Systemic Gene Delivery Expands the Repertoire of Effective Antiangiogenic Agents*

Yong Liu‡, Ann Thor§§, Emma Shtivelman‡, Yihai Cao**, Guanghuan Tu‡, Timothy D. Heath‡‡, and Robert J. Debs‡ §§

From the §Geraldine Brush Cancer Research Institute at the California Pacific Medical Center, San Francisco, California 94115, the ¶Department of Pathology, Northwestern University School of Medicine, Evanston, Illinois 60201, *Laboratory of Angiogenesis Research, Microbiology and Tumor Biology Center, Karolinska Institute, S-171 77, Stockholm, Sweden, and ¶¶School of Pharmacy, University of Wisconsin, Madison, Wisconsin 53706

Cationic liposome-DNA complex (CLDC)-based intravenous gene delivery targets gene expression to vascular endothelial cells, macrophages and tumor cells. We used systemic gene delivery to identify anti-angiogenic gene products effective against metastatic spread in tumor-bearing mice. Specifically, CLDC-based intravenous delivery of the p53 and GM-CSF genes were each as effective as the potent antiangiogenic gene, angioatin, in reducing both tumor metastasis and tumor angiogenesis. Combined delivery of these genes did not increase anti-tumor activity, further suggesting that each gene appeared to produce its antimitastatic activity through a common antiangiogenic pathway. CLDC-based intravenous delivery of the human wild type p53 gene transfected up to 80% of tumor cells metastatic to lung. Furthermore, it specifically induced the expression of the potent antiangiogenic gene, thrombospondin-1, indicating that p53 gene delivery in vivo may inhibit angiogenesis by inducing endogenous thrombospondin-1 expression. CLDC-based delivery also identified a novel anti-tumor activity for the metastasis suppressor gene C3. Thus, CLDC-based intravenous gene delivery can produce systemic antiangiogenic gene therapy using a variety of different genes and may be used to assess potential synergy of combined anti-tumor gene delivery and to identify novel activities for existing anti-tumor genes.

Molecular genetics has identified a large number of genes whose aberrant or loss of expression is correlated with the initiation and/or progression of the malignant phenotype (1, 2). However, it has been difficult to determine whether the overexpression of the normal gene products can produce significant anti-tumor effects in tumor-bearing animals for several reasons. First, it may be difficult to obtain sufficient quantities of secreted gene products to test their potential anti-cancer activity in tumor-bearing animals (3, 4). Second, testing gene products that function only within cells, such as tumor-suppressing DNA-binding nucleoproteins, often requires the use of in vivo gene delivery, since administration of recombinant proteins would not be expected to deliver therapeutic levels of the active molecules to their required tissue and cellular sites of action in tumor-bearing hosts. Similar cellular barriers have limited the in vivo delivery of therapeutic levels of antisense and ribozyme constructs directed against the products of overexpressed transforming genes (5, 6).

Existing in vivo gene delivery systems have been particularly limited in their ability to treat metastatic cancer, which is the major cause of morbidity and mortality in human cancer patients (7). We have developed a redesigned CLDC1-based, intravenous gene delivery approach that produces levels of systemic gene expression from 500 to 1,700-fold higher than intravenous injection of conventional CLDC (8–10). We now show that these redesigned CLDC are able to transfect large numbers of metastatic tumor cells with the wild type human p53 gene following intravenous injection into C57Bl6 mice bearing metastatic B16-F10 melanoma tumors. Furthermore, CLDC-based, intravenous delivery of a variety of different anti-tumor genes each produced significant anti-metastatic activity and concurrently reduced tumor angiogenesis. This approach also permitted the assessment of potential in vivo anti-tumor synergy between combinations of anti-tumor genes, thus helping to determine whether genes are producing their anti-tumor effects by common or independent pathways. It also identified a novel anti-tumor activity for the CC3 metastasis suppressor gene in tumor-bearing hosts.

EXPERIMENTAL PROCEDURES

Plasmids

*p4199—CMV-p53 was constructed by isolating a 1.4-kilobase pair HindIII–SalI fragment containing the wild type human p53 cDNA (a gift from Dr. B. Vogelstein) and ligating it into the HindIII–SalI site of p4136 (11).

*p4305—CMV-angiostatin was constructed by isolating a 1.4-kilobase pair HindIII–XbaI fragment containing the murine angiostatin cDNA (a gift from Dr. J. Folkman) and then ligating it by blunt end ligation into the PciI site of p4109 (11).

*p4442—CMV-BCL-2 was constructed by ligating the human BCL-2 cDNA (a gift from Dr. S. Korsmeyer) into the EcoRV site of pVR1223 (12).

*p4447—CMV-CC3 was obtained by isolating a 0.8-kilobase pair fragment containing the human CC3 and ligating it into the EcoRV site of pVR2223. The construction of p4119, CMV-chloramphenicol acetyl-

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§§ Supported by NIH Grants CA58914, DK45917, DK49550, and HL53702, Xenex, LLC, and by the State of California, Breast Cancer Research Program. To whom correspondence should be addressed: California Pacific Medical Research Institute, 2330 Clay St., Stern Bldg., San Francisco, CA 94115. Tel.: 415-561-1704; Fax: 415-561-1725; E-mail: debs@cooper.cpmc.org.

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transferease (CAT) (11), and p4241, CMV-luciferase (8), have been described. Plasmids were purified as described previously (11).

Preparation of Cationic Liposomes and CLDC

DOTIM:cholesterol multilamellar vesicles (MLV) and pure DOTIM MLV were prepared as described previously (8). CLDC were prepared as described (11).

Tumor Cells and Tumor Inoculation

Murine B16-F10 melanoma cells were grown in RPMI 1640 with 5% fetal bovine serum at 37 °C with 5% CO2. The B16-F10 melanoma cells expressed the wild type p53 gene, as determined by both DNA damage and Western blot analyses (data not shown), and also expressed low levels of the CC3 protein, as determined by Western blot analysis (data not shown), and also expressed low levels of the CC3 protein, as determined by Western blot analysis (data not shown). For tumor cell inoculation, B16-F10 cells were trypsinized, and then 25,000 cells/mouse in 200 μl of culture medium were injected by tail vein into 25-g female C57Bl6 mice (Simonson, Gilroy, CA). B16-F10 melanoma is a highly metastatic subclone of B16 melanoma (13) that kills mice approximately 35 days following intravenous inoculation of 25,000 cells (data not shown).

In Vivo Transfections and Analysis of Anti-tumor Activity

Unless specifically indicated, each mouse received 25 μg of plasmid DNA complexed to DOTIM:cholesterol MLV. The DNA:lipid ratio (μg of DNA/nmol of total lipid) was 1:16 for all DOTIM:cholesterol MLV and 1:26 for pure DOTIMA MLV. Each of these DNA:lipid ratios had previously been determined to produce maximal levels of gene expression following intravenous injection of CLDC (Ref. 8 and data not shown). CLDC were injected into tumor-bearing mice either 3 or 10 days after tumor cell inoculation or, in some cases, once only at day 7 following tumor inoculation. For the immunohistochemistry studies, mice were injected intravenously with CLDC 29 days after tumor cell inoculation. All mice were sacrificed 30 days after tumor cell inoculation, and lungs from each mouse were dissected out, infused transtracheally with 10% neutral buffered formalin (Fisher), and then fixed in 10% neutral buffered formalin. The number and size of the black-appearing tumor nodules were counted two times under a dissecting microscope by an individual blinded to the identity of the groups. Only tumor nodules >2 mm in diameter were included in the analysis, since tumor growth beyond 2–3 mm appears to depend on the presence of tumor angiogenesis (14–17). The potential statistical significance of differences between the various groups was assessed using an unpaired, two-sided Student’s t test.

Immunohistochemistry and Vascular Staining

Formalized paraffin-embedded 4-μm sections were deparaffinized and rehydrated to phosphate-buffered saline. Pretreatments included microwave antigen retrieval in a 10 mM citrate buffer for 10 min at 95 °C. Tumor sections were incubated for 20 min in 0.3% hydrogen peroxide at 37 °C to quench endogenous peroxidases. After washing, AEC (Biomeda Corp., Foster City, CA) was used for 20 min. Rabbit polyclonal anti-factor VIII (von Willebrand factor, 1:250 dilution; Dako Corp., Carpinteria, CA) or anti-thrombospondin (1:35 dilution; Biodesign International, Westbrook, ME). Incubation using polyclonal anti-factor VIII (von Willebrand’s factor, a 1:250 dilution; Dako Corp., Carpinteria, CA) was carried out at room temperature for 1 h. All primary antibodies were washed with 0.2–1% Tween 20 (Fisher), and biotinylated goat-anti-rabbit (1:500; Vector Laboratories, Factor VIII, p53) or biotinylated rabbit-anti-mouse (1:200; Vector Laboratories; thrombospesin; link) was applied for 30 min. Following additional washes, ABC elite (factor VIII, p53, Vector Laboratories) or streptavidin-horseradish peroxidase (1:100; Zymed Laboratories Inc.) was applied for 30 min. After additional washes, AEC (Biomeda Corp., Foster City, CA) was used for 20 min followed by counterstaining with hematoxylin and coverslipping with crystal mount (Biomedica Corp.).

Quantifying Tumor Angiogenesis and the Percentage of Cells Expressing p53 and TSP-1

The intratumoral blood vessels were quantitated using factor VIII, anti-von Willebrand factor’s staining, as described above. For each tumor, high power magnification was used with an intraocular grid. The grid facilitated counting so that no vessel was scored twice. Vessels that were visibly interconnected were scored only once. Neovessels within each tumor >2 mm were counted in as many high powered fields (grids) as could be placed within each tumor (usually two or three, as high as five) based upon the size of the nodule. This differs from vessel counting in larger human tumors (18), because areas of high vascularity were not preselected. Rather, the entire cross section of these tumor nodules was analyzed. The final angiogenesis score for each nodule reflected the summation of all scores (the sum of scores, divided by the total number of fields that were counted). Every tumor nodule of >2 mm that was microscopically visualized from each animal was analyzed for neoangiogenesis. A total of nine tumors from five different mice treated with the CAT gene, nine tumors from four different mice treated with the angiostatin gene, 12 tumors from six different mice treated with the GM-CSF gene, and four tumors from three different mice treated with the GM-CSF gene were analyzed. Thus, 34 individual tumors from 18 individual mice, all derived from experiment 1a were individually counted. To quantitate p53 gene expression, only cells showing the characteristic pattern of nuclear staining were counted as positive, and for TSP-1, only cells showing cytoplasmic reactivity were counted. Scores for p53 and TSP-1 gene expression reflect the percentage of positive cells for each histologically identifiable tumor nodule evaluated. All evaluable tumors on the tissue sections used for the assay were quantitated.

RESULTS

CLDC-mediated, Intravenous Delivery of the Murine Angiostatin Gene, MURINE GM-CSF, or the Wild Type Human p53 Gene Each Produces Significant Anti-metastatic Activity—We first compared the size and number of lung metastases in the CLDC-treated and control C57Bl6 mice 30 days after intravenous injection of 25,000 B16-F10 melanoma cells/mouse. Individual mice in groups of eight received 400 nmol of DOTIM: cholesterol MLV complexed to 25 μg of an HCMV-driven expression plasmid containing the murine angiostatin gene, the murine GM-CSF gene, the human wild type p53 gene, or the CAT reporter gene (mock-treated controls) on day 3 and again on day 10 following tumor inoculation. The control group received B16-F10 cells but no further treatment.

CLDC-mediated, intravenous delivery of the murine angiostatin gene, murine GM-CSF, or the wild type human p53 gene each produced significant anti-metastatic effects, as determined by both the total number of lung metastases (data not shown) and those greater than 2 mm in diameter in tumor-bearing mice, when compared with either reporter gene-treated (p < 0.05) or untreated (p < 0.05) controls by a two-sided Student’s t test (Fig. 1A). The p53-treated, angiostatin-treated, and GM-CSF gene-treated groups each produced similar reductions in the number of lung metastases compared with control groups. Reporter gene (mock)-treated and untreated controls did not differ significantly in either the total number of tumors or the number of tumors greater than 2 mm in diameter (p < 0.375). Previously, intravenous injection of DOTIMA:DOPE small unilamellar vesicles complexed to 10 μg of control (non-coding) DNA produced some degree of nonspecific anti-tumor activity in SCID mice bearing a human breast cancer xenograft when compared with nontreated controls (19). The presence or absence of nonspecific CLDC-mediated anti-tumor activity between the two studies may be due to differences in the tumor lines and/or mouse strains used, the size and composition of the cationic liposomes used, the dose and schedule of CLDC administration, and/or the time of sacrifice.

Co-injecting Combinations of These Genes Does Not Increase Anti-tumor Activity—We next tested whether co-injection of the p53 and GM-CSF genes, the p53 and angiostatin genes, or the angiostatin and GM-CSF genes into individual groups of mice reduced syngeneic tumor activity when compared with injection of the individual genes themselves. CLDC-based intravenous injection of each gene individually, as well as each of the gene combinations, reduced both the total number of lung tumors and the numbers of tumors greater than 2 mm by comparable levels when compared with the control mice (Fig. 1B). Thus, no combination of genes enhanced the level of anti-
tumor activity produced by each gene individually, indicating a lack of synergistic anti-tumor activity. As observed previously in Fig. 1A, the angiostatin, p53, and GM-CSF genes individually each produced similar levels of anti-tumor activity. CLDC-based, intravenous injection of 12.5 μg of CMV-angiostatin plus 12.5 μg of CMV-luciferase produced antimetastatic effects that did not differ significantly from those produced by 25 μg of CMV-angiostatin alone (data not shown). Thus, the failure of angiostatin combined with either GM-CSF or p53 to produce synergistic anti-tumor effects does not appear to be due to the reduced dose of the individual anti-tumor genes injected.

CLDC-based Intravenous Gene Delivery Permits Assessment of the Anti-metastatic Activity of a Variety of Potential Anti-cancer Genes—We then compared the size and number of lung metastases produced in groups of mice injected with CLDC containing the murine angiostatin gene, the human CC3 gene, the human BCL-2 gene, or the luciferase gene (mock-treated controls) on days 3 and 10 after tumor inoculation or no treatment (control group). Additional groups of tumor-bearing mice received a single intravenous injection of CLDC containing either the murine angiostatin gene or the luciferase gene 7 days after receiving B16-F10 melanoma cells in order to determine the effect of delaying CLDC therapy on the level of anti-tumor activity achieved. We also tested the CC3 gene (an apoptosis-inducing metastasis suppressor gene (20)) and the BCL-2 gene (a potent antagonist of apoptosis). Overexpression of BCL-2 has been linked to the development of human tumors (21). CLDC-mediated, intravenous delivery of the murine angiostatin gene and the human CC3 gene at days 3 and 10 each produced significant anti-metastatic effects, when compared with BCL-2 gene-treated mice \( (p < 0.05) \), to luciferase gene (mock)-treated \( (p < 0.05) \) or to untreated \( (p < 0.05) \) controls (Fig. 1C).

A single intravenous injection at day 7 of CLDC containing the murine angiostatin gene also significantly reduced tumor metastases when compared with control groups \( (p < 0.05) \) but was somewhat less effective than two injections of the angiostatin gene initiated at a time when the tumor burden was less extensive. Furthermore, although the B16-F10 line has been cloned as a predominately lung metastatic tumor line (13), we noted the presence of significant numbers of extrapulmonary produces significant antimetastatic effects in tumor-bearing mice (25,000 B16-F10 melanoma cells/mouse intravenously into groups of eight C57BL6 mice on day 0). All mice were sacrificed at day 30 following tumor inoculation, and lung metastases \( > 2 \) mm were counted using a dissecting microscope. Potential significance of differences between the various groups was determined using a two-sided Student’s \( t \) test. a, individual mice in groups of eight were injected intravenously on day 3 and again on day 10 with CLDC containing 400 nmol of DOTIM:cholesterol MLV complexed to 25 μg of a CMV-driven expression plasmid encoding either the murine angiostatin gene, the murine GM-CSF gene, or the human wild type p53 gene, the CAT gene (mock-treated controls), or no treatment (control group). b, individual mice in groups of eight were injected intravenously on day 3 and again on day 10 with CLDC containing 400 nmol of DOTIM:cholesterol MLV complexed to 25 μg of a CMV-angiostatin, CMV-GM-CSF, or CMV-p53 or to 12.5 μg of CMV-angiostatin plus CMV-p53, CMV-angiostatin plus CMV-GM-CSF, or CMV-p53 plus CMV-GM-CSF, respectively. c, individual mice in groups of eight were injected intravenously on day 3 and again on day 10 with CLDC containing 400 nmol of DOTIM:cholesterol MLV complexed to 25 μg of each CMV-angiostatin, CMV-CC3, CMV-BCL-2, CMV-luciferase, or CMV-p53. Individual mice in groups of eight were also injected intravenously on day 7 only with CLDC containing 400 nmol of DOTIM:cholesterol MLV complexed to 25 μg of either CMV-angiostatin, CMV-CC3, CMV-BCL-2, CMV-luciferase, or CMV-p53. Individual mice in groups of eight were also injected intravenously on day 7 only with CLDC containing 650 nmol of pure DOTMA MLV complexed to 25 μg of CMV-angiostatin.

**Fig. 1.** Intravenous injection of CLDC containing the angiostatin gene, the GM-CSF gene, the CC3 gene, or the p53 gene each tumor activity produced by each gene individually, indicating a lack of synergistic anti-tumor activity. As observed previously in Fig. 1A, the angiostatin, p53, and GM-CSF genes individually each produced similar levels of anti-tumor activity. CLDC-based, intravenous injection of 12.5 μg of CMV-angiostatin plus 12.5 μg of CMV-luciferase produced antimetastatic effects that did not differ significantly from those produced by 25 μg of CMV-angiostatin alone (data not shown). Thus, the failure of angiostatin combined with either GM-CSF or p53 to produce synergistic anti-tumor effects does not appear to be due to the reduced dose of the individual anti-tumor genes injected.
B16-F10 metastases in this as well as in prior experiments. Therefore, we counted the number of extrapulmonary metastases present in this experiment. The overall number of extrapulmonary metastases was significantly reduced in the angiostatin-treated (a total of one metastasis) and CC3-treated groups (no metastases) compared with control groups (a total of 8 ± 2.7 metastases) (p < 0.01 for angiostatin and p < 0.005 for CC3). Metastases were present in the liver, gastrointestinal tract, spinal cord, thymus, skin, and lymph nodes. Thus, CLDC-based intravenous gene delivery of active anti-angiogenic and tumor suppressor genes can reduce metastatic spread to a variety of target organs to which solid tumors commonly metastasize (7).

Increasing the Efficiency of CLDC-based Intravenous Delivery of the Angiostatin Gene Further Increases Anti-tumor Activity—To more specifically address the relationship between the level of antiangiogenic expression achieved and of the extent of the anti-tumor activity produced, we tested whether increasing the level of angiostatin gene expression through the use of a more efficient cationic formulation could significantly increase anti-tumor efficacy against B16-F10 metastases. Specifically, we compared the anti-tumor activity against B16-F10 produced by the angiostatin gene delivered by two different cationic liposome formulations: 1) DOTIM:cholesterol liposomes (used in experiments 1A through 1C) or 2) pure DOTMA liposomes have been shown to produce approximately 10-fold higher levels of gene expression than DOTIM:cholesterol liposomes (Ref. 22 and data not shown). We found that intravenous injection of 25 μg of the angiostatin gene complexed to DOTIM: cholesterol liposomes reduced metastases >2 mm by 65% versus control mice, whereas the angiostatin gene complexed to pure DOTMA liposomes reduced metastases by 88% versus control mice (p < 0.025 versus DOTIM:cholesterol liposomes) (Fig. 1D). Thus, the degree of anti-tumor activity achieved appears to depend on the efficiency of gene transfer and expression produced. Increasing the level of angiostatin gene expression further reduced the number of lung metastases to quite low levels.

CLDC-based Intravenous Delivery of the p53, GM-CSF, or Angiostatin Genes Significantly and Comparably Reduces Tumor Angiogenesis—We then attempted to identify a mechanism for the comparable levels of anti-tumor activity produced by the p53, angiostatin, and GM-CSF genes. We determined the mean number of intratumoral blood vessels in p53-, GM-CSF-, angiostatin-, and CAT-treated mice using neangiogenesis counts facilitated by immunoreactivity to Factor VIII-antibody as described under “Experimental Procedures.” Vessel counts were made on a total of nine tumors from five different mice treated with the CAT gene, nine tumors from four different mice treated with the angiostatin gene, 12 tumors from six different mice treated with the p53 gene, and four tumors from three different mice treated with the GM-CSF gene.

| CLDC injected | Total blood vessels/tumor |
|---------------|---------------------------|
| CLDC-angiostatin gene | 8.0 ± 2.2^a |
| CLDC-p53 gene | 7.9 ± 2.3^a |
| CLDC-GM-CSF gene | 8.5 ± 3.4^a |
| CLDC-CAT (control) | 14.2 ± 3.1 |

*p < 0.0005 versus control.

*p < 0.01 versus control.

TABLE I

Intravenous, CLDC-based injection of the angiostatin, p53, or GM-CSF genes each significantly reduces tumor vascularity

Means ± S.D. values are shown. Mice were treated as described in the legend to Fig. 1A. Vessels were stained using a Factor VIII,-anti- von Willebrand's factor antibody as described under “Experimental Procedures.” Vessel counts were made on a total of nine tumors from five different mice treated with the CAT gene, nine tumors from four different mice treated with the angiostatin gene, 12 tumors from six different mice treated with the p53 gene, and four tumors from three different mice treated with the GM-CSF gene.

Since delivery of the p53 gene significantly reduced tumor vascularity (Table I) and since p53 gene expression has been shown to induce expression of the potent antiangiogenic factor, TSP-1, in cultured fibroblasts (24) and epithelial cells (25), we determined whether systemic delivery of the p53 gene altered TSP-1 expression in the lung, using immunohistochemistry. p53-treated mice showed TSP-1 expression within the cytoplasm of the large majority of tumor cells present within the lung as well as in rare normal pulmonary cell types (Fig. 2C). TSP-1 expression was not detected in the lungs of either CAT-treated (Fig. 2D) or untreated mice (data not shown). Thus, p53 gene delivery could specifically induce widespread TSP-1 expression in metastatic tumors in vivo. TSP-1 may mediate both the antiangiogenic (Table I) and antimetastatic (Fig. 1, A and B) activity of p53 in B16-F10 tumor-bearing mice.

The Level of Induction of TSP-1 Expression in Metastatic Tumor Cells Depends on the Level of p53 Gene Expression Produced—To directly assess the dose-response relationship between p53 gene transduction and the induction of TSP-1 gene expression, we quantitated the percentage of tumor cells expressing p53 and also TSP-1, following intravenous injection of DOTIM:cholesterol liposomes complexed to either 40 or 50 μg of the p53 gene. Increasing the DNA dose from 40 to 50 ng of the p53 gene reproducibly increases the level of expression of de- livered genes between 5- and 10-fold following intravenous injection of CLDC containing DOTIM:cholesterol liposomes (data not shown). Injecting CLDC containing 40 μg of the p53 gene transduced 29.6 ± 3.7% of B16-F10 tumor cells metastatic to the lung with p53 and induced TSP-1 expression in 39.2% ± 3.4% of these cells, whereas injecting CLDC containing 50 μg of the p53 gene transduced 82.1% ± 2.7% of lung tumor cells with p53 and induced TSP-1 expression in 98.6% ± 0.6% of these cells (Table II). (No positive staining for p53 or TSP-1 gene containing either the wild type p53 gene or the CAT gene. These mice were injected with B16-F10 cells on day 0 and subsequently with CLDC on day 29, a time when lung metastases were at an advanced stage. Essentially all tumors examined in the lungs of p53-treated mice showed p53 expression within the nucleus of transfected cells (Fig. 2A). Although the staining for p53 immunoreactivity varied in intensity (Fig. 2A), large numbers of tumor cells examined showed some degree of positive staining for p53. Immunopositivity of wild type p53 suggested a high level of p53 gene expression, since the wild type p53 generally does not stain positively in this assay because of rapid degradation of the encoded protein (25). Positivity was also noted in some macrophage and endothelial cell nuclei (Fig. 2A). p53 expression was not seen in either tumor cells or in normal cells in the lungs of either CAT-treated (Fig. 2B) or untreated mice (data not shown).
expression was detected in either B16-F10 or normal cells from the lungs of mice injected with CLDC containing 50 μg of either CMV-p53 or CMV-CAT intravenously on day 29 and were sacrificed 24 h later. The lungs were infused with 10% neutral buffered formalin, quick frozen, and then processed as described under "Experimental Procedures." A, high power (40×) magnification of a metastatic pulmonary nodule from CMV-CAT-injected mouse, immunostained for p53. Over 90% of nuclei from this pulmonary tumor nodule demonstrate nuclear reactivity for p53, with 10% of nuclei intensely positive, 10% moderately positive, and another 70% weakly positive for p53 expression. B, high power (40×) magnification of a metastatic pulmonary nodule from CMV-CAT-injected mouse, immunostained for p53. No specific nuclear stain for p53 is shown, similar to all other tumor nodules in both CAT-treated and untreated (data not shown) control animals. The counter stain faintly demonstrates the tumor cell outlines. C, high power (× 40) magnification of a metastatic pulmonary nodule (center of field) surrounded by pulmonary tissue from a p53-treated mouse. This tissue has been immunostained for thrombospondin-1 and demonstrates cytoplasmic immunoreactivity (red) in greater than 90% of the tumor cells in this nodule. D, high power (× 40) magnification of a metastatic pulmonary nodule from a CAT gene-treated mouse that has been immunostained for TSP-1. No cytoplasmic immunoreactivity for TSP-1 is detectable.

**TABLE II**

| CLDC-DNA dose injected | p53-positive cells | TSP-positive cells | % |
|------------------------|--------------------|--------------------|---|
| 40 μg of DNA           | 29.6 ± 3.7         | 39.2 ± 3.4         |   |
| 50 μg of DNA           | 82.1 ± 2.7         | 98.6 ± 0.6         |   |

*Fold increase vs. 40-μg dose 2.8 2.5

*p < 0.0005 vs the respective 40 μg DNA-injected groups.

**DISCUSSION**

Anti-tumor genes have been classified into multiple different functional categories, including tumor suppressor genes, immunostimulatory genes, anti-oncogenes, and anti-angiogenic genes. However, it has become evident that individual genes may produce anti-tumor effects by several different mechanisms. As examples, the wild type p53 gene has been shown to function as a potent tumor suppressor gene (28, 29). More recently, p53 has also been shown to exert angiogenic activity in tumor-bearing mice (30), as well as to induce the production of the anti-angiogenic protein TSP-1 in cultured cells (26, 27). Furthermore, stable transfection of tumor cells with the cytokine gene GM-CSF has been shown to exert host-

**FIG. 2.** Intravenous injection of CLDC containing the p53 gene transfects large numbers of metastatic tumor cells and induces expression of the endogenous TSP-1 gene. Groups of four mice that had received 25,000 B16-F10 cells intravenously on day 0 received CLDC containing 400 nmol of DOTIM:cholesterol MLV complexed to 50 μg of either CMV-p53 or CMV-CAT intravenously on day 29 and were sacrificed 24 h later. The lungs were infused with 10% neutral buffered formalin, quick frozen, and then processed as described under "Experimental Procedures." A, high power (40×) magnification of metastatic tumor in p53-treated mouse immunostained for p53. Over 90% of nuclei from this pulmonary tumor nodule demonstrate nuclear reactivity for p53, with 10% of nuclei intensely positive, 10% moderately positive, and another 70% weakly positive for p53 expression. B, high power (40×) magnification of a metastatic pulmonary nodule from CMV-CAT-injected mouse, immunostained for p53. No specific nuclear stain for p53 is shown, similar to all other tumor nodules in both CAT-treated and untreated (data not shown) control animals. The counter stain faintly demonstrates the tumor cell outlines. C, high power (× 40) magnification of a metastatic pulmonary nodule (center of field) surrounded by pulmonary tissue from a p53-treated mouse. This tissue has been immunostained for thrombospondin-1 and demonstrates cytoplasmic immunoreactivity (red) in greater than 90% of the tumor cells in this nodule. D, high power (× 40) magnification of a metastatic pulmonary nodule from a CAT gene-treated mouse that has been immunostained for TSP-1. No cytoplasmic immunoreactivity for TSP-1 is detectable.

**Increasing p53 gene expression increases the induction of endogenous TSP-1 gene expression in metastatic tumors**

Means ± S.E. values are shown. Groups of three mice, 29 days after intravenous injection of 25,000 B16-F10 melanoma cells, were injected intravenously with CLDC containing either 40 or 50 μg of the p53 gene complexed to DOTIM: cholesterol liposomes at a 1:16 DNA: lipid ratio and were sacrificed 24 h later. p53 and TSP-1 immunohistochemistry and quantitation of positive tumor cells were performed as described under "Experimental Procedures.”
based intravenous gene delivery targets gene expression to macrophages, as well as to vascular endothelial cells (8, 29), the use of intravenous, CLDC-based GM-CSF gene delivery may shift the balance of GM-CSF’s pro- and anti-angiogenic activities toward anti-angiogenesis when compared with the administration of recombinant GM-CSF protein.

Our results show that CLDC-based intravenous delivery of the GM-CSF gene, the p53 gene, and the anti-angiogenic angiotatin gene each reduces both tumor angiogenesis and metastatic spread by comparable levels (Fig. 1, A and B). Furthermore, CLDC-based delivery of combinations of these genes failed to significantly alter anti-tumor activity when compared with injection of each gene individually (Fig. 1B), suggesting that these genes are producing their anti-tumor effects via a common pathway. Therefore, our results are consistent with the interpretation that the products of the p53, GM-CSF, and angiotatin genes each produce anti-metastatic activity primarily as anti-angiogenic agents following intravenous, CLDC-based delivery. In support of this hypothesis, the level of reduction of tumor angiogenesis produced by CLDC-based intravenous injection of the murine angiotatin gene was similar to that produced by implantation of murine fibrosarcoma cells stably transfected with the murine angiotatin gene (Table I and Ref. 22). In addition, we have also shown that CLDC-based intravenous delivery of the wild type human p53 gene can specifically induce the production of TSP-1 in metastatic tumor cells in tumor-bearing mice (Fig. 2), indicating that p53-mediated antiangiogenesis in vivo may operate at least in part through the induction of TSP-1 expression (26, 27).

Vascular endothelial cells, macrophages, and tumor cells have been shown to be the three principal cell types involved in controlling the angiogenic phenotype (14–17, 36). Previously, CLDC-based intravenous gene delivery has been shown to target the expression of transferred genes to vascular endothelial cells (8, 33) and macrophages (8, 35). Our results now indicate that intravenously injected CLDC containing the p53 gene can transfect up to 80% of all lung tumor cells in mice bearing advanced metastatic disease (Table II). Thus, the use of CLDC may produce high levels of antiangiogenic gene products at their specific cellular sites of action. However, although CLDC-based delivery of the angiotatin gene reduced lung metastases by as much as 90% compared with control mice (Fig. 1D) and brought about significant reductions in tumor vascularization (Table I), it did not cause complete tumor regression, as previously reported following twice daily administration of high doses of recombinant angiotatin or implantation of tumor cells stably transfected with the angiotatin gene (3, 4, 24). Since high levels of angiotatin protein must be administered on a daily basis for prolonged periods in order to achieve complete tumor regression (3, 4), the degree of anti-tumor activity produced by only one or two doses of CLDC over the 35-day period of this study is encouraging.

Our results also suggest that the overexpression of selected genes, which are aberrantly expressed only in a limited number of tumor types, may exert more generalized anti-tumor activity via CLDC-based, systemic gene delivery. For example, highly metastatic small cell lung carcinoma cells often lack CC3 expression. Stable transfection of these highly aggressive small cell lung cancer cells with the CC3 gene was shown to suppress their metastasis in mice (20). We found that CLDC-based systemic delivery of the CC3 gene produced significant anti-metastatic activity against B16-F10 melanoma, which expresses low levels of CC3 protein (Fig. 1C). This finding indicates that enforcing expression of higher levels of CC3 in metastatic cells that are not originally CC3-negative can produce significant antitumor effects. CLDC-based delivery of other such genes into tumor-bearing hosts may reveal novel tumor targets not identified by more traditional molecular genetic approaches. Furthermore, similar to existing chemotherapy of cancer, the combined anti-tumor activities of several different gene products, expressed simultaneously, may be required for therapeutic efficacy, particularly in patients with extensive tumor burdens. CLDC-based intravenous gene delivery may also be used to assess whether combinations of putative anti-cancer genes can produce synergistic anti-tumor activities in tumor-bearing hosts.

Metastatic spread causes death in the overwhelming majority of patients dying from the most common forms of human cancer (7). Significant reductions in cancer mortality will thus require the development of new systemic therapies that can prevent and/or reverse the metastatic spread of cancer. The ability to transfer and express anti-tumor genes by intravenous administration offers a novel approach to the treatment of cancer. In addition, it may permit the identification of novel anti-cancer genes as well as reveal anti-cancer genes that act synergistically in tumor-bearing hosts. Ultimately, it may create the possibility of treating metastatic cancer by specifically correcting the molecular alterations responsible for producing the malignant phenotype.

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