Endostatin-Cytosine Deaminase Fusion Protein Suppresses Tumor Growth by Targeting Neovascular Endothelial Cells

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Abstract

Endostatin, an angiogenesis inhibitor tested in multiple clinical trials, selectively targets neovascular endothelial cells, suppressing tumor growth. To enhance the therapeutic efficacy of endostatin, we fused endostatin with cytosome deaminase, which converts a prodrug 5-flucytosine into a cytotoxic 5-fluorouracil. This therapeutic strategy was developed based on the observation that the endostatin-green fluorescence protein gene and endostatin-luciferase gene selectively target to endothelial cells in vitro and to the tumor site in vivo, respectively. When we used the endostatin-cytosine deaminase fusion protein to treat s.c. grafted tumors or experimental metastasis tumors, our results showed that endostatin-cytosine deaminase treatment provided stronger tumor growth suppression and increased mean survival time of the mice compared with the treatments of endostatin alone, cytosome deaminase alone, or endostatin plus cytosome deaminase. The endostatin-cytosine deaminase protein significantly inhibited the growth of endothelial cells and preferentially induced tumor cell apoptosis. This endostatin-cytosine deaminase fusion approach opens an avenue for cancer-targeting therapy.

Introduction

Angiogenesis inhibitors, a new class of antitumor therapy associated with low toxicity and low drug resistance, suppress tumor growth by blocking the formation of new blood vessels, which provide oxygen and nutrients for growth (1, 2). One such inhibitor is endostatin, a 20-kDa fragment cleaved from a collagen XVIII COOH terminus that inhibits endothelial cell proliferation, migration, invasion, and tube formation (3) and that has shown angiogenesis and antitumor effects in animal models (4). It specifically binds to neovascular endothelial cells through its interaction with the integrin receptors αvβ3 and αvβ5 (5, 6). αvβ3 has been implicated in tumor metastasis, and the selective αvβ3 inhibitor has been shown to reduce the occurrence of osteolytic breast cancer metastases (7). Furthermore, endostatin labeled with a near-IR probe was shown to selectively accumulate in the tumor site (8). These results strongly suggest that endostatin has a unique ability to target neovascular endothelial cells and therefore may be useful in antiangiogenesis therapy.

The targeted delivery of therapeutic agents to the tumor site has been achieved by using cancer antigen-specific antibody fusion proteins, such as IL12-L19 (9) and A33scFV-cytosine deaminase (10), or cancer-specific ligand fusion proteins, such as the prolactin antagonist G129R-endostatin (11). In another approach, embryonic endothelial progenitor cells have been genetically engineered to express thymidine kinase, a suicide gene that suppresses lung metastases (12). However, nonspecific toxicity is a major drawback of all these approaches. Cytosome deaminase is a suicide enzyme capable of converting prodrug 5-flucytosine (5-FC) into a cytotoxic 5-fluorouracil (5-FU) and has also been tested in multiple clinical trials (13). We reasoned that an endostatin-cytosine deaminase fusion protein should possess tumor-targeting property of endostatin to allow selective tumor-killing effect of cytosome deaminase in the presence of 5-FC, in addition to antiangiogenesis activity of endostatin, and thus might produce potent antitumor activity.

Materials and Methods

Cell lines. Human embryo kidney 293 (HEK293) cells, murine CT26 colon adenocarcinoma cells, murine 4T1 breast adenocarcinoma cells, murine SV40 transformed endothelial cells (SVEC), and murine NIH3T3 and human WI38 fibroblast cells were maintained at 37°C in a 5% CO2 incubator with DMEM/F-12 plus 10% fetal bovine serum. Human umbilical vein endothelial cells (HUVEC) were maintained in endothelial cell medium-2 (Cambrex, East Rutherford, NJ).

Cloning of endostatin fusion protein. PCR was used to generate the green fluorescence protein (GFP), firefly luciferase, and the cytosome deaminase gene. The individual gene was sequentially assembled with a COOH terminus of the endostatin gene by appending a short VPVG amino acid linker sequence.

Expression of endostatin fusion proteins. HEK293 cells were transfected with the constructs, and stable transfectants were selected in the presence of blasticidin (6 µg/mL). The fusion proteins were purified from a cell culture medium and concentrated using a Centricson-10 concentrator (Amicon, Billerica, MA). Secretion of endostatin-GFP and secreted GFP were confirmed by fluorescent microscope and Western blot (Fig. L4).

In vitro endothelial cell targeting. Established HEK293 transfectants endostatin-GFP or secreted GFP were seeded onto a 10-cm plate. At the same time, different cell lines of CT26 colon adenocarcinoma, 4T1 breast adenocarcinoma, NIH3T3 fibroblast, WI38 fibroblast, SVEC mouse endothelial cells, and HUVECs were seeded onto a cover slide. After the cells became attached, the targeted cell slides were placed into the 10-cm endostatin-GFP plate and enough medium was added to cover all the cells. After incubation for 24 hours, the cover slide was washed with PBS, and the green fluorescence was observed under a fluorescence microscope.

In vivo tumor targeting. CT26 parental cells (2 × 106) were s.c. injected into the left flank of 6-week-old BALB/c female mice. Group of three mice...
were used and randomly divided. The CT26 endostatin-luciferase stable cells were injected into the right flank of the mice in the experimental group, and CT26-secreted luciferase stable cells were used for the control group. The level of luciferase expression was similar between CT26 endostatin-luciferase and CT26-secreted luciferase (Fig. 1C) and endostatin-luciferase expression was confirmed by Western blot (Fig. 1C). After 14 days, luciferase activity was detected using the in vivo imaging system (Xenogen, Alameda, CA).

Endothelial tube assay. Matrigel (50 μL; BD Biosciences, San Jose, CA) was added to each well of a 96-well plate and allowed to polymerize. A suspension of 5 × 10^6 HUVEC cells was passed into a Matrigel-coated well. The cells were treated with a conditioned medium collected from different plasmids, including endostatin, endostatin-cytidine deaminase, and cytosine deaminase–transfected HEK293 cells. All assays were done in triplicate. The cells were incubated for 24 hours at 37°C and viewed under a microscope. Five fields were viewed, and tubes were counted and averaged.

Migration assay. The inhibitory effect of endostatin on vascular endothelial growth factor–induced chemotaxis was tested on HUVECs using an 8-μm Boyden chamber (Costar, Acton, MA) assay. Cells (1 × 10^5) were seeded on the upper chamber wells together with supernatant collected from HEK293 cells transfected with different plasmids, including endostatin, endostatin-cytidine deaminase, and cytosine deaminase (Invitrogen, San Diego, CA). M199 medium containing 2% fetal bovine serum plus 10 ng/mL vascular endothelial growth factor was placed in the lower chamber as a chemotactrant. The chamber was incubated at 37°C for 8 hours. After the nonmigrated cells were discarded and the upper wells were washed with PBS, the filters were scraped with a plastic blade and the cells were fixed in 4% formaldehyde in PBS and stained with 4,6-diamidino-2-phenylindole fluorescent dye. Five fields were viewed under a fluorescence microscope, and the cells were counted and averaged.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to measure the cytotoxic effect of 5-FU converted from 5-FC by microsomes of CT26 cells. The CT26 cells were incubated for 1 hour, after which 100 μL of different concentrations of 5-FU or 5-FC were added 24 hours after transfection. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (20 μL; 5 mg/mL, Sigma, St. Louis, MO) was added into the cell culture and the cells were incubated for 1 hour, after which 100 μL of DMSO (Sigma) was added to each well. The light absorbance was measured at 570 nm immediately after using a multwell scanner (LabSystems, Helsinki, Finland).

Tumor models. Two syngeneic mouse tumor models of murine 4T1 breast cancer and murine CT26 colon cancer have been used. Murine CT26 cells (2 × 10^6) were injected s.c. into the right flank of BALB/c female mice. Murine 4T1 breast adenocarcinoma cells (2 × 10^6) were injected s.c. into the right flank of 6-week-old BALB/c female mice. Murine 4T1 breast adenocarcinoma cells (2 × 10^6) were injected into the mammary fat pad of 6-week-old BALB/c female mice. Group of five mice were used and randomly divided. Five to 7 days after inoculation, the tumors averaged 3 to 5 mm in diameter. The plasmid DNA and cationic liposome complexes were injected i.v. into the tail vein (14). The treatment was repeated twice weekly for 3 weeks. The day after the DNA-liposome injection, 5-FC (500 mg/kg) was injected i.p.

Immunofluorescence staining of vessels in tumor and normal tissue. Briefly, 4-μm frozen sections were fixed in cold (−20°C) 100% acetone for 5 minutes and then air dried. After immersion in 1× PBS for 15 minutes, the slides were incubated for 18 hours with rat monoclonal anti-CD31 antibody (BD Biosciences) at 4°C and rinsed with 1× PBS and incubated with goat anti-rat immunoglobulin G conjugated to Texas red (1:200; Jackson ImmunoResearch Laboratory, Inc., West Grove, PA) in the dark for 60 minutes at ambient temperature. The CD31-positive blood vessels were counted in 10 to 30 fields at ×200 magnification in a blinded fashion. In each sample, five fields were randomly counted for the CD31-positive cells. At least three tumor tissues in each group were stained and samples were examined by two individuals, including one pathologist.

In vivo apoptotic (terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling) assay. For in vivo apoptotic assay, tumors were fixed in 10% formalin and embedded in paraffin blocks. Tissue sections were incubated with proteinase K (20 mg/mL in 10 mmol/L Tris-HCl (pH 7.4–8.0) for 15 minutes at 37°C), permeabilized in 0.1% Triton X-100 in 0.1% sodium citrate, and then labeled with the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling reaction mixture (Promega, Madison, WI) according to the manufacturer’s protocol. Briefly, biotinylated nucleotide mix and terminal deoxynucleotidyl transferase enzyme were added and incubated for 1 hour at 37°C; slides were washed in PBS, blocked in hydrogen peroxide, and incubated in streptavidin horseradish peroxidase. The slides were developed in 3,3′-diaminobenzidine and counterstained with hematoxylin. The apoptotic cells (brown staining) were counted under a microscope. In each sample, five fields were randomly counted for the apoptotic cells. At least three tumor tissues in each group were stained and samples were examined by two individuals, including one pathologist.

Results
Endostatin fusion proteins specifically target the tumor. To investigate the possibility of using endostatin-cytosine deaminase for cancer-targeting therapy, we first tested whether endostatin was capable of transferring a fusion protein to endothelial cells. To this end, two stable cell lines, one that expressed endostatin-GFP and another that expressed secreted GFP, were generated from parental HEK293 cells and designated HEK293-endostatin-GFP and HEK293-secreted GFP, respectively. The expression of endostatin-GFP and secreted GFP was confirmed by fluorescence microscopy and Western blot analysis (Fig. 1A). When cocultured with endothelial cells, the endostatin-GFP, but not the secreted GFP, bound to endothelial cells, HUVEC and SVEC (Fig. 1B, top). Although endostatin-GFP bound strongly to HUVECs and SVECs, it bound weakly to the epithelial cell lines 4T1 and CT26 and fibroblast cell lines NIH3T3 and WI38 (Fig. 1B, bottom). As we expected, the secreted GFP did not bind to the epithelial cells or the fibroblast cells (data not shown). These results showed that endostatin is able to carry the fusion GFP selectively to endothelial cells in vitro.

We then evaluated the endostatin protein for its ability to selectively deliver a fusion protein into the tumor site in vivo. Using the parental CT26 cells, we generated two lines, one that stably expressed endostatin-luciferase and another that stably expressed secreted luciferase, which we designated CT26-endostatin-luciferase and CT26-secreted luciferase, respectively. The levels of luciferase activity in CT26-endostatin-luciferase and CT26-secreted luciferase cells were similar (Fig. 1C, left) and the expression of endostatin-luciferase in CT26-endostatin-luciferase was confirmed by Western blot analysis (Fig. 1C, right). In a subsequent experiment, six BALB/c mice that carried CT26 xenografts in their left flank were randomly divided into two groups. One group of mice was inoculated with CT26-endostatin-luciferase on the right flank and the other with CT26-secreted luciferase. As shown in Fig. 1D, the tumors derived from CT26-endostatin-luciferase had growth delays, indicating the ability of endostatin-luciferase to selectively target the parental CT26 tumor. We reasoned that the observed accumulation of endostatin-luciferase at a tumor site was due to the targeting of endostatin-luciferase to endothelial cells because endostatin-GFP bound not to CT26 cancer cells (Fig. 1B, bottom) but to SVEC, a mouse endothelial cell line (Fig. 1B, top).

Biological function assay of endostatin-cytosine deaminase protein. We then studied whether endostatin could be used as a tumor-targeting protein to lead the cytotoxic suicide gene product
Figure 1. Endostatin fusion proteins target the tumor. 

A, characterization of endostatin-GFP and secreted GFP protein. Left, HEK293-endostatin-GFP (HEK293-Endo-GFP) and HEK293-secreted GFP (HEK293-sGFP) under fluorescence or light microscope; right, Western blot analysis of secreted GFP (sGFP) and endostatin-GFP (Endo-GFP) from a culture medium with endostatin (left) and GFP (right) antibodies. IB, immunoblot. 

B, endostatin-GFP selectively bound to endothelial cells (HUVECs and SVECs) but not to the epithelial (CT26 and 4T1) or fibroblast (NIH3T3 and WI38) cells (bottom). The experiment was done thrice independently. 

C, characterization of endostatin-luciferase and secreted luciferase protein. Left, luciferase activities of CT26-endostatin-luciferase (CT26-Endo-Luc) and CT26-secreted luciferase (CT26-sLuc) lines were quantified by the Xenogen Living Image System. RLU, relative light unit. Right, Western blot analysis of endostatin-luciferase and secreted luciferase. IB: Endostatin; IB: E-actin. The experiment was done thrice independently. 

D, endostatin-luciferase selectively accumulated at the tumor site. Left, black circle, tumor inoculated by CT26-endostatin-luciferase or CT26-secreted luciferase; red circle, tumors inoculated with parental CT26 without luciferase expression; right, the relative light units of the circled areas (left) in the mice were quantified by the Xenogen Living Image System (bar graphs). The experiment was done thrice independently.
cytosine deaminase to the tumor site to kill cancer cells. The expression of endostatin, secreted cytosine deaminase, and endostatin-cytosine deaminase proteins collected in a culture medium was determined by Western blot analysis (Fig. 2A, right inset). To assess whether endostatin-cytosine deaminase also retains its 5-FC prodrug-converting enzyme activity, we used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and relative cell viability to measure the 5-FC prodrug-converting enzyme activity (Fig. 2A). This showed that there was a marked decrease in cell viabilities in the groups receiving the 5-FU and endostatin-cytosine deaminase plus 5-FC treatments (Fig. 2A, left). This endostatin-cytosine deaminase–mediated suppression of cell viability was clearly evident when compared with that mediated by secreted cytosine deaminase and endostatin (Fig. 2A, right). To show that the endostatin-cytosine deaminase fusion protein possessed antiangiogenic activity, we did endothelial tube assays on Matrigel-coated wells and migration assays with vascular endothelial growth factor–attracted cells and determined that the angiogenesis inhibitory effects of endostatin-cytosine deaminase were similar to those of endostatin (Fig. 2B). In particular, there were significant decreases in tube formation (top) and the numbers of migrated cells (bottom) in the groups treated with endostatin and endostatin-cytosine deaminase compared with those receiving the mock treatment (culture medium).

**Endostatin-cytosine deaminase suppresses tumor growth.** To investigate the effect of the endostatin-cytosine deaminase gene on large tumors, we carried out gene therapy experiments using BALB/c mice bearing s.c. CT26 xenografts. The therapeutic effect of endostatin-cytosine deaminase was determined following systemic delivery of the DNA lipoplex i.v. through tail vein injection. The group treated with endostatin-cytosine deaminase in the presence of the 5-FC prodrug showed significant tumor stabilization or regression compared with the groups receiving the other treatments 1 month after tumor inoculation (Fig. 3A, left). Consistently, the endostatin-cytosine deaminase therapeutic effect is dependent on 5-FC prodrug (Fig. 3A, right). In line with the tumor growth suppression results, endostatin-cytosine deaminase expression and 5-FC treatment prolonged the life span of the mice carrying colon cancer xenografts. As shown in the Fig. 3B, the mean survival time of the group treated with endostatin-cytosine deaminase plus 5-FC (96 days) significantly increased compared with that of the other groups, such as the one treated with endostatin-cytosine deaminase without 5-FC (57 days) or cytosine deaminase plus 5-FC (58 days).

To ensure the targeting effect of endostatin-cytosine deaminase, we s.c. delivered the DNA lipoplex to treat a CT26 xenograft tumor. The CT26 tumor cells were inoculated in the right flank of the mice, and the various DNA lipoplexes were given in the left flank. The results showed that the endostatin-cytosine deaminase had better therapeutic efficacy than either endostatin or cytosine deaminase alone (Fig. 3C). Interestingly, the cytosine deaminase treatment that could not selectively target cancer neovascular endothelial growth factor–attracted cells (bar graphs). Bars, SD. All experiments were done thrice in triplicate.
Figure 3. Endostatin-cytosine deaminase suppresses tumor growth. A, left, endostatin-cytosine deaminase showed superior suppression of CT26 colon tumor growth. 5% Dextrose (D5W) was used as a control. All treatments included i.p. injection of 5-FC (500 mg/kg) 1 day after DNA-liposome delivery unless noted. Arrows, i.v. DNA lipoplex injection; asterisk, date of the last treatment. Five mice were used in each group. Bars, SD. Right, endostatin-cytosine deaminase therapeutic efficacy depends on 5-FC. B, endostatin-cytosine deaminase + 5-FC prolonged the mean survival time of mice. The treatment protocol is the same as in (A). Ps < 0.05, statistically significant difference between endostatin-cytosine deaminase + 5-FC versus endostatin-cytosine deaminase without 5-FC and endostatin-cytosine deaminase + 5-FC versus cytosine deaminase + 5-FC. C, s.c. injection of the endostatin-cytosine deaminase DNA lipoplex suppressed CT26 tumor growth. CT26 colon cancer cells were first inoculated in the right flank of mice. When the tumor reached 3 to 5 mm in diameter, the mice were treated with a s.c. injection of endostatin-cytosine deaminase DNA lipoplex in the left flank. All treated groups were also treated with the i.p. injection of 5-FC (500 mg/kg). D, right, endostatin-cytosine deaminase showed better therapeutic efficacy than the combination therapy of endostatin with cytosine deaminase in an orthotopic 4T1 mammary tumors. Both groups were treated with i.p. injection of 5-FC (500 mg/kg). All animal experiments have been carried out thrice by two individuals independently.
cells had no therapeutic effect supporting the importance of the endostatin-targeting activity from endostatin-cytosine deaminase.

To test whether this therapeutic approach can be extended to other tumor models, we chose an orthotopic mammary tumor model of 4T1 breast adenocarcinoma in a mammary fat pad. We first systemically delivered the DNA lipoplex i.v. The i.v. endostatin-cytosine deaminase gene therapy effectively suppressed tumor growth in the mammary tumor model (Fig. 3D, left), as it did in the CT26 xenograft tumor model, and the therapeutic efficacy also depended on the presence of the 5-FC prodrug (Fig. 3D, right). Furthermore, the endostatin-cytosine deaminase was much more effective than the combination of endostatin and cytosine deaminase in suppressing tumor growth (Fig. 3E), indicating that the therapeutic efficacy of endostatin-cytosine deaminase is greater than the sum of endostatin plus cytosine deaminase.

**Endostatin-cytosine deaminase reduced tumor endothelial cell numbers and induced tumor cell apoptosis.** In support of the tumor suppression results, CD31-positive staining showed that...
the number of endothelial cells in tumor tissues was greatly reduced in the endostatin-cytosine deaminase–treated group than in the other treatment groups (Fig. 4A, top). In addition, the endostatin-cytosine deaminase plus 5-FC treatment again showed a much more potent effect in reducing the numbers of endothelial cells compared with the endostatin-cytosine deaminase without 5-FC treatment (Fig. 4A, bottom). To examine whether the tumor growth suppression was caused by endostatin-cytosine deaminase–induced apoptosis, we did the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay. The results showed that the number of apoptotic cancer cells treated with endostatin-cytosine deaminase is significantly higher than other treatments (Fig. 4B, top and bottom). Importantly, virtually no apoptosis was detected in normal tissues under the same experimental conditions (Fig. 4B, bottom), supporting the tumor-targeting nature of endostatin.

Discussion

Angiogenesis is involved in distant metastases (15). By targeting angiogenesis, endostatin gene therapy directly suppresses not only the growth of solid tumors but also the occurrence of metastases (16). Use of endostatin for tumor vascular targeting avoids the pitfalls of delivering a high molecular weight agent to the tumor interstitially, because endothelial cells are easier to access than tumor cells. In addition, endothelial cells are considered to be genetically stable and are less likely to become drug resistant.

Cytosine deaminase, a therapeutic suicide gene for tumors, converts 5-FC into 5-FU. 5-FU is a standard chemotherapeutic agent for colon cancer and inhibits RNA and DNA synthesis during the S phase of the cell cycle. Continuous low dosage of chemotherapy (metronomic chemotherapy) is able to suppress endothelial cell growth and to induce cell apoptosis or cell growth arrest (17). The metronomic chemotherapy also suppresses the endothelial cell mobilization and induces angiogenesis inhibitors, such as thrombospondin. Similarly, endostatin has been shown to down-regulate several proangiogenic signaling pathway components, such as activator protein-1, nuclear factor-κB, and signal transducers and activators of transcription, and to up-regulate several antiangiogenic genes, such as THBS-1, vasostatin, kininogene, AT3, and maspin (18). The endostatin gene and cytosine deaminase gene that converted 5-FC into 5-FU may act in concert to inhibit angiogenesis while also providing direct inhibition to tumor cell growth.

Targeting angiogenesis using embryonic endothelial progenitor cells armed with a suicide gene has recently been shown to selectively target hypoxic lung metastases and efficiently eradicate the tumor (12). However, the use of therapeutic progenitor cells for cancer therapy still needs to overcome a few concerns, such as the potential adverse effect of treatment with engineered endothelial progenitor cells. As reported, the mice receiving the engineered endothelial progenitor cells alone actually died sooner than those receiving the control treatment (19). In this regard, by overcoming the nonspecific and poor delivery of a therapeutic gene to the tumor region, the current endostatin-cytosine deaminase treatment strategy seems to be more advantageous than the engineered endothelial progenitor cells. The endostatin-cytosine deaminase therapy could be worthwhile for further development into clinical trials.

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