the media of cells transfected with the pSecTagA plasmid without the K1–3 insert. Based upon the average yield of several protein preparations, 1 mg of purified K1–3 protein was obtained from the media bathing 1.2 × 10⁸ transfected cells for 3 days. LPS levels in Ni²⁺ column-purified protein solutions, which were <1.2 ng/mg K1–3, were markedly below growth inhibitory levels (data not shown; 23).

Appropriate folding of the kringle domains is essential for the antiangiogenic activity of angiostatin (7). In the active conformation, the kringle 1 domain binds lysine (24, 25). To test for functional lysine binding, medium from transfected HEK 293 cells was subjected to affinity chromatography over lysine-Sepharose columns. After extensive washing, bound protein was eluted from the column by ε-amino-N-caproic acid and analyzed by Western blot, revealing a 32-kDa protein (Fig. 2, A and B).

The stability of purified K1–3 was tested by adding purified protein (1.6 μg) to the media of HUVE cells. Western blot analysis demonstrated that K1–3 was not significantly degraded for up to 72 h (data not shown).

Suppression of HUVE Cell Proliferation in Vitro. To determine whether recombinant K1–3 protein had antiangiogenic activity, cultured HUVE cells were incubated with increasing concentrations of Ni²⁺ affinity column-purified K1–3 protein. After 72 h, control-treated HUVE cells had doubled in number from 3.2 × 10³ cells to 6.3 × 10³ cells (Fig. 3). In contrast, in the presence of purified K1–3, the HUVE cell number was reduced in a dose-dependent manner with an IC₅₀ of 50 nM. A significant 37% reduction in cell number was seen at 47 nM K1–3 protein (P = 0.02), with a maximal reduction of 87% obtained at 125 nM K1–3 (P = 0.004). Suppression of HUVE proliferation was not seen if the recombinant K1–3 protein was boiled for 10 min prior to use (data not shown).

Suppression of Intracerebral Glioma Growth. One day after implantation of 9L glioma cells into the right basal ganglia, rats were treated with s.c. injections of 100 μg of purified K1–3 protein. Animals were dosed every 12 h and were treated for 10 consecutive days.

Analysis of tumor size demonstrated that K1–3-treated animals had tumors that were significantly smaller than control-treated animals (Table 1). The suppression of tumor growth by K1–3 was not markedly dependent upon tumor size. Animals implanted with 0.5 × 10⁵ 9L cells and treated with K1–3 had tumors that were 85% smaller than matched controls.
implanted with \(1 \times 10^5\) 9L cells had tumors that were 74% smaller than controls.

**Tumor Vascularization.** To study the effect of K1–3 on the tumor microvasculature, representative sections of all tumors were examined for expression of CD31, a platelet endothelial cell adhesion molecule found on the surface of small and large blood vessels in pathological and normal tissue (26). After CD31 immunoreactive hot-spots were identified at low power, microvessels were then counted at \(\times 400\). The average number of blood vessels observed in tumors from K1–3-treated animals was significantly less \(15 \pm 2\) (\(n = 12\)) per high power field than in tumors from control-treated animals \(22 \pm 2\) (\(n = 10\)) per high power field \(P = 0.01\; \text{(Fig. 4)}\). There were no obvious differences in the morphology of the microvasculature between the treated and control animals or in the localization of the microvessels within the tumor.

**DISCUSSION**

During tumorigenesis, there is disruption of the normal physiological balance between angiogenic inhibitors and stimulators leading to the rapid proliferation of endothelial cells (27, 28). Since the discovery of the angiogenic growth factors and the endogenous angiogenic inhibitor, angiostatin, many studies have focused on manipulating these pathways with the goal of inhibiting tumor angiogenesis (17, 29, 30).

Angiostatin is derived from an internal fragment of plasminogen and contains four kringle domains (looped disulfide-linked structures; Ref. 1). Most of the antiproliferative activity of angiostatin resides in kringle domains 1, 2, and 3 (7). To produce human angiostatin, plasminogen has been purified from plasma and enzymatically digested. The fragment comprising angiostatin is isolated and purified by affinity chromatography on lysine-Sepharose. This process, when successful, results in low yields of active compound making it inadequate to produce sufficient quantities for clinical testing (3).

In the present study, we demonstrated that a recombinant protein, K1–3, containing plasminogen kringles 1–3 can be produced and secreted by mammalian cells. Upon secretion, the recombinant K1–3 protein folds appropriately, as demonstrated by its ability to bind to lysine-Sepharose, a property of native angiostatin (24, 25). In culture, a single dose of recombinant K1–3 protein significantly suppressed the proliferation of HUVE cells, confirming that the recombinant K1–3 protein is expressed in a conformation that is similar to native angiostatin.

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To test the antiangiogenic activity of the K1–3 protein in vivo, we used a rat model of intracranial brain tumors formed by syngeneic 9L glioma cells (31). These cells were originally derived from a nitrosourea-induced rat glioma, and in vitro are not affected by incubation with K1–3 (data not shown). Tumors

![Fig. 3 Suppression of HUVE cell proliferation by recombinant K1–3.](image)

HUVE cells were incubated with increasing concentrations of Ni²⁺ column-purified K1–3 for 3 days. For each concentration of K1–3, a minimum of six experiments performed in triplicate were evaluated. Cell growth curves were evaluated for the determination of IC₅₀ by the MTS assay. The black bar represents the number of cells at the start of the experiment; bars, SE. *, \(P < 0.02\).

<table>
<thead>
<tr>
<th>No. of 9L cells implanted</th>
<th>Mean tumor volume (mm³)</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0.5 (\times 10^5)</td>
<td>6.0 ± 1.5</td>
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<tr>
<td>((n = 5))</td>
<td>((n = 5))</td>
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<tr>
<td>1 (\times 10^5)</td>
<td>14.0 ± 4.1</td>
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<td>((n = 5))</td>
<td>((n = 7))</td>
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\(^* P = 0.011.\; \ ^b P = 0.016.\)

![Fig. 4 Immunohistochemical detection of CD31 in control- (A) and K1–3-treated (B) intracerebral 9L tumors. No counterstain (\(\times 400\)).](image)
Mutations with loss of p53 function are found in cells expressing a mutant form of the p53 tumor suppressor gene induced by bFGF is inhibited by angiostatin (1). In addition, 9L vascular endothelial cell growth are unknown, angiogenesis although the exact mechanism(s) by which angiostatin inhibits glioblastoma cells with loss of p53 function express strong angiogenic activity, whereas expression of wild-type p53 results in the secretion of an antiangiogenic factor (34). These properties would be expected to render 9L cells relatively resistant to antiangiogenic therapy. However, systemic injections of recombinant K1–3 were very effective in suppressing the growth of intracranial 9L tumors. This was associated with a decrease in tumor vascularity measured by CD31 immunostaining of microvessels, which has been used to quantify blood vessels in many tumor types (26). Based upon the levels of LPS measured in the K1–3 preparations, it is unlikely that contaminating LPS was responsible for the tumor growth suppression that was seen (23). These data are consistent with angiogenesis inhibition as the mechanism of tumor growth suppression rather than as an effect on tumor cell proliferation.

Although physiologically active recombinant angiostatin derivatives have therapeutic potential, there are limitations to the isolated use of angiostatin. One problem is the need for chronic treatment; studies in a variety of animal tumor models have demonstrated that if angiostatin treatment is discontinued, tumor growth resumes (35). One approach to achieving sustained drug delivery is the injection of viral vectors directly into the tumor bed. However, if the tumor cells are transduced and secreting the active agent, there could be significant loss of drug expression as the transduced cells die. In addition, the length of time that viral vectors express their transgene is transient and variable (36–38). Both of these issues make it likely that a patient would require repeat intracranial administration of vector, which could substantially add to morbidity. Another drawback is that the most commonly used retroviral vectors are relatively inefficient at transducing tumors in vivo. Although adenoviral vectors are very efficient at transducing target cells in vivo (39), their use in immune-competent animals has been limited due to the development of an inflammatory response and the production of antiadenoviral antibodies (38, 40). The long-term use of an antiangiogenic peptide is clinically feasible. Prefilled injectors can simplify the process and slow release formulations, or the use of pumps could avoid the need for daily injections (41, 42). As an outpatient procedure, this form of drug delivery is economically practical.

In summary, the clinical use of angiostatin has been limited by difficulties in producing large quantities of active compound. In the current study, we demonstrate that a recombinant angiostatin derivative with antiangiogenic activity in vitro and in vivo can be easily produced and purified. The ability of K1–3 to suppress intracerebral glial tumor growth is particularly attractive. The large majority of glial tumors are malignant and, to date, remain incurable. Except in unusual cases or for particular subtypes of brain tumors, current systemic or local chemotherapeutic regimens do not significantly affect survival (43, 44). These studies should encourage the development of similar antiangiogenic agents for clinical trials.

REFERENCES


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