Antiangiogenic Gene Therapy for Cancer via Systemic Administration of Adenoviral Vectors Expressing Secretable Endostatin

CHeauYun T. Chen,1 Jane Lin,1 Qin Li,1 Sandrina S. Phipps,1 John L. Jakubczak,1 David A. Stewart,1 Yelena Skripchenko,1 Suzanne Forry-Schaudies,1 Jeanette Wood,2 Christian Schnell,2 and Paul L. HalleNbeck1

ABSTRACT

A growing number of antiangiogenesis strategies have been investigated for the treatment of cancer and other angiogenesis-dependent diseases. One of the most promising strategies is to systemically administer one or more antiangiogenic proteins frequently enough to achieve a sufficient long-term steady state level of the protein(s) to achieve the maximum beneficial effect. However, the utility of this strategy is limited because of many technical difficulties, including obtaining both the quantity and quality of the protein(s) necessary for optimal therapeutic benefit. To overcome these difficulties, we hypothesized that a single administration of a replication-defective adenoviral vector expressing a secretable antiangiogenic protein could achieve an optimal long-term systemic concentration. We constructed a recombinant adenoviral vector, Av3mEndo, which encodes a secretable form of murine endostatin. We demonstrated secretion of endostatin from several cell lines transfected with Av3mEndo. Partially purified endostatin secreted from Av3mEndo-transduced mammalian cells was shown to potently inhibit endothelial cell migration in vitro. A single intravenous administration of Av3mEndo in mice was shown to result in (1) prolonged and elevated levels of circulating endostatin, (2) partial inhibition of VEGF-induced angiogenesis in a VEGF implant angiogenesis model, and (3) prolonged survival and in 25% of mice the complete prevention of tumor growth in a prophylactic human colon/liver metastasis xenograft murine model. These results support our contention that adenoviral vector-mediated expression of an antiangiogenic protein(s) represents an attractive therapeutic approach to cancer and other angiogenesis-dependent diseases.

OVERVIEW SUMMARY

We constructed an E1, E1a, and E3-deleted adenoviral vector, Av3mEndo, which expressed a secretable form of murine endostatin from the RSV promoter. Transfection of Hep3B (human hepatoma) and S8 (human lung carcinoma-derived) cell lines by Av3mEndo resulted in the efficient expression and secretion of murine endostatin. The secreted endostatin was biologically active as shown by its potent inhibition of VEGF-induced HUVEC migration. The effects of a single intravenous administration of Av3mEndo were tested in a murine VEGF implant angiogenesis model and an orthotopically implanted human colon/liver metastasis xenograft model. Treatment demonstrated (1) prolonged and elevated levels of circulating endostatin in all tested models, (2) correlation of liver transcription and circulating endostatin levels, (3) partial inhibition of VEGF-induced angiogenesis in a VEGF implant angiogenesis model, and (4) prolonged survival and in 25% of mice the complete prevention of tumor growth in the prophylactic human colon/liver metastasis xenograft murine model.

1Gentic Therapy, a Novartis Company, Gaithersburg, MD 20878.
2Oncology Research, Novartis, CH-CH002 Basel, Switzerland.
INTRODUCTION

Angiogenesis, the process of new blood vessel formation from existing vessels, is a fundamental process in cancer development (Folkman, 1971; Hanahan and Folkman, 1996). Many proteins possessing an angiogenic activity have been shown to elicit antitumor effects, including thrombospondin (Good et al., 1990; Volpert et al., 1998), interferon α (Dvorak and Greser, 1988; Singh et al., 1995), and platelet factor 4 (Maione et al., 1990). Application of angiogenesis strategies for cancer therapy have been extensively studied and reviewed by Zetter (1998). Among them, endostatin, originally discovered from conditioned medium of murine hemangioendothelioma cells (O'Reilly et al., 1997), was shown to elicit potent antitumor effects in experimental murine tumor models (Boehm et al., 1997). Several cycles of prolonged treatment utilizing systemic administration of purified endostatin resulted in complete tumor regression in several murine tumor models with no drug resistance or side effects (Boehm et al., 1997). However, this protein-based therapy is likely to require repeated and long-term administration of high-quality endostatin for optimal therapeutic benefit.

Endostatin is a carboxy-terminal peptide of collagen XVIII (O'Reilly et al., 1997). The crystal structure of murine endostatin (Hoben et al., 1998) and human endostatin (Ling et al., 1998) has been well characterized. Both proteins have been shown to inhibit endothelial cell proliferation and migration, to induce G1 arrest and apoptosis of endothelial cells in vitro, and to have a potent antitumor effect in vivo in several independent studies (O'Reilly et al., 1997; Blezinger et al., 1999; Chen et al., 1999; Dhanabal et al., 1999a-c; Yamaguchi et al., 1999). In a transgenic mouse model of pancreatic islet cell carcinoma (RIP1-Tag2), the murine version of the protein was shown to block initial tumor formation and progression. Endostatin had no effect on solid tumor regression by itself, but in conjunction with angiostatin the protein reduced solid tumor regression by 58.9% (Bergers et al., 1999).

Although an angiogenic gene therapy approach has been reported in several studies with various angiogenic inhibitors, e.g., angioptatin (Grisselli et al., 1998; Nguyen et al., 1998; Tanaka et al., 1998), platelet factor 4 (Tanaka et al., 1999), endostatin (Nguyen et al., 1998; Blezinger et al., 1999; Chen et al., 1999), antisense mRNA against vascular endothelial growth factor (VEGF) (Nguyen et al., 1998), and soluble RANTES (Kong et al., 1998), most of them were designed for in situ delivery, which at best would be expected to affect only the tumor where the vector was injected. One report (Blezinger et al., 1999) showed that intramuscular injection of a plasmid encoding endostatin could produce a concentration of circulating endostatin of 8 ng/ml. Intravenous delivery of liposome-complexed plasmid DNA (Chen et al., 1999) also demonstrated a systemic level of endostatin at 33 ng/ml. However, in both studies the elevation in circulating endostatin was transient. Not surprisingly, the effects on tumor growth were moderate, which seems likely to be due to the low and transient level of circulating endostatin observed.

Intravenous delivery of recombinant adenoviral vectors has previously been shown to result in sustained levels of factor VIII expression and phenotypic correction (Connelly et al., 1996). We hypothesized that the same gene delivery system would be useful to achieve a prolonged and high concentration of a circulating angiogenic protein such as endostatin, which has been previously shown to be necessary for optimal therapeutic benefit.

The current study was designed to test whether this angiogenesis gene therapy strategy could have the potential to effectively treat primary and metastatic tumors. We have constructed a replication-deficient (E1-, E2A-, E3-deleted) adenovirus serotype 5 (Ad5)-based vector, Av3mEndo, to express and secrete murine endostatin from vector-transduced cells. Cell lines transduced with Av3mEndo were shown to secrete high levels of endostatin. Partially purified endostatin secreted from Av3mEndo-transduced cells was shown to potently inhibit endothelial cell migration in vitro. A single intravenous delivery of Av3mEndo in mice resulted in a circulating level of endostatin of up to 700 ± 435 ng/ml. A single intravenous administration of Av3mEndo was also shown to inhibit VEGF-dependent angiogenesis in an in vivo growth factor implant model and significantly prolonged survival in a proangiogenic human colon/cancer metastasis xenograft mouse model. In fact, mice killed 4 months after the death of all controls in the metastasis model showed no primary or metastatic tumors. These results support our contention that adenoviral vector-mediated expression of an angiogenic protein(s) represents an attractive therapeutic approach to cancer and other angiogenesis-dependent diseases. These results indicated that the angiogenic gene therapy strategy described herein may be therapeutically beneficial for the treatment of disseminated cancer and potentially many other angiogenesis-related diseases.

MATERIALS AND METHODS

Cell lines

Human umbilical vein endothelial cells (HUVECs) were obtained from Cascade Biologies (Portland, OR), and were cultured in M200 supplemented with low serum growth supplement (LSGS; Cascade Biologies). A549 (human lung carcinoma), Hep3B (human hepatocellular carcinoma), and 293 (human embryonic kidney) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and routinely cultured in Richter's complete medium (CM) with 5% fetal bovine serum (FBS); Eagle's minimal essential medium (EMEM) with 10% FBS; and Richter's CM with 10% FBS, respectively. S8 (derived from A549 cells and described in PCT Application No. WO97/25446, published July 17, 1997) were made at Genetic Therapy, Inc. (GTT, Gaithersburg, MD) and routinely cultured in Richter's CM with 5% FBS.

PCR and assembly of murine endostatin cDNA and Ig-k leader sequence

The mouse endostatin (mEndo) cDNA was amplified by polymerase chain reaction (PCR) from mouse collagen XVIII clone ID 748987 from Genome Systems (St. Louis, MO) with the primers 5'-ACT GGT GAC GCG GCC CAT ACT G-3' and 5'-AAG GGC TAT CCA TCT AGG TGG CAG AGG CCT AT-3' (598-bp Fl fragment).
ANTIANGIOGENIC GENE THERAPY FOR CANCER

The mouse immunoglobulin κ chain leader sequence (Igk leader) was PCR amplified from cDNA (Avitrogen, Carlsbad, CA) with the primers S-CAC TGG TTA CTG CCT TAT CG-3’ and S-CAG TGG CAG AGG CRT CTG ACC AGT G3-3’ (147-bp F2 fragment). PCR was carried out with Pfu DNA polymerase (Stratagene, La Jolla, CA) for 35 cycles under the following conditions: 95°C for 30 sec, 95°C denaturation for 1 min, 55°C annealing for 1 min, and 72°C extension for 2 min. The DNA fragments were gel purified.

The sig-mEndo chimeric DNA (718 bp) was generated by PCR with overlap extension (Horton et al., 1990) with F1 and F2 DNA fragments generated above as templates to assemble mouse Igk leader sequence and murine endostatin cDNA. PCR was carried out with the primers S-CAC TGG TTA CTG CCT TAT CG-3’ and S-AAG GCC TAT CGA TCT AGG TGG CAG AGG CCT AT-3’, using Pfu DNA polymerase (Stratagene). PCR was run for 35 cycles under the following conditions: 95°C hot start for 3 min, 95°C denaturation for 1 min, 60°C annealing for 1 min, and 72°C extension for 2 min.

The pAmvEndo+Lrr adenoviral shuttle plasmid was constructed by inserting the 718-bp sig-mEndo chimeric DNA into the NheI and ClaI sites of adenoviral shuttle plasmid, pAv2995, which is downstream of the Rous sarcoma virus (RSV) promoter and upstream of the simian virus 40 (SV40) polyadenylation signal. The shuttle plasmid contains a LoxP site for Cre/lox-mediated recombination. The entire region of the sig-mEndo in the pAvEndo+Lrr adenoviral plasmid was confirmed by direct sequencing analysis by the Gene Therapy Core Technologies Molecular Core Laboratory at GTI.

Construction of recombinant adenoviral vector Av3mEndo

The recombinant Av3mEndo (with E1, E2a, and E3 deleted), encoding the sig-mEndo chimera, was generated with two plasmids, pSQ3 and pAvEndo+Lrr, by Cre/lox-mediated recombination. The pSQ3 plasmid contains a loxP site followed by the Av3 gene deletion of the region from the left end inverted terminal repeat (ITR) to the end of E1a. pAvEndo+Lrr and pSQ3 were first linearized with NstI and ClaI restriction enzymes, respectively. A transient transfection was performed with 293 cells (4 x 10⁶ cells per well of a six-well plate), using the calcium phosphate mammalian transfection system (Promega, Madison, WI). The calcium phosphate-DNA precipitate was prepared with 4.8 g of linearized pAmvEndo+Lrr, 12 g of linearized pSQ3, 6 g of pcmVE2a, and 6 g of pcmVE30 in a total volume of 1.8 ml. A 0.6 ml calcium phosphate-DNA precipitate was added to each well. The 293 cells were incubated with calcium phosphate-DNA precipitate at 37°C for 16 hr. The precipitate was removed and the cells were washed with phosphate-buffered saline (PBS). Fifteen days posttransfection, cytopathic effect (CPE) was observed. The cells and the medium were then harvested by scraping. The crude viral lysate was prepared by five cycles of freezing and thawing. The Av3mEndo vector was reamplified in S3 cells with 0.5 mM dexamethasone in Richter’s CM containing 5% FBS until CPE was observed. The adenoviral vector titer (particles per milliliter) and biological titer (plaque-forming units [PFU] per milliliter) were determined as described (Mirraeder et al., 1995). The correct genome structures of the purified Av3mEndo and control Av3Null were confirmed by restriction digests and Southern blot analysis. The Av3mEndo seedlot was confirmed to be negative for replication-competent adenovirus (RCA) by the Gene Therapy Core Technologies Molecular Core Laboratory at GTI.

Preparation of mEndo and Null supernatant protein from vector-transduced cells

The mEndo and Null supernatant proteins were prepared from Av3mEndo- and Av3Null-transduced S3 cells, respectively. The cells were incubated with vectors at a particle-to-cell ratio of 750 for 3 hr. The infection medium containing the vector was then removed and replenished with fresh medium. Seventy-two hours posttransfection, the supernatant was collected and filtered through a 2-μm pore size filter. Each 40 ml of supernatant was passed through a 1-mL heparin-Sepharose 6B column (Amersham Pharmacia Biotech, Piscataway, NJ) equilibrated with 50 mM Tris·HCl (pH 7.5), 0.1 M NaCl, and 10% glycerol. After the unbound protein was washed with 50 mM Tris·HCl (pH 7.5), 0.1 M NaCl, and 10% glycerol, the heparin column-bound protein was eluted with 4 ml of buffer containing 300 mM Tris·HCl (pH 7.5), 1 M NaCl, and 20% glycerol. The protein concentration was determined with a Bio-Rad (Hercules, CA) protein assay kit. The supernatant protein was aliquoted and stored at -70°C until use. The protein was routinely dialyzed against Hanks’ balanced salt solution (HBSS) before analysis.

SDS-PAGE and N-terminal protein sequencing analysis

The partially purified supernatant mEndo and Null proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Each 60 μg of supernatant protein was mixed with Laemmli sample buffer (Sigma, St. Louis, MO) and heated at 95°C for 3 min. The denatured protein was loaded on a 4-12% linear gradient precast gel (Bio-Rad). The gel was stained with GelCode blue stain reagent (VWR Scientific Products, Willard, OH) to visualize the protein bands. For N-terminal protein sequencing analysis, the mEndo protein was analyzed by SDS-PAGE performed in a similar way, except that the protein was transferred to polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was stained with 0.1% Coomassie Blue R-250 in 40% methanol and 1% acetic acid for 2 min, followed by four destaining washes with 50% methanol for 15 min per wash. The membrane was air dried and the 20-kDa protein band corresponding to the expected molecular mass of murine endostatin was subjected to N-terminal protein sequencing analysis by automated Edman degradation, using a Perkin-Elmer Biosystems (Foster City, CA) protein sequencer (Protein Sequencing Midwest Analytical, St. Louis, MO).

ELISA detection of mEndo secretion

Secretion of murine endostatin was routinely determined with a murine endostatin enzyme-linked immunosorbent assay (ELISA) kit (ACCUCYTE murine endostatin; Cytimmune Sciences, College Park, MD) according to the manufacturer procedure.
Migration assay

Cell migration was assessed in 48-well chemotaxis chambers (NeuroProbe, Cabin John, MD) as described (Polyverine et al., 1991). Polycarbonate (8 μm) membrane (VWR Scientific Products) was coated with bovine collagen type I (0.1 mg/ml; Becton Dickinson Labware, Bedford, MA) in 0.2 N acetic acid according to the following procedure. Polycarbonate membrane was soaked in 0.3 N acetic acid overnight. The membrane was rinsed with PBS. Collagen type I (bovine) was digested in 0.2 N acetic acid to a final concentration of 0.1 mg/ml. The membrane was soaked in collagen (0.1 mg/ml) for 30 min and air dried. Low-passage (passage 2 or 3) HUVEC's were cultured in M200 supplemented with L-SDS (Cascade Biologics) until the migration assay. Cells were suspended in migration assay medium, M199 plus 1% FBS, to a cell density of 2 × 10^6 cells/ml and preincubated in the presence or absence of the partially purified supernatant protein from either Av3mEndo- or Av3null-transduced S5 cells as described above. Cell mixtures were then incubated at 37°C with 5% CO2 for 30 min. VEGF165 (R&D Systems, Minneapolis, MN) at the indicated concentrations was prepared in migration assay medium and added to the bottom chamber. After assembly with a collagen type I-coated polycarbonate membrane between the top and bottom chambers, 50 μl of the preincubated cell suspension (equivalent to 10,000 cells) described above was added to the top chamber. The membrane was removed after 5 hr of incubation and was stained with Diff-Quik (VWR Scientific Products). The nonmigrated cells in the top chamber were removed by wiping with tissue. Cells that migrated through the membrane to the bottom chamber were quantified by counting cell nuclei under a microscope. The basal migration was determined with migration assay medium in the absence of any test substance, mEndo, Null, or angiogenic factor, VEGF165. Assays were carried out in quadruplicate under each condition.

Colon liver metastasis model

Male athymic CD-1 nude mice between 4 and 5 weeks of age were purchased from Charles River Laboratories (Wilmington, MA). The mice were maintained in a high-efficiency particle-arresting (HEPA) filtered environment with cages, food, and bedding sterilized by autoclaving. Animal diets were purchased from Harlan Teklad (Madison,WI). Ampicillin (5%, v/v; Sigma) was added to the autoclaved drinking water. Mice were treated with Av3mEndo vector or the control vector Av3null both at 2 × 10^11 particles/mouse, by tail vein injection in a final volume of 100 μl. The controls were also carried out with HBSS saline alone at 100 μl per mouse. The steady state concentration of endostatin in the circulation was determined 1 day prior to vector injection and 10 days after vector injection, utilizing the murine endostatin ELISA kit (Cytimmune). Thirteen days after vector injection, tumor fragments derived from poorly differentiated human stage IV colon adenocarcinoma (T3N1M1) were transplanted by surgical orthotopic implantation as described by Sun et al. (1995). After tumor implantation, there were 11, 17, and 16 mice surviving in HBSS, Av3Null, and Av3mEndo-treated groups, respectively. The animal survival was then monitored throughout the study. The percent survival was calculated relative to the number of mice surviving the orthotopic surgical tumor implantation procedure (100%). Mouse survival was analyzed by using a Kaplan-Meier survival plot followed by log-rank (Mantel-Cox) test to identify significant differences in survival between groups. The significance level was at p < 0.05.

In vivo growth factor implant angiogenesis model

The angiogenic effects of the vector-generated endostatin were tested in an in vivo growth factor implant model. Female: C3HBL/6 mice were treated with Av3mEndo at 2 × 10^11 particles/mouse by tail vein injection in a final volume of 100 μl. The controls were treated identically with Av3null vectors at 2 × 10^11 particles/mouse or HBSS alone at a final volume of 100 μl per mouse. Thirteen days after vector injection, the parous Tacon chambers filled with 0.3% agar containing heparin (20 U/ml) and VEGF165 (3 μg/ml) were subcutaneously implanted. Seven days after chamber implantation, animals were killed with 3% isoflurane and pentobarbital (210 mg/kg) injected intraperitoneally. Chambers were then removed from each animal. The vascularized fibrous tissue formed around each implant was then removed from each chamber and weighed (wet weight) immediately. Tissue samples were then homogenized after addition of 2 ml of distilled water. The samples were then centrifuged for 1 hr at 7000 rpm. The supernatant was filtered through a 0.45-μm GH Cellulose filter (Acrodisc GF; Gelman Sciences, Ann Arbor, MI) to avoid contamination. The amount of hemoglobin present in the supernatant was determined by spectrophotometric analysis at 540 nm, using a Drabkin reagent kit (Sigma hemoglobin 525; Sigma Chemical, Poole, Dorset, UK). An aliquot of the filtrate (100 μl) was added to 1 ml of Drabkin's solution and the mixture was incubated for 15 min at room temperature. The absorbance at 540 nm is proportional to the hemoglobin concentration. The hemoglobin measurements were then converted to a blood volume measurement (microliters), using a calibration curve with known standards from a donor mouse. The statistical significance of inhibition was compared between groups by Dunnett's test. The significance was set at p < 0.05. Plasma was collected at the time of sacrifice and endostatin concentrations were determined with the murine endostatin ELISA kit (Cytimmune).
ANTIANGIOGENIC GENE THERAPY FOR CANCER

A

\[\text{Diagram showing the process of gene therapy for cancer.}\]

B

\[\text{Diagram showing different Av3 constructs.}\]
Liver transduction analysis

Male C57BL/6J mice between 8 and 9 weeks old were purchased from Harlan Sprague Dawley (Indianapolis, IN). Mice were then intravenously injected via the tail vein with Av3mEndo or the control Av3Null vector at 2 x 10^11 particles/mouse in 100 μl, or with the equivalent volume of PBS. Sixteen days after vector injection, all mice were killed. Liver samples were collected to determine the Av3mEndo DNA content by Southern blot analysis. Blood was collected for endostatin ELISA analysis.

Genomic DNA was isolated from frozen liver samples of Av3mEndo-, Av3Null-, or HBSS-treated mice, using the genomic DNA isolation kit from Qiagen (Valencia, CA). Frozen liver was minced and treated with protease at 35°C for 18 hr in a hybrid oven. After centrifugation, DNA was then isolated from the supernatant by using the Qiagen column. DNA was digested with NcoI and resolved on a 1% agarose-TAE gel. After transfer to a nylon membrane, the membrane was prehybridized in 5X Denhardt's, 6X SSC (saline-sodium citrate), 10 mM EDTA, 0.5% SDS, and salmon sperm DNA (0.1 mg/ml) at 68 ± 2°C for 2 hr. The membrane was then hybridized with a 32P-labeled sig-mEndo internal probe at 68 ± 2°C and washed in SSC/SSC-containing buffers at 68 ± 2°C according to the standard protocol. For quantitative analysis, various amounts of pAvmEndoLXR shuttle plasmid were added to 10 μg of genomic DNA isolated from the livers of treated mice to establish a standard curve. The 32P radioactivity of the sig-mEndo chimeric DNA band was determined by PhosphoImager (Molecular Dynamics, Sunnyvale, CA) analysis. The Av3mEndo DNA content and the copy number of sig-mEndo chimeric DNA per hepatocyte were determined by interpolation from the standard curve.

RESULTS

Generation of a recombinant adenoviral vector encoding murine endostatin

The murine endostatin gene cassette was PCR amplified from a plasmid containing the murine α1 (XVIII) collagen gene. The DNA was assembled with a murine Ig-k secretion signal leader to generate a secretable form of endostatin (sig-mEndo) by PCR splicing overlap extension (Horton et al., 1990). The expected sig-mEndo chimeric DNA sequence was confirmed by sequencing and subsequently cloned into the adenoviral shuttle plasmid to create pAvmEndoLXR. The adenoviral vector encoding the sig-mEndo chimeric sequence was generated by transient transfection with pSG3 and pAv3mEndoLXR plasmids in 293 cells through Cre/lox-mediated recombination as shown in Fig. 1A. The Av3mEndo and control Av3Null vectors are schematically shown in Fig. 1B. Vector construction was confirmed by restriction analysis and Southern blots (data not shown).

Av3mEndo-mediated endostatin expression and secretion

Av3mEndo-mediated murine endostatin expression was determined by Northern blot analysis of vector-transduced A549 cells (data not shown). The expected sig-mEndo mRNA was detected in Av3mEndo-transduced but not in untransduced or Av3Null vector-transduced cells. The expression and secretion of murine endostatin protein were characterized further in vector-transduced S8 cells. Supernatant from Av3mEndo- and Av3Null-transduced S8 cells was collected 72 hr posttransduction and partially purified from a heparin-Sepharose column as described in Materials and Methods. The partially purified proteins were subjected to SDS-PAGE (Fig. 2). A distinct 20-kDa protein band corresponding to the expected molecular mass of murine endostatin was observed in Av3mEndo-transduced cells but not in Av3Null-transduced cells. This 20-kDa protein was subjected to N-terminal protein sequence analysis and it was demonstrated that the cleavage of the signal peptide from murine endostatin occurred at the expected sites. To quantify the amount of murine endostatin that was secreted, we measured the concentration of murine endostatin in Av3mEndo-transduced Hep3B cells. We demonstrated that Av3mEndo could elicit the production of 1-2 μg of murine endostatin in 10^6 transduced Hep3B cells per 24 hr (Fig. 2B).

Functional characterization of murine endostatin in vivo

To determine if the secreted endostatin was biologically active, we prepared partially purified protein from the supernatants of either Av3mEndo- or Av3Null-transduced S8 cells as described above. The partially purified mEndo protein potently inhibited VEGF165-induced HUVEC migration, whereas no effect was observed with Null supernatant protein (Fig. 3A and B). The median effective concentration (EC50) for inhibition of migration for the partially purified mEndo protein was 0.17 ± 0.05 ng of total protein/ml as determined from three independent experiments. Similar results were observed with transduced Hep3B cells (results not shown). We also examined whether basic fibroblast growth factor (bFGF)-stimulated HUVECs or CPAE (a bovine pulmonary arterial endothelial cell line) cell proliferation could be inhibited by the partially purified endostatin. Our partially purified mammalian expressed endostatin potently inhibited bFGF-stimulated CPAE cells at 10 ng/ml, confirming previous reports (Dhanabal et al., 1999a). However, we were not able to demonstrate inhibition of bFGF-stimulated HUVECs at 1 μg/ml, as previously reported (Yamaguchi et al., 1999). These differences could most likely be attributed to the source of endostatin, among other possibilities as well.

Dose-dependent expression in vivo of endostatin after systemic Av3mEndo administration

To determine whether the Av3mEndo vector could also elicit the secretion of murine endostatin in vivo we injected 2 x 10^11 particles of Av3mEndo or Av3Null, or an equal volume of HBSS, via the tail vein into C57BL/6j male mice. Sixteen days after vector injection, all mice were killed. The sig-mEndo chimeric DNA in the liver was determined by Southern blot analysis. All Av3mEndo-treated mice showed sig-mEndo gene transduction in the liver, while no sig-mEndo liver transduction was shown in Av3Null- or HBSS-treated mice. As shown in Table 1, Av3mEndo-treated mice had a serum endostatin concentration of 709 ± 435 ng/ml, while the Av3Null- and HBSS-treated mice had serum concentrations of 56 ± 19 and
The concentration of circulating endostatin was also shown to be dependent on the amount of liver transduction as depicted in Fig. 4. Trend-line statistical analysis demonstrated that there was a positive correlation between the amount of Av3mEndo liver transduction and the amount of circulating endostatin. Thus, a single systemic administration of Av3mEndo resulted in high levels of circulating murine endostatin approximately 12- to 14-fold above the endogenous level in this strain of mice.

In vivo growth factor implant angiogenesis model

To examine the effect of systemic administration of Av3mEndo on angiogenesis, we examined VEGF-induced an

FIG. 2. Adenovirus-mediated expression and secretion of murine endostatin in vector-transduced cells. (A) The mEndo (lane 2) and Null (lane 3) partially purified supernatants from vector-transduced S8 cells were analyzed by SDS-PAGE (see Materials and Methods). Sixty micrograms of each partially purified supernatant protein was analyzed on 4 to 12% linear gradient precast gels. The protein standard was run in lane 1. The gel was stained with GelCode blue stain reagent to visualize the protein bands. The arrow marks the expected mass of murine endostatin (20 kDa). After being transferred to a PVDF membrane from a duplicate SDS-polyacrylamide gel, the 20-kDa protein band was excised and subjected to N-terminal protein sequencing analysis. The determined protein sequence is shown with arrows marking the beginning of the N termini of two major secreted proteins. Eighty percent contained additional amino acid residues (DAA) upstream of the reported N terminus of endostatin, and 20% contained an A residue from the murine Igk signal peptide. (B) Hep3B cells (10^5) were transduced by the Av3mEndo vector at 10 FFU/cell. The collected culture medium was analyzed by murine endostatin ELISA (Materials and Methods).
FIG. 3. Inhibition of HUVEC migration by secreted murine endostatin. (A) VEGF165 at various concentrations was added to induce HUVEC migration. The migrated HUVECs were determined after 5 hr of incubation (Materials and Methods). The fold increase in migrated HUVECs was calculated relative to the migrated HUVECs in the absence of VEGF165. (B) Supernatant proteins were prepared from S8 cells transduced with Av3mEndo or Av3Null vectors and partially purified as described in Materials and Methods. The migrated HUVECs in the absence of VEGF165 were subtracted to determine the percentage of HUVECs induced to migrate by VEGF165. Percent migration was normalized on the basis of 100% migration in the presence of VEGF165 at 10 ng/ml and in the absence of Null or mEndo supernatant proteins. Results represent means ± standard deviation of four replicates.
**Table 1. Blood Levels of Endostatin**

<table>
<thead>
<tr>
<th></th>
<th>Colon liver metastasis model</th>
<th>Growth factor implant angiogenesis model</th>
<th>Liver transduction analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ng/ml</td>
<td>N</td>
<td>ng/ml</td>
</tr>
<tr>
<td>Prebleed</td>
<td>42.4±11.2</td>
<td>11</td>
<td>ND</td>
</tr>
<tr>
<td>HBSS</td>
<td>39.2±11.5</td>
<td>11</td>
<td>14.4±9.5</td>
</tr>
<tr>
<td>Av3Null</td>
<td>68.2±20.1</td>
<td>11</td>
<td>25.2±7.3</td>
</tr>
<tr>
<td>Av3mEndo</td>
<td>327.9±126.6</td>
<td>16</td>
<td>276.8±146.2</td>
</tr>
</tbody>
</table>

Strain: Athymic CD-1 nude mice
Day (after vector injection): 10

*Mouse serum or plasma was collected as described in text. Levels of murine endostatin were determined with a murine endostatin ELISA kit as described. The prebleed was collected 2 days prior to vector injection from randomly selected mice. Results represent means ± standard deviation, which were calculated from the number of mice in each group (N represents number of mice per group). ND, Not determined.

Angiogenesis by utilizing an *in vivo* VEGF implant angiogenesis model. Female C57BL/6 mice were treated with Av3mEndo or Av3Null vector at 2 × 10⁹ particles/mouse, or with an equal volume of HBSS, by a single tail vein injection. Porous chambers containing 0.8% agar or 0.8% agar plus 2 mg of VEGF were subcutaneously implanted in mice on day 14 after vector injection (Fig. 5A). Tissue surrounding the implanted chamber was collected for angiogenesis analysis 21 days after vector injection. Serum was also collected on day 21 to measure the amount of circulating murine endostatin in this model. Intravenous delivery of Av3mEndo was shown to inhibit angiogenesis *in vivo* by reducing the VEGF-induced blood weight and blood volume in the implanted chambers by 55 and 66%, respectively (Fig. 5B and C, p < 0.05). Av3Null had no effect.

**FIG. 4.** Dose-dependent expression of endostatin after systemic Av3mEndo administration *in vivo*. Male C57BL/6J mice were treated with Av3mEndo or the control Av3Null vectors at 2 × 10⁹ particles/mouse, or with HBSS at 100 μl/mouse, by tail vein injection (Materials and Methods). Sixteen days after vector cell injection, all mice were killed. Liver transduction (copy number of sig-mEndo gene per hepatocyte) was determined by Southern blot analysis. Blood endostatin concentration was determined by murine endostatin ELISA analysis (Materials and Methods). Av3Null- and HBSS-treated mice showed no sig-mEndo gene in the liver. The amount of circulating endostatin in the blood stream was plotted against the copy number of vectors per hepatocyte. Each measurement represents the average of three determinations from each Av3mEndo-treated mouse. Trend-line statistical analysis was performed to determine whether there was a linear dose response.
A

Schedule

Day 0
i.v. vector injection
2 x 10^{11} particles per mouse

Day 14
Subcutaneous implantation of VEGF porous tissue chambers

Day 21
Angiogenesis analysis of blood weight and content

B

![Graph showing weight (mg) vs. treatment groups]

C

![Graph showing blood content (µl) vs. treatment groups]

FIG. 5. In vivo growth factor implant angiogenesis model. (A) Female C57BL/6 mice were treated with Av3mEndo or control Av3Null vectors at 2 x 10^{11} particles/mouse, or an equal volume of HBSS (control), by tail vein injection. The porous tissue chambers filled with 0.8% agar and VEGF165 (2 µg/ml) were subcutaneously implanted. Seven days after chamber implantation, the chamber was removed. The angiogenic response was quantified by measuring (B) the weight of the vascularized tissue that grows around the implant and (C) the blood volume of this tissue as described (p < 0.05 in comparison with HBSS-treated group and Av3Null-treated group).

on VEGF-induced angiogenesis in this model. As shown in Table 1, Av3mEndo-treated mice had elevated levels of endostatin with a mean of 277 ± 146 ng/ml. In contrast, Av3Null- and HBSS-treated mice had endostatin levels below 40 ng/ml.

Naturally occurring colon liver metastasis model

Intravenous delivery of adenoviral vectors into mice via tail vein injection has been previously shown to result in efficient transduction of the liver and persistent concentration of circulating levels of secretable gene products expressed from the vector (Smith et al., 1993; Connelly et al., 1996). Thus, we expected that the highest concentration of endostatin expressed subsequent to tail vein injection of the Av3mEndo vector might be in the liver. In addition, we hypothesized that endostatin may have the most profound effect on liver metastases that have to induce angiogenesis in order to grow (Hanahan and Folkman, 1996). Thus we reasoned that systemic injection of the Av3mEndo vector might have the most profound effect on liver metastases. To determine if the systemic delivery of Av3mEndo can inhibit liver metastases, we tested a single tail vein injection of Av3mEndo in a naturally occurring colon liver metastasis model (Sun et al., 1999) in a prophylactic manner (Fig. 6A). Male athymic CD-1 nude mice were treated with Av3mEndo or Av3Null vector at 2 x 10^{11} particles/mouse or an equal volume of HBSS by tail vein injection. Tumor fragments established from poorly differentiated human stage IV colon adenocarcinoma (T3N1M1) were implanted by surgical orthotopic implantation onto the top of the ascending colon of vector-treated mice on day 13 after vector injection. In independent studies, this tumor model showed that microscopic liver metastasis developed 7-10 days after orthotopic transplantation, with a few cases of lymph node metastasis. Mice died of serious late-stage liver metastasis (Sun et al., 1999). The control mice treated with Av3Null (17 of 17) or HBSS (12 of 12) all died 2 months after tumor implantation (Fig. 6B). In contrast, Av3mEndo-treated mice survived significantly longer, with 25% of Av3mEndo-treated mice still alive 4 months after all other animals succumbed to metastases in the liver (Fig. 6C) (p < 0.05). These mice were then killed on day 188 after t-
A Schedule

(Day 10 post vector injection)

Day -13
i.v. vector injection
2 x 10^11 particles/mouse

Day -3
Serum collection

Day 0
Tumor implantation

Monitor survival

B

% Survival

Days post tumor implantation

FIG. 6. Colon liver metastasis model. (A) Athymic nude mice were treated with Av3mEndo or control Av3Null vectors at 2 x 10^11 particles/mouse, or with an equal volume of HBSS, by tail vein injection. Tumor fragments derived from a poorly differentiated human stage IV colon adenocarcinoma were implanted by surgical orthotopic implantation in the colon as described in Materials and Methods. (B) Survival was monitored throughout the study and normalized to the number of mice surviving tumor implantation (p < 0.05 in comparison with Av3Null-treated group and HBSS-treated group).

To determine the circulating endostatin level, blood samples were collected 10 days after vector injection (day -3 of tumor implantation) and the concentration of circulating endostatin was determined. As shown in Table 1, mice treated with Av3mEndo vector showed elevated levels of endostatin, with a mean of 328 ± 127 ng/ml, which is five- to eight-fold higher than in Av3Null- or HBSS-treated mice, respectively (Table 1).

The relationship between circulating endostatin levels on day 10 after vector injection (day -3 of tumor implantation) and survival is shown in Table 2. HBSS-treated mice had a mean survival of 36.6 days, and Av3Null-treated mice had a mean survival of 30.2 days. In contrast, Av3mEndo-treated mice showed a significantly longer mean survival, of 84.8 days (p < 0.05). Of particular note, 25% of the Av3mEndo-treated mice lived 124 days after all controls had died and on sacrifice showed no sign of primary or metastatic tumors at necropsy. There was also a correlation between endostatin serum levels and survival (Table 2). Mice with endostatin serum levels below 200 ng/ml had a mean survival of 35.8 days whereas mice with levels between 200 and 300 ng/ml and between 300 and 576 ng/ml had a mean survival of 96.4 days (p < 0.05) and 69.7 days (p < 0.05), respectively (Table 2).

DISCUSSION

Delivery of an antiangiogenic protein by intravenous injection of an adenoviral vector expressing a secretable form of the antiangiogenic protein was shown to be a promising strategy for the treatment of cancer metastases on the basis of several findings. Intravenous delivery of Av3mEndo resulted in at least a partially effective concentration of endostatin. We demonstrated that the amount of circulating endostatin reached 327.9 ± 126.6 ng/ml in nude mice 10 days after vector administration and 708.5 ± 435.4 ng/ml in C57BL mice 16 days after vector administration. In addition, a single systemic administration of Av3mEndo in C57BL mice resulted in the systemic inhibition of VEGF-induced angiogenesis in vivo and signi-
TABLE 2. MEAN SURVIVAL OF MICE IN COLON LIVER METASTASIS MODEL

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Mean survival (days)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBSS</td>
<td>36.6 ± 38.2</td>
<td>11</td>
</tr>
<tr>
<td>Av3Null</td>
<td>30.2 ± 20.9</td>
<td>17</td>
</tr>
<tr>
<td>Av3MEndo</td>
<td>84.8 ± 89.3</td>
<td>16</td>
</tr>
</tbody>
</table>

**Blood endostatin**

<table>
<thead>
<tr>
<th>Level (ng/ml)</th>
<th>Mean survival (days)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Below 200</td>
<td>35.8 ± 16.3</td>
<td>29</td>
</tr>
<tr>
<td>200 to 300</td>
<td>96.4 ± 76.0</td>
<td>9</td>
</tr>
<tr>
<td>300 to 576</td>
<td>69.7 ± 60.7</td>
<td>6</td>
</tr>
</tbody>
</table>

*Survival day was determined relative to tumor implantation. Mice with long-term survival were included with survival of 188 days (day of sacrifice). Results represent mean ± standard deviation, which was calculated from the number of mice in each group (N represents number of mice per group). A Student unpaired t test was performed.

Significant difference from controls, with p < 0.05.

Significant difference from endostatin below 200 ng/ml, with p < 0.05.

candy-prolonged survival of nude mice subjected to orthotopic implantation onto the colon of a tumor that rapidly metastasizes to the liver. In the liver metastasis model, we demonstrated that a prophylactic intravenous injection of Av3MEndo prolonged survival to a mean of 85 days as compared with the control groups, which survived 30–37 days (p < 0.05) (Table 2). In addition, those animals that had circulating levels of endostatin of <200 ng/ml had a mean survival of only 36.6 days whereas those that had levels of 200–300 and 300–576 ng/ml had a mean survival of 96.4 days (p < 0.05) and 69.7 days (p < 0.05), respectively. In addition, 25% of mice (all in the group of animals that expressed between 200 and 300 ng of endostatin per milliliter) lived more than 4 months longer than all controls and on sacrifice and necropsy showed no evidence of metastases or primary tumor in the liver or elsewhere. Further studies will need to be done to determine if there is an optimal level of endostatin for maximal therapeutic benefit.

It has previously been reported that subcutaneous injections of partially denatured endostatin protein resulted in the complete regression of subcutaneous tumors in 100% of treated mice in several murine tumor models (Boehm et al., 1997). Why, then, did this gene therapy approach (or others) not show results as dramatic as was previously shown with the purified protein? There are a number of possibilities. The sources of endostatin protein are quite different, one being a partially denatured form derived and purified from Escherichia coli (O'Reilly et al., 1997) versus in vivo expression of endostatin with a partial secretion signal. Each form could have its own unique set of biological characteristics, as has been suggested here and elsewhere. In addition, the tumor cell environment, i.e., liver versus subcutaneous, is quite different, which could dramatically alter the dependence of a tumor cell on supporting endothelial cells (Fukumura et al., 1997). In the liver there are already a multitude of endothelial sinusoidal cells that may supply nutrients to at least small tumors. In subcutaneous tumors, both the tumor and supporting endothelial cells within the tumors grow quite rapidly and therefore might be more sensitive to angiogenic therapy. In addition, it is now becoming quite well established that endothelial cells from different organs are quite different with respect to their biological properties, including what receptors are expressed (Marti and Risdal, 1998). Thus it is certainly possible that endostatin could act more potently or on one subset of endothelial cells (e.g., within tumors in the subcutaneous environment versus tumors within the liver). In addition the circulating dose of endostatin was not determined in previous studies, and thus there is no way to compare therapeutic doses of circulating endostatin.

Finally, it should be appreciated that tumor metastases are a much more serious form of cancer than localized tumors in general, and are thus much more difficult to treat. Ultimately, the only way to address which method of delivering endostatin is better (if either) is to directly compare both gene therapy and protein approaches in both the subcutaneous tumor and liver metastases models. Ultimately, each of several approaches to the production and delivery of endostatin or other angiogenic proteins may have its own unique advantages depending on the stage, type, and location of the cancer to be treated.

Various endostatin-based gene therapy strategies have been reported, including naked plasmid DNA (Blazinger et al., 1999), liposome-formulated plasmid DNA (Chen et al., 1999), and adenovirus-associated viral vector (Nguyen et al., 1998). Similar to our study, all these approaches have demonstrated limited efficacy as compared with the protein approach reported previously (Boehm et al., 1997). As discussed above, there are many reasons why all these endostatin-based gene therapy approaches have demonstrated only limited antitumor activity. However, our adenovirus-based approach did significantly improve the amount of circulating endostatin relative to the plasmid DNA (Blazinger et al., 1999) or liposome-formulated plasmid DNA (Chen et al., 1999) approaches and did result in efficacy in a prophylactic liver metastasis model, which has not been previously reported. Ultimately, there are a variety of vector improvements that could result in safer, higher, prolonged, and regazable levels of expression of endostatin. In addition, different vector delivery methods could be used to optimize the treatment for liver metastases and other cancers. Studies utilizing many of these strategies are currently in progress here and elsewhere.

Of primary interest was the fact that the endostatin expressed from Av3MEndo-transduced mammalian cells was an extremely potent inhibitor against VEGF-induced HUVEC migration. Our results consistently demonstrated that murine endostatin potently inhibited VEGF-induced HUVEC migration. Our partially purified endostatin contained only 14% murine endostatin as determined by ELISA. On the basis of the corrected endostatin concentration, the ED50 of inhibition by murine endostatin is in the range of 0.01–0.1 ng/ml, suggesting that mammalian expressed endostatin has potentially unique and potent properties. Yamaguchi et al. (1999) have reported that mammalian expressed endostatin purified from human embryonic kidney (293-EBNA) cells potently inhibited the VEGF-induced migration of HUVECs, with an ED50 range from 0.01 to 0.1 ng/ml, consistent with our results. The fact that mammalian expressed endostatin could be more potent than another source is also supported by Yamaguchi et al. (1999), who demonstrated that mammalian expressed endostatin required...
ANTIANGIOGENIC GENE THERAPY FOR CANCER

1000-fold less dose for tumor regression in the experimental RC-9 tumor model than endostatin produced from either E. coli inclusion bodies (O'Reilly et al., 1997) or Pichia pastoris (Dhanabal et al., 1999a).

We also demonstrated that mammalian cell-produced endostatin could inhibit non-VEGF-induced migration since the maximum inhibition by endostatin was consistently greater than VEGF-induced migration. Among several possibilities for the inhibition of non-VEGF-dependent migration is that endostatin elicited apoptosis of endothelial cells, as has been previously reported (Dhanabal et al., 1999b).

The fact that systemic delivery of the adenoviral vector encoding endostatin demonstrated antitumor effects against liver metastases might be due to high local expression and secretion in the liver since murine livers are highly transduced after tail vein injection of adenoviral vectors (Smith et al., 1993). However, a systemic administration of AvF3Endo did result in high circulating levels of endostatin that have previously been shown to have systemic effects. In addition, we showed that systemic administration of AvF3Endo did result in the reduction of angiogenesis in VEGF-containing agar implanted subcutaneously in mice. Regardless of the mechanism, treatment of liver metastases is an important goal for cancer therapy. Liver is the prime target for cancer metastases for a large number of invasive cancers including colon, prostate, gastrointestinal, bronchogenic, and certain gynecological tumors (Jiang, 1998). Antiangiogenesis gene therapy using the current system could have the potential for the development of liver-directed secretion of antiangiogenic factors against liver metastases and possibly systematically disseminated disease, as well. A conclusion similar to ours has been reported in that study investigators (Li et al., 1999) demonstrated that the systemic delivery of Adr NazATF, an adenoviral vector expressing the amino-terminal fragment of mouse urokinase-type plasminogen activator, efficiently protected the liver from metastases challenge through an antiangiogenesis process. In conclusion, attenuated adenoviral vectors expressing one or more secretable antiangiogenic proteins could have several benefits over the existing protein-based strategy, including target specificity, less frequent administration, mammalian cell expression with full bioactivity, and prolonged and high-level circulation of the inhibitor, and may be a promising strategy for future cancer treatment.

ACKNOWLEDGMENTS

We are grateful to GTRi/Systemix Core technology for the vector preparation and the Southern blot analysis of liver transduction. We also thank AntiCancer for the orthotopic tumor model studies reported herein.

REFERENCES


LI, H., GRISCELLI, F., LINDENMEYER, F., OPOLON, P., SUN,


Address reprint requests to:
Dr. Paul L. Hallenbeck
Genetic Therapy, Inc.
A Novartis Company
9 West Watkins Mill Road
Gaithersburg, MD 20873