Inhibition of Tumor Growth Correlates with the Expression Level of a Human Angiostatin Transgene in Transfected B16F10 Melanoma Cells

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ABSTRACT

Although the therapeutic value of angiostatin, a proteolytic fragment of plasminogen, has been recognized for the treatment of cancer, the production of bioactive angiostatin remains a difficult task. Here we report that expression of a cDNA encoding a secreted, four-kringle human angiostatin inhibited tumor growth of B16F10 melanoma cells in mice but did not suppress tumor cell growth in culture. After transfection and selection, stable expression of the angiostatin cDNA was demonstrated in several B16F10 clones by quantitative mRNA analysis using the Taqman method. Cells that expressed angiostatin at either a low, medium, or high level were injected into C57BL/6 mice. s.c. Growth of B16F10 tumors was diminished by the angiostatin transgene, and the inhibition was directly proportional to the expression level of angiostatin in the transfected cells. However, suppression of s.c. tumor growth was transient, and eventually, tumors emerged with a strongly decreased expression of the transgene. Angiostatin expression also reduced lung metastasis from i.v.-injected B16F10 cells. Our data indicate that a cDNA encoding bioactive human angiostatin is potentially useful for gene therapy of human cancers, but the delivery of the transgene may require repeated dosing to achieve sustained dormancy of primary tumors and cancer metastases.

INTRODUCTION

Formation of new blood vessels is thought to be essential for tumor growth. This neovascularization process can be induced by tumor cells, which stimulate normally quiescent endothelial cells to proliferate and differentiate (1, 2). However, endothelial cells are responsive to both positive and negative regulators. Solid tumors also release factors that are potent inhibitors of endothelial cell growth and migration (3–5). Several antiangiogenic proteins have been identified that suppress the growth of cancer metastases while being released into the blood flow from a primary tumor site. Among these, thrombospondin is secreted by cancer cells (5), and the expression can be increased in tumor cells can inhibit both s.c. and metastatic tumor growth of B16F10 melanoma cells (6). Angiostatin was found in the blood and urine of mice bearing Lewis lung carcinoma (3), and endostatin was isolated from animals with hemangioendotheliomas (4). Both proteins are produced in tumors, but they are not secreted by the cancer cells. Recently, tropolin I was added to the list of angiogenesis inhibitors that have been shown to inhibit metastatic tumor growth (7).

Angiostatin and endostatin are fragments of larger proteins, and they are generated in the extracellular compartment by proteolytic digestion of plasminogen (3, 8) or collagen XVIII (4), respectively. Release of a protease, metalloelastase, from infiltrating macrophages can produce angiostatin in Lewis lung carcinoma (9), and a similar activation mechanism may exist for endostatin. The tumor suppressor gene (6). Angiostatin was found in the blood and urine of mice bearing Lewis lung carcinoma (3), and endostatin was isolated from animals with hemangioendotheliomas (4). Both proteins are produced in tumors, but they are not secreted by the cancer cells. Recently, tropolin I was added to the list of angiogenesis inhibitors that have been shown to inhibit metastatic tumor growth (7).

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48 h, and medium was collected for Western blot analysis. Attached cells were washed with 1× PBS and lysed in 2 ml of 100 mM sodium phosphate buffer (pH 7.2), 150 mM NaCl, 1% Triton X-100, 20 mg/liter phenylmethylsulfonyl fluoride, 20 mg/liter aprotinin, 20 mg/liter leupeptin, and 2 mg/liter pepstatin.

**Western Blot Analysis of HA-tagged Angiostatin Protein.** Rabbit polyclonal anti-HA antibody (2.5 μg; Santa Cruz Biotechnology, Santa Cruz, CA) and 50 μl of protein A/G-Sepharose (Santa Cruz Biotechnology) were added to either 1 ml of conditioned culture medium or 0.5 ml of cell extract and incubated on ice for 1 h. The antibody-Sepharose complex was spun down, washed twice in lysis buffer, and denatured at 95°C in loading buffer. After separation on an 8% SDS-polyacrylamide gel, proteins were transferred to a polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL). Angiostatin was detected with a mouse monoclonal anti-HA antibody (Boehringer Mannheim, Indianapolis, IN) diluted to 5 μg/ml in PBS + 0.1% Tween 20 (TPBS). After washing with TPBS, membranes were incubated with a horse-radish peroxidase-coupled secondary antibody (Amersham). Blots were developed with the Pierce Supersignal chemiluminescence system (Pierce, Rockford, IL).

**Stable Clones of B16F10 that Overexpress Untagged Human Angiostatin.** Subconfluent B16F10 cells were cotransfected with both the angiostatin expression vector (pMB0388) and a RSVneo expression vector using LipofectAMINE (Life Technologies, Inc.). Transfected cells were selected at 2 mg/ml G418 in MEM containing 10% FBS + nonessential amino acids for 2 weeks. The mass culture was split and cultured with G418 at a lower density for an additional 2 weeks. Single cell clones were picked and expanded in the presence of G418. The cell pellets were then prepared for the analysis of angiostatin mRNA expression.

**Detection of Angiostatin mRNA by Quantitative RT-PCR using the Taqman Method.** Total RNA was isolated from cell pellets or tumor samples with the BION101 FastRNA kit (Vista, CA). Samples were homogenized in a mixture of 8% glycerol; 100 mM nonessential amino acids for 2 h. The reaction was repeated 39 times. RT-PCR mixture consisted of 100 ng of RNA started at 60°C for 1.5 min and completed at 95°C for 15 s. The two-step PCR of standards, and absolute copy numbers were determined with a standard curve. The mass culture was split and cultured with G418 at a lower density for an additional 2 weeks. Single cell clones were picked and expanded in the presence of G418. The cell pellets were then prepared for the analysis of angiostatin mRNA expression.

**RESULTS**

**Secretion of Angiostatin after Transient Transfection of 293 Cells.** Codon-optimized cDNAs of murine and human angiostatin were synthesized and ligated into plasmid, pMB75.6, which provided a CMV-driven transgene expression. Both cDNAs encoded proteins that matched the kringle 1–4 region of plasminogen fused in-frame to an NH2-terminal immunoglobulin κ chain secretion signal. A HA-tag was added to the COOH terminus of the murine protein. Intracellular production of HA-tagged murine angiostatin and the secretion of the protein into the cell culture medium were investigated after transient transfection of 293 cells with the expression construct. Cell culture medium and protein extracts were collected 24 h after transfection and analyzed by immunoblotting. Western blot analysis with an anti-HA antibody revealed that a Mr. 50,000 angiostatin protein was produced and accumulated within the cells (Fig. 1A). Angiostatin was also secreted into the cell culture medium (Fig. 1B). The protein was not detected in B16F10 cells transfected with an expression vector for green fluorescent protein or in immunoprecipitations with an anti-Flag control antibody (Fig. 1, A and B). Sequence analysis of the secreted angiostatin protein at the NH2 terminus showed that the immunoglobulin κ chain signal peptide was cleaved correctly (data not shown).

**Constitutive Expression of Untagged Human Angiostatin in B16F10 Melanoma Cells.** To study the biological activity of the vector-encoded angiostatin, the CMV-driven expression vector for constitutive expression of human angiostatin was transfected into B16F10 melanoma cells. The CMV-driven expression vector for...
manner, and the inhibition was statistically significant (Fig. 3). The effect of angiostatin, mRNA concentrations were measured in tumor cells to examine whether these B16F10 transfectants became resistant to the angiostatin transgene expression (Fig. 3). Thus, antiangiogenic inhibition of tumor growth, as opposed to a direct effect of angiostatin, mRNA concentrations were significantly reduced in all tumors and varied from 0.35–1.6% (n = 18) of the mRNA concentration found in the three angiostatin-expressing cell clones before injection into the mice. The results indicate that angiostatin inhibition of tumor growth of B16F10 melanoma cells is directly proportional to the transgene expression and that expression of angiostatin can select for non-angiostatin-expressing tumor cells.

Inhibition of Metastatic Tumor Growth. Because inhibition of metastatic tumor growth is still a major challenge for cancer treatment, we investigated the inhibition of experimental lung metastasis by the angiostatin transgene. G418-selected B16F10 cells with undetectable angiostatin expression and cells with high expression of the transgene (3719 RU) were injected into the tail vein of C57BL/6 mice, and the number of lung nodules was counted 26 days later. Angiostatin significantly decreased the number of B16F10 nodules in the lung after injection of $2.5 \times 10^5$ cells/mouse (Fig. 4). None of the animals that received the angiostatin-expressing cells developed more than 3 nodules/lung, and the few nodules that developed expressed less than 1% of the transgene mRNA level found in the same cells before injection. In contrast, 50% of the mice that received the vector control-transfected B16F10 cells had more than 150 nodules/lung. The experiment was repeated with $5 \times 10^4$ cells/mouse and 10 animals/clone, and similar results were obtained (data not shown). The significant difference in metastatic growth was not triggered by divergent growth properties of the two cell clones because both clones had the same growth rate in the MTT proliferation assay (Fig. 2).

**DISCUSSION**

Notwithstanding advances in cancer therapy, treatment of most advanced cancers is unsuccessful, and melanomas are particularly resistant to conventional cancer treatment. In the search for more efficient therapeutics, a novel protein, angiostatin, was identified that inhibited tumor growth by blocking tumor neovascularization (3). Unfortunately, high doses of purified human angiostatin protein were needed to achieve a tumoricidal effect in mice (12, 13, 20), in part because human angiostatin appears to be rapidly cleared from the circulation (13). As an alternative to administration of purified, recombinant angiostatin protein, protocols were designed to establish angiostatin secretion in the vicinity of tumors by gene transfer of an angiostatin cDNA (16–18).

As a first step toward the use of human angiostatin as a plasmid-based gene therapeutic, we have tested a plasmid construct carrying a CMV-driven transgene encoding a secreted, kringle 1–4 human angiostatin. We specifically chose to express a four-kringle angiostatin instead of a smaller, three-kringle protein because stable expression of a transgene encoding kringles 1–4 of murine angiostatin was found to be a potent inhibitor of tumor growth in mice (16). It was also shown that kringle 4 is required for inhibition of endothelial cell migration by angiostatin (15). However, the antitumor effect of a cDNA that encoded only kringle 1–3 of human angiostatin has recently been reported (18), suggesting that inhibition of endothelial cell migration may not be essential for the tumor suppressor activity of angiostatin. To achieve secretion of the angiostatin protein after gene delivery, we fused the angiostatin cDNA sequence in-frame with a cDNA sequence encoding the murine immunoglobulin κ chain secretion signal. Although we did not select the plasminogen secretion signal used by others (16–18), a protein sequence analysis of secreted angiostatin showed that the immunoglobulin κ chain signal peptide was correctly cleaved and assured that secreted angiostatin did not have extra amino acids that could potentially trigger an immune response or hinder activity. Immunoblotting experiments detected a secreted, $M_r$ 50,000 samples from the angiostatin-expressing clones. Transgene mRNA concentrations were significantly reduced in all tumors and varied from 0.35–1.6% (n = 18) of the mRNA concentration found in the three angiostatin-expressing cell clones before injection into the mice. The results indicate that angiostatin inhibition of tumor growth of B16F10 melanoma cells is directly proportional to the transgene expression and that expression of angiostatin can select for non-angiostatin-expressing tumor cells.

**Expression of Human Angiostatin cDNA Does Not Inhibit Tumor Cell Growth in Culture.** The specificity of angiostatin for antiangiogenic inhibition of tumor growth, as opposed to a direct effect of angiostatin, cell clone that did not express angiostatin, a clone with high angiostatin expression (3719 RU), and parental B16F10 cells were seeded in 96-well plates, and cell growth was determined using the MTT cell proliferation assay. The three cell lines exhibited the same growth rate (Fig. 2). Thus, the expression of the angiostatin transgene does not intrinsically inhibit the growth of B16F10 cells.

**Inhibition of s.c. Tumor Growth Correlates with Angiostatin Expression.** The growth of angiostatin-expressing B16F10 cells in syngeneic C57BL/6 mice was compared with the growth of three vector control transfectants. Hence, $1 \times 10^6$ cells of either vector controls or stable transfectants with low (514 RU), medium (1707 RU), and high (3719 RU) angiostatin expression were s.c. implanted into the flanks of mice to monitor tumor growth. Tumors developed earliest in mice inoculated with vector control-transfected B16F10 cells that were G418-selected but did not express the transgene (controls; Fig. 3 A-C). At the same time, tumors also developed in mice implanted with B16F10 cells that exhibited low angiostatin transgene expression (Fig. 3A). However, tumors grew at a slower rate in this group than in the control groups. Subsequently, tumors were found in mice implanted with B16F10 cells that had medium angiostatin transgene expression (Fig. 3, A-C). The last group of mice to develop tumors carried the B16F10 cell clone with the highest angiostatin transgene expression (Fig. 3, A-C). Thus, angiostatin reduced tumor frequency (Fig. 3A) and volume (Fig. 3B) in a dose-dependent manner, and the inhibition was statistically significant (Fig. 3C). To examine whether these B16F10 transfectants became resistant to the effect of angiostatin, mRNA concentrations were measured in tumor cells, and stable clones were generated. Using the Taqman technology to measure mRNA concentrations, we identified three clones that had a low, medium, or high level of angiostatin transgene mRNA. Angiostatin expression was determined as the transgene copy number per 1000 copies of murine actin (in RU) and ranged from 514 RU for the clone with low angiostatin expression to 1707 RU for the clone with medium angiostatin expression to 3719 RU for the clone with the highest angiostatin expression.

![Cell Number vs. Day](image-url)
protein after transient transfection of 293 cells with a plasmid encoding the HA-tagged version of angiostatin. Proteolytic digestion of plasminogen was found to produce a \( M_r \) 38,000 angiostatin peptide (3), but other investigators reported a larger size, ranging from \( M_r \) 50,000 – 58,000, for recombinant four-kringle angiostatin (16, 20, 21). Thus, our results indicate that the engineered angiostatin protein was correctly secreted upon transgene expression in mammalian cells.

Previous studies showed that angiostatin protein selectively inhibited proliferation of endothelial cells but not of other cell types, including tumor cells (3, 16). We similarly found that expression of a high, constitutive level of untagged human angiostatin had no direct effect on tumor cell growth in culture. Expression of angiostatin in the B16F10 cells did not completely block s.c. and metastatic B16F10 tumor growth in mice but prolonged survival by significantly slowing tumor growth. Similar effects on tumor growth were obtained with angiostatin-transfected T241 fibrosarcoma cells (16) and after transduction of human cancer cells with retroviral (17) and adenoviral (18) vectors that carried the angiostatin transgene.

The suppression of primary B16F10 melanomas correlated directly with transient expression of the angiostatin transgene in vivo. Of note, angiostatin expression prolonged the survival of mice bearing s.c. B16F10 tumors without any direct effect on tumor growth. In contrast, angiostatin expression significantly inhibited the growth of metastatic B16F10 melanomas in mice but did not completely block tumor growth.

**Angiostatin Expression**

Fig. 3. Expression of human angiostatin inhibits s.c. tumor growth of B16F10 melanoma cells in a dose-dependent manner. Three clones with low (514 RU; ×), medium (1707 RU; +), and high expression (3719 RU; •) of untagged human angiostatin and three vector control clones (■, ▲, and ◆) were injected into the flank of C57BL/6 mice (1 × 10⁶ cells/injection; 11 mice/cell clone). Tumor frequency (A) and growth (B) were continuously monitored. In A, \( n = 11 \) for each point. In B, mean values of tumor volumes are shown (\( n = 10 \) for controls, \( n = 6 \) for 514 RU, and \( n = 5 \) for 1707 RU). Tumor growth of angiostatin-expressing clones was compared with tumor growth of vector controls at day 21 after cell injection (C); \( P = 0.007 \), pooled controls (\( n = 27 \)) versus 514 RU (\( n = 9 \)); \( P = 0.001 \), pooled controls (\( n = 27 \)) versus 1707 RU (\( n = 9 \)); \( P < 0.001 \), pooled controls (\( n = 27 \)) versus 3719 RU (\( n = 11 \)); Mann Whitney U rank-sum test. Box, 25–75% distribution. Bars, Minimum-Maximum.

Fig. 4. Angiostatin inhibits lung metastasis of B16F10 cells. A G418-selected B16F10 cell clone that did not express angiostatin (0 RU; ■) and a clone with a high angiostatin expression level (3719 RU; □) were injected into the tail vein of C57BL/6 mice (2.5 × 10⁶ cells/animal; 10 mice/group). The number of lung nodules was counted 26 days later. None of the animals died before the analysis.
with transgene expression levels. This was observed in repeated experiments. A relationship between angiostatin expression level and tumor growth inhibition has not been described previously. The level of transgene mRNA expression was notably high in the stable transfectants and ranged from 50–370% of the endogenous mRNA concentration. Because a relationship between angiostatin mRNA abundance and tumoristic activity was still encountered at this level of transgene expression, our data suggest that a high angiostatin expression level is needed to achieve tumor remission. In comparison, inhibition of primary tumor growth required the administration of 50–100 mg/kg/day of purified angiostatin (12, 13, 20) and was not seen below 10 mg/kg/day (13), whereas metastatic tumor was already affected at a dose of 1–2 mg/kg/day (13, 20).

Growth inhibition of angiostatin-transfected B16F10 tumors was transient and coincided with a dramatic decrease of angiostatin expression in those tumors. Escape from dormancy was also observed with angiostatin-transfected fibrosarcomas (16). It is possible that promoter silencing or a loss of the transgene could have led to the drop in transgene expression in the B16F10 tumors. B16F10 cells are genomically unstable (22), which could explain a loss of the transgene. Because genomic instability is found in many cancers, the gene therapeutic use of angiostatin may therefore require either repetitive delivery of the cDNA or selective transgene expression in genomically stable tumor endothelial cells to prevent the regrowth of tumors. Cationic liposome-plasmid DNA complexes have been shown to specifically target endothelial cells in tumors (23, 24) and may be used to deliver a plasmid-based expression vector. For optimal clinical benefits, plasmid DNA therapeutics could also be used in combination with established treatment procedures. An additive effect between angiostatin therapy and ionizing radiation has already been demonstrated (25).

In summary, expression of an angiostatin transgene can inhibit the growth of a melanoma cell line in mice, and delivery of angiostatin by gene therapy may offer a new opportunity to counter the growth of cancers that have been resistant to current cancer therapies, such as metastatic melanomas.

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