Dual Targeting of Tumor Angiogenesis and Chemotherapy by Endostatin–Cytosine Deaminase–Uracil Phosphoribosyltransferase

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Abstract

Several antiangiogenic drugs targeting VEGF/VEGF receptor (VEGFR) that were approved by the Food and Drug Administration for many cancer types, including colorectal and lung cancer, can effectively reduce tumor growth. However, targeting the VEGF signaling pathway will probably influence the normal function of endothelial cells in maintaining homeostasis and can cause unwanted adverse effects. Indeed, emerging experimental evidence suggests that VEGF-targeting therapy induced less tumor cell–specific cytotoxicity, allowing residual cells to become more resistant and eventually develop a more malignant phenotype. We report an antitumor therapeutic EndoCD fusion protein developed by linking endostatin (Endo) to cytosine deaminase and uracil phosphoribosyltransferase (CD). Specifically, Endo possesses tumor antiangiogenesis activity that targets tumor endothelial cells, followed by CD, which converts the nontoxic prodrug 5-fluorocytosine (5-FC) to the cytotoxic antitumor drug 5-fluorouracil (5-FU) in the local tumor area. Moreover, selective targeting of tumor sites allows an increasing local intratumoral concentration of 5-FU, thus providing high levels of cytotoxic activity. We showed that treatment with EndoCD plus 5-FC, compared with bevacizumab plus 5-FU treatment, significantly increased the 5-FU concentration around tumor sites and suppressed tumor growth and metastasis in human breast and colorectal orthotropic animal models. In addition, in contrast to treatment with bevacizumab/5-FU, EndoCD/5-FC did not induce cardiotoxicity leading to heart failure in mice after long-term treatment. Our results showed that, compared with currently used antiangiogenic drugs, EndoCD possesses potent antitumor activity with virtually no toxic effects and does not increase tumor invasion or metastasis. Together, these findings suggest that EndoCD/5-FC could become an alternative option for future antiangiogenesis therapy.

Introduction

Several anticancer drugs that target angiogenesis by blocking VEGF/VEGF receptor (VEGFR) have shown promise and have been approved by the Food and Drug Administration (FDA) for clinical treatment of various cancer types (1). These drugs include bevacizumab (Avastin), a humanized VEGF-neutralizing monoclonal VEGF antibody, and sorafenib and sunitinib, small molecular inhibitors that block VEGFR tyrosine kinase activity. Although these agents have successfully inhibited primary tumor growth and progression, there is no curative potential activity (2, 3). In addition to causing severe adverse effects, such as cardiotoxicity, gastrointestinal perforation, poor wound healing, and hypertension, induced by antiangiogenic treatment (4, 5), recent reports also indicate that anti-VEGF therapy enhances tumor progression by increasing invasion and metastasis (6, 7).

Tumors require the VEGF pathway for progression; however, stimulation of other angiogenesis factors may occur after VEGF/VEGFR-targeted therapy and induce tumor resistance to antiangiogenesis monotherapy (8). For example, in addition to VEGF-stimulated angiogenesis, 6 other proangiogenic growth factors contribute to various stages of breast cancer progression (9); in addition, several intracellular molecules have been identified that modulate tumor angiogenesis (10). Because it is not sufficient to inhibit tumor growth with the use of antiangiogenesis monotherapy, a combination approach with
chemotherapy is commonly used to improve the clinical benefits of antiangiogenic drug treatment (11). Although combination therapy may reduce tumor resistance and enhance therapeutic efficiency compared with use of a single antiangiogenic drug, increased toxicity results in shorter progression-free survival (12). Initially, anti-VEGF/VEGFR antiangiogenic drugs were not expected to cause toxic effects or drug resistance; however, increasing numbers of clinical reports have shown that inhibition of the VEGF signaling pathway has led to off-target effects that influenced normal body homeostasis (13). Some adverse effects include potentially life-threatening complications such as gastrointestinal perforation, with short-term treatment, and a reduced left ventricular ejection fraction (LVEF) in the heart, with long-term treatment.

Drugs that block angiogenesis can be classified as either direct or indirect inhibitors (2). Compared with direct angiogenic inhibitors, indirect anti-VEGF/VEGFR agents are more likely to induce drug resistance because they target genetically altered and unstable tumor cells rather than genetically stable endothelial cells (2, 14). Endostatin (Endo), an angiogenesis inhibitor that targets unique proliferating endothelial cells and inhibits proliferation, migration, and invasion (15) through interaction with the α5β3 and αvβ3 integrin receptors (16), was tested in multiple clinical trials (17, 18) without causing severe adverse effects or drug resistance. However, because of poor clinical response and its short protein half-life, endostatin was not pursued further in clinical trials after phase II studies in the United States (19, 20).

Cytosine deaminase is an enzyme capable of converting the 5-fluorocytosine (5-FC) prodrug into cytotoxic 5-fluorouracil (5-FU; ref. 21). A fusion gene consisting of cytosine deaminase and uracil phosphoribosyltransferase (for simplicity, we refer to this fusion gene as CD throughout this article) was reported to increase the 5-FC/5-FU conversion rate to 10,000 times the rate observed with the use of a single enzyme alone (22) and is well known to produce bystander effect in cancer cell killing in a gene therapy setting (23, 24). However, because it is difficult to target chemotherapy specifically to the tumor site, systemic treatment will have off-target issues and cause unwanted adverse effects.

In this study, we report an engineered EndoCD (25) fusion protein that provides a wider therapeutic window, higher therapeutic efficacy and safety, and lower drug resistance potential for cancer treatment. We compared the therapeutic efficacy of EndoCD plus 5-FC (EndoCD/5-FC) and bevacizumab plus 5-FU (bevacizumab/5-FU) and found that EndoCD/5-FC surpassed bevacizumab/5-FU in several aspects: (i) EndoCD/5-FC exhibited potent antitumor activity by increasing 5-FU concentration locally at the tumor site, resulting in higher therapeutic efficacy; (ii) EndoCD/5-FC decreased metastasis; (iii) EndoCD/5-FC did not induce cardiotoxicity or cardiac function failure as monitored by MRI over a prolonged period. Our findings show that EndoCD fusion protein is an excellent candidate as an antiangiogenic inhibitor to be tested in future clinical trials.

Materials and Methods

Detailed methodology is described in the Supplementary Methods.

Cell lines

MDA-MB-231 and murine 4T1 breast adenocarcinoma cell lines were maintained in Dulbecco’s Modified Eagle’s F12 Medium supplemented with 10% FBS. Human umbilical vein endothelial cells were cultured in endothelial cell medium-2 (Cambrex). The 620-L-1 colon cancer cell line was generated by our laboratory after several cycles of preselection from an orthotopic colon model that produced 100% liver metastasis and was maintained by G418 selection. All cell lines were validated by short tandem repeats (STR) DNA fingerprinting by using the AmpFISTR Identifiler PCR Amplification Kit according to the manufacturer’s instructions (Applied Biosystems; catalogue no. 4322288). The STR profiles were compared with known American Type Culture Collection (http://www.atcc.org/) fingerprints to the Cell Line Integrated Molecular Authentication Database (CLIMA) version 0.1.200808 (http://bioinformatics.istge.it/clima/; ref. 26) and to the MD Anderson fingerprint database. The STR profiles matched known DNA fingerprints or were unique.

Recombinant protein purification

The coding sequence of the human Endo was amplified from pPICZαA/hE (EntreMed) by PCR and cloned into the pET28 bacterial expression vector (Novagen) to generate pET28Endo. The yeast CD was subcloned from pORF5-Fcy::Fur into pET28 (pET28CD). Recombinant proteins endostatin, CD, and EndoCD were expressed from pET28Endo, pET28CD, and pET28EndoCD, respectively, and purified from a liter of isopropyl β-thiogalactopyranoside (IPTG)-induced bacterial culture according to procedures previously described (27). The molecular weights of endostatin, CD, and EndoCD are 20, 40, and 60 kDa, respectively. Therefore, an equimolar ratio (1:2:3) of the proteins was used for all experiments.

Animal models

All animals were maintained in the animal facility, and experiments were carried out according to The University of Texas MD Anderson Cancer Center guidelines. For the syngeneic model, BALB/c mice were inoculated (mammary fat pad) with 1 × 10⁶ 4T1 murine breast adenocarcinoma cells. After the tumor volume reached 3 to 5 mm in diameter, equal molar amounts of proteins (endostatin, CD, and EndoCD) were injected via tail vein. One hour after protein treatment, all groups received 5-FC (500 mg/kg) by intraperitoneal (i.p.) injection. For the orthotopic xenograft model, nude mice were inoculated...
with $3 \times 10^6$ MDA-MB-231 human breast cancer cells in the mammary fat pad or $3 \times 10^6$ 620-L-1 human colon cancer cells in the cecal wall. Mice received 10 mg/kg of bevacizumab (once every 2 weeks; the clinical dose and schedule used in treating breast and colon cancer) or 60 mg/kg of EndoCD (twice per week; the protein dosage was based on the endostatin clinical dose of 20 mg/kg and the schedule was based on protein stability) via tail-vein injection. For mice treated with EndoCD, 500 mg/kg of 5-FC was given 1 hour later by i.p. injection. Mice treated with bevacizumab were given 15 mg/kg 5-FU i.p. (clinical dose) once per week. Tumor volume was monitored by measuring luciferase signals using IVIS-200 Optical In Vivo Imaging System (Xenogen). In a reduced-treatment experiment, the number of treatments given was decreased from 10 to 5. All protein treatments were given i.v., whereas chemical drugs were administered by i.p. injection.

**Immunofluorescence staining**

Frozen sections (4 μm) were fixed in cold 100% acetone for 5 minutes and then air-dried. After immersion in 1 x PBS for 15 minutes, the slides were incubated with rat monoclonal anti-CD31 antibody (Becton Dickinson Biosciences) at room temperature for 1 hour, rinsed with 1 x PBS and then incubated with goat anti-rat immunoglobulin G conjugated to Texas Red (1:200; Jackson ImmunoResearch Laboratory) in the dark at ambient temperature for 60 minutes. CD31-positive blood vessels were counted in 10 to 30 fields at x200 magnification in a blinded fashion.

**Results**

Previously, we developed a gene therapy–based EndoCD treatment that was both antiangiogenic and cytotoxic and had significant antitumor effect in vivo (25). However, because the FDA has not approved a single human gene therapy as a drug to date, a protein-based therapy of EndoCD would be more practical for obtaining FDA approval. To achieve this goal, we constructed a recombinant protein expression vector containing the human endostatin and yeast CD fusion gene that was engineered to express EndoCD as a single polypeptide (Fig. 1A). As an endothelial cell–targeting agent, endostatin would bring CD to the tumor site as Endo targets tumor vasculature (28), and once at the tumor site, CD could then convert 5-FC to cytotoxic 5-FU and induce apoptosis. Because of the differences in the molecular weight of endostatin, CD, and EndoCD (20, 40, and 60 kDa, respectively), we used a ratio of 1:2:3 of the proteins for all the experiments in this study to ensure equal molarity (Fig. 1B).

To determine the stability of the purified protein in serum, 12.5 μmol/L of each purified, His-tagged protein (endostatin, CD, and EndoCD) was mixed immediately...
with mice serum, incubated at 37°C for the number of days indicated, and analyzed by immunoblotting with use of anti-His-tag antibody (Supplementary Fig. S1A). We found that, although Endo had a short protein half-life of less than 1 day, which is consistent with previous clinical reports (29), the EndoCD fusion protein had a much longer protein stability with a half-life of about 3 days than Endo in the presence of mice serum (Supplementary Fig. S1B). After we validated the protein stability of EndoCD in serum, we tested its antiangiogenic activity and cytotoxicity in vitro. As shown in Supplementary Fig. S2, and consistent with the previously established EndoCD gene therapy, purified EndoCD protein significantly decreased tube formation and the number of migrated cells and suppressed cell growth. As expected, EndoCD/5-FC and CD/5-FC showed similar cell-killing effect in mouse 4T1 breast cancer cell line. However, we observed a slight difference in cell killing activity between EndoCD/5-FC and CD/5-FC in human MDA-MB-231 cells for which the mechanism is unclear. Nonetheless, these results show that the purified EndoCD fusion protein maintained the function of endostatin and CD in vitro.

To determine the antitumor activity of EndoCD in vivo, we first injected 4T1 mammary tumor cells into the mammary fat pad of BALB/c mice to establish a syngeneic mouse model. When the tumor grew 3 to 5 mm in diameter, 2.5 mg/kg of endostatin, 5 mg/kg of CD, or 7.5 mg/kg of EndoCD was administered by i.p. injection of mice for a total of 10 treatments (indicated by arrows), with i.p. injection of 500 mg/kg 5-FC (22) given 1 hour after protein treatment. We selected 2.5 mg/kg for Endo as the starting treatment dose because it was previously shown to effectively inhibit tumor growth (15). Mice that received EndoCD/5-FC showed more significant tumor regression (Fig. 1C) and had a prolonged overall survival rate (Fig. 1D) compared with those that received Endo/5-FC or CD/5-FC. These results indicate that the EndoCD fusion protein did not alter the original biological function of either endostatin or CD and inhibited tumor growth more effectively than did the 2 proteins alone.

To characterize the biological effects of EndoCD-mediated antitumor and antiangiogenic activities in vivo, we harvested tumor samples from the treated mice described above to examine in vivo angiogenesis inhibition as well as induction of cell death induced by the purified fusion proteins. Immunofluorescence staining of tumor tissue with use of CD31 antibody (a marker for endothelial cells) as well as use of the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) assay showed that EndoCD/5-FC reduced tumor vascular density and induced endothelial and cancer cell apoptosis. On the basis of the merged image of CD31 and apoptosis double staining in EndoCD/5-FC–treated tumor samples, the majority of the apoptotic signal was found in and around endothelial cells (TUNEL/CD31 panel and inset; Fig. 2A). The signal (green) from the TUNEL assay overlapped with the CD31 signal (red) in the tumor of EndoCD/5-FC–treated mice, indicating that the endothelial cells underwent active apoptosis. Moreover, the presence of TUNEL signal (green) around the endothelial cells (Fig. 2A, inset) only in EndoCD/5-FC- but not Endo/5-FC- or CD/5-FC–treated cells suggests that it most likely is from apoptotic tumor cells. These observations suggest that the cytotoxic effect is a result of increased local concentration of 5-FU in the tumor microenvironment.

To determine the effect of protein treatments in cell proliferation, MDA-MB-231 breast cancer cells were first injected into the mammary fat pad of nude mice, and when tumors grew to 10 mm in diameter, mice were treated once only with purified Endo (20 mg/kg), CD (40 mg/kg), or EndoCD (60 mg/kg) proteins plus 500 mg/kg 5-FC, a clinically sufficient dose of 5-FU (15 mg/kg; 1 × 5-FU), or 10 times the clinically sufficient dose (150 mg/kg; 10 × 5-FU). The choice of 20 mg/kg Endo was based on a previous preclinical study (15) and is also within the dose tested in the phase I clinical trial (ref. 18; 15–600 mg/m² in human is equivalent to 4.8–194.4 mg/kg in mouse; ref. 30). Tumors were harvested from mice 48 hours after treatment and labeled with bromodeoxyuridine (BrdU) antibody for in vivo BrdU incorporation analysis. The results show that EndoCD/5-FC most significantly reduced cancer cell proliferation (Fig. 2B) compared with all other treatment groups.

The potent inhibitory activity of EndoCD/5-FC treatment on cancer cell proliferation in vivo, which is even stronger than that of 10 × 5-FU treatment (Fig. 2B), prompted us to measure the local concentration of 5-FU in the tumor sites. To this end, we used liquid chromatography/tandem mass spectrometry (LC/MS-MS) to measure 5-FU concentration in the tumor microenvironment. First, tumor-bearing mice were given 500 mg/kg 5-FC, 10 × 5-FU, or 60 mg/kg EndoCD plus 500 mg/kg 5-FC. Then, tumors were removed 2 hours after drug treatment, and 5-FU was directly extracted from tumor samples for detection by LC/MS-MS. The 5-FU concentration in tumors from the EndoCD/5-FC–treated group was approximately 7-fold higher than that from the 10 × 5-FU treatment group (Fig. 2C). Together, the results show that EndoCD/5-FC can reduce tumor-site vascular density and increase 5-FU concentration around tumor sites to enhance apoptosis in both tumor and tumor endothelial cells.

To investigate the acute toxicity of EndoCD, mice were administered 60 mg/kg EndoCD with 500 mg/kg 5-FC given 1 hour after the protein treatment. Blood samples were collected every other day for 7 days from all mice treated with Endo, CD, or EndoCD fusion protein and analyzed for changes in the serum level of liver aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, and creatinine. Clearly, aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen, and creatinine levels in EndoCD-treated mice were all within the normal range (31; Supplementary Fig. S3). In addition, no mice in the EndoCD/5-FC treatment group...
displayed any obvious symptoms such as loss of appetite or inactivity or died more than 2 months after initial treatment, when the experiment was terminated (data not shown). Thus, these results suggest that treatment with EndoCD/5-FC produced virtually no toxicity or nondetectable toxicity in mice. To further determine the antitumor activity of EndoCD/5-FC, we compared the therapeutic efficacy
of EndoCD/5-FC and bevacizumab/5-FU in orthotopic mouse models of human colorectal cancer with liver metastasis (620-L-1) and human breast cancer (MDA-MB-231). Seven days after injection, when tumors were established, EndoCD (60 mg/kg, twice per week) or bevacizumab (10 mg/kg, once every 2 weeks; ref. 32) was i.v. injected, and 5-FU (15 mg/kg, once per week; ref. 31) or 5-FC (500 mg/kg; given 1 hour after EndoCD treatment) was administered by i.p. injection. For practical clinical reasons, the treatment protocols for bevacizumab and 5-FU were essentially derived from previously established clinical doses and schedules (32).

We observed significantly better tumor suppression in mice treated with EndoCD/5-FC than in those treated with bevacizumab or 5-FU alone (Fig. 3A and B). Although we did not observe a significant difference in tumor reduction between EndoCD/5-FC and bevacizu-

Discussion

VEGF/VEGFR serves an important role in regulating tumor angiogenesis initiation and in controlling human homeostasis. In addition to its role in angiogenesis, VEGF...
Figure 3. Therapeutic efficacy of EndoCD/5-FC and bevacizumab/5-FU in an orthotopic animal model. In a metastatic colorectal cancer model (A) and a human breast cancer model (B), 60 mg/kg of EndoCD proteins were injected i.v. twice per week. An hour after protein treatment, mice were given 500 mg/kg of 5-FC by i.p. injection. For bevacizumab treatment, 10 mg/kg of the drug was injected i.v. once every 2 weeks and mice were given with 15 mg/kg of 5-FU by i.p. injection once per week. C, EndoCD/5-FC–treated mice (from A) exhibited prolonged overall survival. EndoCD/5-FC showed better antitumor activity than bevacizumab/5-FU in mice treated only for 3 weeks for a total of 5 treatments in a metastatic colorectal cancer model (D) and breast cancer model (E). F, EndoCD/5-FC reduced liver metastasis in a metastatic colorectal cancer model. Bec, bevacizumab. Arrows represent each protein treatment.

Dual-Targeted Therapy of EndoCD

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also maintains normal biological functions including blood pressure, kidney function, and blood coagulation (13). Thus, starvation of tumor growth by systemically blocking VEGF/VEGFR would also influence human homeostasis and induce off-target adverse effects. Although inhibition of a tumor angiogenesis pathway by a single drug alone is not sufficient to block redundancies in tumor angiogenesis regulators, leading to potential increases in tumor relapse, invasion, and metastasis (38), a combination of antiangiogenic drugs and chemotherapy has been shown to causes more life-threatening adverse effects (12). Therefore, by targeting unique endothelial cells through the use of an endogenous antiangiogenic molecule, the off-target adverse effects and drug-induced resistance may be reduced. With one exception, most endogenous antiangiogenesis proteins are low in protein stability as well as cytotoxic antitumor activity (39).

Figure 4. In vivo cardiac function detection. A, hydroxyproline content was measured and normalized to heart tissue weight. The hydroxyproline content in the bevacizumab/5-FU treatment group was significantly higher than that in control mice of the same age, and there was no significant difference between levels in the EndoCD/5-FC group and control mice. B, the presence of fibrosis is shown in blue by trichrome staining of heart histologic section. C, mice serum VEGF level decreased in bevacizumab/5-FU–treated mice but not in EndoCD/5-FC–treated mice. D, representative example of vascular density immunostaining by CD31 antibody (red) of heart tissue. Bevacizumab/5-FU–treated mice showed significantly reduced vascular density in the heart tissue. E, after drug treatment, LVEF was significantly decreased in the bevacizumab/5-FU group, whereas there was no significant difference of LVEF in the EndoCD/5-FC group. The pretreatment percentage was used as the basal level of LVEF. All mice described in A–E were treated with 10 mg/kg of bevacizumab plus 15 mg/kg of 5-FU or 60 mg/kg of EndoCD plus 500 mg/kg of 5-FC. EDV, end-diastolic volume; ESV, end-systolic volume; NS, not significant.
high-dose- and site-specific chemotherapeutic effect, thereby offering potentially curative therapeutic benefits. Because the VEGF pathway is also essential for normal biological functions, inhibitors that interrupt it would, no doubt, produce off-target side effects in normal organs. Instead, the use of an approach that targets proliferating endothelial cells would most probably decrease the adverse effects (14). Therefore, by bringing chemotherapeutic drugs to the tumor site, treatment with drugs such as EndoCD further increases the local cytotoxic effect.

Recently, some studies have reported that cancer stem cells transdifferentiate into endothelial cell phenotypes to form tumor vasculature, explaining the failure of anti-angiogenic drugs in the clinic and of proposed strategies to specifically target tumor endothelial cells to block angiogenesis in cancer treatment (40–42). However, it is not yet clear whether transdifferentiation of stem-like cancer cells into endothelial cells also takes place in solid tumors such as breast or colon cancer. This is an interesting issue to be addressed in a systematic approach in the future. In this study, we provided evidence that EndoCD/5-FC not only targets tumor angiogenesis specifically but also reduces the number of surviving tumor cells, thus decreasing the incidence of tumor invasiveness and metastasis. Altogether, the dual-targeting antiangiogenic and chemotherapeutic strategy we have developed may provide a new therapy to prevent tumor recurrence, decrease tumor metastasis, and eliminate unwanted cytotoxic adverse effects.

Disclosure of Potential Conflicts of Interest

M-C. Hung is an inventor on patents.

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In Memoriam

We recognize Mrs. Serena Lin-Guo for her courageous fight against breast cancer.

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References


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