Enhanced inhibition of murine tumor and human breast tumor xenografts using targeted delivery of an antibody-endostatin fusion protein

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Abstract

Endostatin can inhibit angiogenesis and tumor growth in mice. A potential limitation of endostatin as an antitumor agent in humans is the short serum half-life of the protein that may decrease effective concentration at the site of tumor and necessitate frequent dosing. In an effort to improve antitumor activity, endostatin was fused to an antibody specific for the tumor-selective HER2 antigen to create an antibody-endostatin fusion protein (anti-HER2 IgG3-endostatin). Normal endostatin rapidly cleared from serum in mice (T1/2 = 0.6–3.8 hours), whereas anti-HER2 IgG3-endostatin had a prolonged half-life (90% intact; T1/2 = 40.2–44.0 hours). Antigen-specific targeting of anti-HER2 IgG3-endostatin was evaluated in BALB/c mice implanted with CT26 tumors or CT26 tumors engineered to express the HER2 antigen (CT26-HER2). Radio-iodinated anti-HER2 IgG3-endostatin preferentially localized to CT26-HER2 tumors relative to CT26 tumors. Administration of anti-HER2 IgG3-endostatin to mice showed preferential inhibition of CT26-HER2 tumor growth compared with CT26. Anti-HER2 IgG3-endostatin also markedly inhibited the growth of human breast cancer SK-BR-3 xenografts in severe combined immunodeficient mice. Anti-HER2 IgG3-endostatin inhibited tumor growth more effectively than endostatin, anti-HER2 IgG3 antibody, or the combination of antibody and endostatin. CT26-HER2 tumors treated with the endostatin fusion protein had decreased blood vessel density and branching compared with untreated CT26-HER2 or CT26 treated with the fusion protein. The enhanced effectiveness of anti-HER2 IgG3-endostatin may be due to a longer half-life, improved serum stability, and selective targeting of endostatin to tumors, resulting in decreased angiogenesis. Linking of an antiangiogenic protein, such as endostatin, to a targeting antibody represents a promising and versatile approach to antitumor therapy.

Introduction

Antiangiogenic therapy designed to block neovascularization is an evolving anticancer strategy (1, 2). Antiangiogenic tumor therapies have recently attracted intense interest because of their broad-spectrum of action, low toxicity, and absence of drug resistance (1). Tumor angiogenesis is regulated by a balance of stimulators [vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), etc.] and inhibitors of angiogenesis (angiostatin, endostatin, etc.). The efficacy of a humanized anti-VEGF antibody (bevacizumab, Avastin, recombinant humanized monoclonal antibody-VEGF; Genentech, South San Francisco, CA) when used in combination with chemotherapy reported in a phase III trial in metastatic colon carcinoma suggests that antiangiogenic approaches may be used to augment existing antitumor strategies (3–5).

Endostatin is a 20-kDa fragment of the α1 chain of type XVIII collagen with potent antiangiogenic and antitumor properties (6, 7). The antitumor activity of endostatin may in part be due to inhibition of the proliferation and migration of endothelial cells (8, 9). In addition, endostatin may down-regulate VEGF expression in tumor cells (10). Systemic therapy with endostatin caused primary tumor regression in murine models without obvious toxicity (1, 2). Systemic endostatin also suppressed the growth of human renal cell cancer xenografts in a nude mouse model (11, 12). Antiangiogenic therapy with endostatin has been shown to block tumor growth with no evidence for emergence of resistance despite multiple cycles of therapy (2, 11). In some murine models, repeated treatment with endostatin...
resulted in permanent eradication of tumor (1, 2, 11, 13). These results show a potential advantage of antiangiogenic therapy because a major cause of treatment failure with contemporary cytotoxic agents is the development of acquired resistance (11).

In early human trials, endostatin administration at a dose of 240 mg/m^2/d that were effective in tumor xenograft studies did not produce significant changes in biological end points (14). “Modest” clinical benefit was observed in 3 of 15 patients (14). In a phase I trial using dose levels up to 600 mg/m^2/d, minimal antitumor activity was seen in 25 patients despite circulating levels that had been effective in mouse models (15). Two of 25 patients (1 with sarcoma and 1 with melanoma) showed antitumor activity. A third phase I trial of endostatin administered as a 1-hour i.v. infusion daily for a 28-day cycle at doses of 30 to 300 mg/m^2 showed that endostatin was free of significant drug-related toxicity, but no clinical responses were observed in all 21 patients (16). These phase I clinical trials have proven that endostatin is a very safe drug in a variety of dose schedules. However, these results did not show substantial antitumor activity. We believe that dosing and schedules may have been suboptimal due to the short half-life of endostatin and/or that bulky disease in advanced and heavily pretreated patients may not be optimally responsive to recombinant human endostatin. Alternatively, the number of endostatin receptors may be diminished in established human tumors when compared with murine tumors, and/or endostatin-induced signaling may be different in human tumors than in murine tumors resulting in different therapeutic outcomes.

The HER2 antigen encodes a receptor-like transmembrane protein with a tyrosine kinase activity (17–19) and is expressed at increased levels on several human cancers (30–40% of breast and ovarian cancers; ref. 20) and has already been successfully targeted in patients (Herceptin; humanized anti-HER2 IgG1; trastuzumab). In the present study, to increase the efficacy of endostatin, we constructed an anti-HER2 IgG3-C^H3-endostatin fusion protein to assemble entire extracellular domain.

Materials and Methods

Cell Lines and Animals
CT26, CT26-HER2 (21), human embryonic kidney 293, and transfected Sp2/0 cells were cultured in Iscove’s modified Dulbecco’s medium (Cellgro, Mediatech, Inc., Herndon, VA) with 5% calf serum (Life Technologies-Invitrogen Corp., Carlsbad, CA). Female BALB/c mice (4–6 weeks) and severe combined immunodeficient (SCID) mice (4–6 weeks) were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were done in compliance with the NIH Guides for the Care and Use of Laboratory Animals and were approved by the University of Miami Institutional Animal Care and Use Committee.

Construction, Expression, and Characterization of αHER2-Endo Fusion Protein

To obtain active endostatin, a mouse endostatin expression vector (pFLAG-CMV-1-endostatin) was cotransfected with pcDNA3.1 (Clontech, Palo Alto, CA) into human embryonic kidney 293 cells, and G418 (0.6 μg/mL)-resistant cells were selected as described previously (22). Secreted endostatin was harvested from serum-free conditioned medium and purified in a heparin-Sepharose CL-6B column.

Experimental murine endostatin gene originated from pcDNA-CMV-1-endostatin by PCR using primers 5'-CCCCCTCAGGATATCATACTCATCGAGCTTCCAGCC and 5'-CCCCGAATTCTGAACCCTTTGGAAGAAGTAGTCATGAAGCC (22). PCR products were subcloned into pGEM-T Easy Vector (Promega, Madison, WI). The subcloned endostatin gene was ligated in frame to the carboxyl end of the heavy chain constant domain (C_\(\text{H}_3\)) of human IgG3 in the vector pAT135 as described previously (23) and the endostatin heavy chain constant region was then joined to an anti-HER2 variable region of a recombinant humanized monoclonal antibody 4D5-8 (HER2, trastuzumab; Genentech) in the expression vector (psV2-his) containing His\(_\text{D}\) gene for eukaryotic selection (24, 25).

The αHER2-Endo fusion protein construct was stably transfected into Sp2/0 myeloma cells stably expressing the anti-HER2 \(\kappa\) light chain to assemble entire αHER2-Endo fusion proteins as described previously (26). The αHER2-Endo fusion proteins were biosynthetically labeled with \(^{[35]S}\)methionine (Amersham Biosciences, Piscataway, NJ) and analyzed by SDS-PAGE (26). The endostatin fusion protein was purified from culture supernatants using protein A immobilized on Sepharose 4B fast flow (Sigma, St. Louis, MO).

To confirm that the endostatin moiety was present in the αHER2-Endo protein by Western blotting, rabbit anti-endostatin (BodyTech, Kangwon-Do, Korea) was used as the primary antibody, and mouse anti-rabbit IgG conjugated with horseradish peroxidase (Sigma) was used as the secondary antibody. Goat anti-human IgG conjugated with horseradish peroxidase was used to detect human antibody.

Chorioallantoic Membrane Assay
The ability of αHER2-Endo to block VEGF/bFGF–induced angiogenesis was tested by chorioallantoic membrane assay, which employed Leghorn chicken embryos (Charles River SPAFAS, Wilmington, MA) at 12 to 14 days of embryonic development (12, 27). Vitrogen gel pellets (Collagen Biomaterials, Palo Alto, CA) were supplemented...
with (a) vehicle (0.1% DMSO) in PBS alone (negative control), (b) VEGF/bFGF (100 ng and 50 ng/pellet, respectively; positive control), or (c) VEGF/bFGF and either αHER2-Endo, anti-HER2 IgG3, or endostatin at various concentrations (0.5, 1, 5, and 10 μg/pellet) as described (28). Polymerized mesh was placed onto the outer region of the chorioallantoic membrane of the embryo and incubated for 24 hours. To visualize vessels, FITC-dextran (400 μL; 100 μg/mL; Sigma) was injected in the chick embryo bloodstream. Fluorescence intensity was analyzed with a computer-assisted image program (NIH Image 1.59). We calculated the two-tailed Student’s t test for the overlapping concentrations tested for all proteins.

**Pharmacokinetic and Biodistribution of αHER2-Endo**

Anti-HER2 IgG3 (100 μg), αHER2-Endo (100 μg), anti-dansyl IgG3 (100 μg), and endostatin (100 μg) were iodinated with 0.5 mCi 125I (Amersham Biosciences) by the chloramine T method, which preferentially labels tyrosine groups (29). BALB/c mice (4–6 weeks) were injected s.c. with either 1 × 10^6 CT26-HER2 or CT26 cells or left un.injected. Groups of three mice with either CT26-HER2 or CT26 tumors or no tumor were injected i.v. with either 30 μCi [125I]αHER2-Endo, 32 μCi [125I]anti-HER2 IgG3, 24 μCi [125I]endostatin, or 32 μCi [125I]anti-dansyl IgG3. Blood samples were serially obtained at various intervals ranging from 15 minutes to 96 hours from the retro-orbital plexus of mice injected with either αHER2-Endo, anti-HER2 IgG3, or anti-dansyl IgG3. Mice injected with endostatin alone were bled within 15 seconds to 60 minutes after the i.v. injection.

To monitor stability of the endostatin fusion protein, the iodinated fusion proteins in serum were resolved through SDS-PAGE and compared by molecular size with the iodinated antibody controls. The trichloroacetic acid–precipitable radioactivity in each blood sample was measured in a gamma counter. The pharmacokinetic variables were calculated by fitting plasma radioactivity data to a biexponential model with derivative free nonlinear regression analysis (PARBMDP, Biomedical Computer P Series Program developed at University of California at Los Angeles Health Sciences Computing Facilities). The data were weighed using weight = 1 / (concentration)^2, where concentration was expressed as 1 / counts per minute (cpm) per microliter (μL) or percentage of injected dose (%ID) per milliliter. The pharmacokinetic variables, such as plasma clearance, initial plasma volume, systemic volume of distribution, steady-state area under the plasma concentration curve (AUC₀→∞), and mean residence time, were calculated from the slopes and intercept of the biexponential equation as described previously (30).

Following the pharmacokinetic experiments, mice were ex-sanguinated by perfusion with 20 mL PBS for measurement of the tissue distribution of 125I-labeled antibody-endostatin fusion protein. The heart, lung, liver, spleen, kidney, muscle, and tumor were removed, weighed, and gamma counted and the %ID/g of tissue was calculated. Specific tumor targeting is expressed as the radiolocalization index (the %ID/g in tumor divided by the %ID/g in blood). To determine the distribution and localization of the 125I-labeled proteins in mice simultaneously implanted with CT26 and CT26-HER2 tumors on opposite flanks, groups of three mice were injected i.v. with either 5 μCi [125I]αHER2-Endo fusion protein or 5 μCi [125I]anti-HER2 IgG3. The animals were sacrificed at different times (6, 24, and 96 hours) after injection of labeled protein, and organs (e.g., lung, liver, kidney, spleen, muscle, CT26 tumor, CT26-HER2 tumor, blood, and urine) were isolated after perfusion of the mouse with PBS, weighed, and counted in a gamma scintillation counter. The %ID/g for each organ was determined as above.

**In vivo Antitumor Effects**

Murine colon adenocarcinoma CT26 cells were transduced with the gene for HER2 antigen as described previously (21). The CT26-HER2 cells that were used in these studies proliferate at the same rate in vitro as parental CT26 cells (data not shown). The in vivo antitumor efficacy of αHER2-Endo was examined using the CT26 or CT26-HER2 cell lines implanted in the syngeneic BALB/c mice. To determine targeting and efficacy of αHER2-Endo, BALB/c mice (8 per group, 4–6 weeks) were injected s.c. with 1 × 10^6 CT26-HER2 cells in the right flank and/or control CT26 cells in the left flank. On day 7, mice (8 per group) were injected i.v. with the αHER2-Endo fusion proteins (42 μg/injection, 2 × 10^{-10} mol, equimolar to 8 μg endostatin), anti-HER2 IgG3 alone (34 μg/injection, 2 × 10^{-10} mol), endostatin alone (8 μg/injection, 4 × 10^{-10} mol), or the combination of anti-HER2 IgG3 (34 μg) and endostatin (8 μg) or PBS as a control. All mice received seven injections delivered at 2-day intervals. Tumor size was measured with calipers and growth rates were recorded and calculated using the following equation: tumor volume \((mm^3) = 4 / 3 \times 3.14 \times [(long \ axis + short \ axis) / 4]^3\).

The human breast cancer SK-BR-3 xenograft model in SCID mice was also used to evaluate antitumor activity of αHER2-Endo fusion protein. SK-BR-3 cells (1 × 10^6 per mouse) were implanted s.c. in the flank of SCID mice. On day 15, mice (8 per group) were injected i.v. with the αHER2-Endo fusion proteins (42 μg), anti-HER2 IgG3 (34 μg), the combination of anti-HER2 IgG3 (34 μg) and endostatin (8 μg), or endostatin (8 μg). This treatment was repeated every other day for 11 doses. Tumor growth was analyzed as described above.

**Immunohistochemistry and Image Analysis of Blood Vessel Formation**

Mice were killed at the end of the experiments and frozen tumors were stored at −80°C until further use. For conventional immunohistochemistry, 5 μm tissue sections were cut using a cryostat (Shandon, Pittsburgh, PA) and placed on positively charged slides (Fisher Scientific, Pittsburgh, PA; ref. 31). To analyze the microvessel formation in tumors, sections were stained with a rat anti-mouse platelet-endothelial cell adhesion molecule-1 (CD31) monoclonal antibody (PharMingen, San Diego, CA) and subsequently with the avidin-biotin complex method (Vector Laboratories, Burlingame, CA). HER2 expression on tumors has been examined by staining tumor sections
with anti-HER2 IgG3. All sections were counterstained with hematoxylin (Sigma). Positively stained vascular endothelial cells (brown) were visualized and imaged using a digital camera attached to a Zeiss microscope (Carl Zeiss, Thornwood, NY).

For confocal microscopic analysis, 30 µm cryosections were cut and stained with a rat anti-mouse CD31 monoclonal antibody (31–33). Blood vessels were visualized with anti-rat IgG-Alexa 594 (Molecular Probes, Eugene, OR). Fluorescent blood vessels were then viewed via LSM5 confocal microscope (Carl Zeiss), and 14 to 21 digital images were obtained per section. These digital images have been composed as one image per each section to measure blood vessel density, and blood vessel area (pixel²) was then computed from the composite images and averaged to measure blood vessel density per tumor. Images were analyzed using NIH ImageJ version 1.31 software by color to form a binary image of the tumor blood vessels.

Statistical Analysis
Antiangiogenic activity, pharmacokinetics, biodistribution, and tumor growth are presented as the mean ± SE. Statistical analysis was done using ANOVA and Student’s t test. Differences were considered statistically significant at P < 0.05.

Results
Production and Antiangiogenic Activity of αHER2-Endo
The αHER2-Endo fusion protein construct was stably transfected into Sp2/0 myeloma cells and the secreted [35S]methionine-labeled protein has a molecular weight of ~220 kDa under nonreducing conditions (data not shown), the size expected for a complete antibody (170 kDa) with two molecules of endostatin (25 kDa each) attached (Fig. 1A). Following reduction, heavy and light chains of the expected molecular weight were observed (data not shown). We confirmed the presence of endostatin in the fusion protein by Western blotting with a rabbit anti-endostatin antiserum and goat anti-human IgG (Fig. 1B). αHER2-Endo was identified at the molecular weight of 220 kDa by both anti-human IgG and anti-endostatin antibody. Following reduction, the heavy chain band from αHER2-Endo migrated at the expected size of 85 kDa (data not shown). Integrity and Figure 1. Characterization of αHER2-Endo. A, schematic diagram of αHER2 IgG3 and αHER2-Endo. B, Western blot analysis of αHER2-Endo. The purified αHER2-Endo, anti-dansyl IgG3, and endostatin were resolved under nonreducing conditions and transferred onto a nylon membrane. To detect the endostatin moiety, rabbit anti-endostatin was used as a primary antibody, and mouse anti-rabbit IgG conjugated with horseradish peroxidase was used as a secondary antibody. Goat anti-human IgG conjugated with horseradish peroxidase was used to detect human antibody. C, inhibition of the angiogenic response induced by VEGF/bFGF. Purified αHER2-Endo preparation 1 (●) and preparation 2 (▲) were added to an aliquot of Vitrogen supplemented with a combination of VEGF and bFGF, and the mixture was placed on a nylon mesh. Impregnated mesh were then placed on the chick embryo and incubated as described in Materials and Methods. New vessel growth was visualized with FITC-dextran and measured by fluorescent intensity. Anti-HER2 IgG3 (▲) and endostatin (△) are included for comparison. Positive control group (■) contains VEGF/bFGF alone, and negative control group (□) contains only vehicle. Points, mean (n = 5); bars, SE.
purity of αHER2-Endo protein purified using protein A affinity columns was tested under nonreducing conditions by SDS-PAGE (data not shown). αHER2-Endo was identified as a single band at the molecular weight of 220 kDa (data not shown).

The ability of endostatin to block VEGF/bFGF–induced angiogenesis in vitro was tested using the chorioallantoic membrane assay. Pellets containing Vitrogen and VEGF/bFGF (100 ng and 50 ng/pellet, respectively) and either anti-HER2 IgG3 (0.5, 1, 5, or 10 μg/pellet: 2.95, 5.9, 29.5, or 59 pmol/pellet), αHER2-Endo (0.5, 1, 5, or 10 μg/pellet: 2.25, 4.5, 22.5, or 45 pmol/pellet), or endostatin (0.5, 1, 5, or 10 μg/pellet: 20, 40, 200, or 400 pmol/pellet) were measured for invasion by newly formed capillaries (Fig. 1C). Two independent preparations of anti-HER2 antibody-endostatin fusion protein were able to suppress the angiogenic response mediated by VEGF/bFGF in a dose-dependent manner with a specific activity similar to that seen with endostatin (P = 0.566 and 0.516; Fig. 1C) and showed significantly better antiangiogenic activity than anti-HER2 IgG3 (P = 0.034 and 0.050; Fig. 1C). Therefore, genetically engineered αHER2-Endo is able to inhibit the angiogenic response mediated by VEGF/bFGF, similar to native endostatin.

Serum Clearance and Stability of αHER2-Endo

To characterize the pharmacokinetics of αHER2-Endo, mice with or without implanted tumors (CT26 or CT26-HER2) were injected i.v. with [125I]αHER2-Endo, anti-HER2 IgG3, endostatin, or a control anti-dansyl IgG3 and clearance of injected radiolabeled proteins was measured. Representative results from mice implanted with CT26-HER2 tumors are shown graphically in Fig. 2, whereas normal mice were used as a control and the pharmacokinetic data for all mice are summarized in Table 1. [125I]Endostatin was rapidly removed from the plasma compartment in mice with or without tumors (T1/2 elimination, 0.6–3.8 hours), whereas the clearance rate of [125I]αHER2-Endo (T1/2 40.2–44.0 hours) was similar to or slightly increased to that of [125I]anti-HER2 IgG3 (T1/2 39.9–63.0 hours) and anti-dansyl IgG3 (T1/2 43.7–46.5 hours; Fig. 2A; Table 1). Therefore, endostatin fused with antibody has a serum half-life of at least 10-fold greater than endostatin alone.

Although the AUC of αHER2-Endo (218% ID h/mL) was reduced ~6-fold compared with anti-HER2 IgG3 (1,243% ID h/mL) in mice bearing CT26-HER2 tumors (Table 1), AUC of αHER2-Endo was increased by a factor of 56 compared with endostatin (3.9% ID h/mL) as a consequence of both a longer half-life of elimination (70-fold increase: 44.0 versus 0.63 hours) and an increased mean residence time (56-fold increase: 46.7 versus 0.83 hours). Endostatin was very rapidly removed from serum within 30 minutes, principally by glomerular filtration and renal clearance, but αHER2-Endo showed much slower clearance from serum than endostatin, albeit slightly faster than clearance that seen for anti-HER2 IgG3 and anti-dansyl IgG3. The difference in AUC between αHER2-Endo and antibodies (anti-HER2 IgG3 and anti-dansyl IgG3) might be due to the endostatin domain in fusion proteins, which may cause preferential deposition in tissues expressing high levels of endostatin-binding proteins, such as the αv and α5 integrins, and/or proteoglycans containing heparan sulfate (34, 35).

To analyze the serum stability of 125I-labeled anti-HER2 IgG3, αHER2-Endo, endostatin, and anti-dansyl IgG3, Figure 2. Serum clearance and stability in mice bearing CT26-HER2 tumors. Serum clearance (A) and serum trichloroacetic acid (TCA) precipitability (B) of 125I-labeled anti-HER2-Endo (●), anti-dansyl IgG3 (○), anti-HER2 IgG3 (●), and endostatin (○) were measured. Measurements of anti-HER2 IgG3 and αHER2-Endo were made up to 96 h after i.v. injection and those of endostatin up to 60 min. Points, mean (n = 3 BALB/c mice); bars, SE. Statistical analysis of serum stability at 5 min to 1 h was done using Student’s t test (paired, two-tailed distribution). Serum samples of 125I-labeled proteins were analyzed by SDS-PAGE (C). Each iodinated initial protein was used as an initial control for its own serum samples. 125I-labeled anti-HER2 IgG3 was also used as a control.
plasma samples were trichloroacetic acid precipitated and counted (Fig. 2B). Ninety-six hours following injection, ~90% of the anti-HER2 IgG3 and αHER2-Endo in serum remained intact. For endostatin, ~90% were intact 2 minutes after injection and only 55% remained intact at 60 minutes. αHER2-Endo cleared much more slowly in the circulation than endostatin (P = 0.0014, comparing 5-minute time point with 1-hour time point).

Biodistribution and Bio localization of αHER2-Endo
To measure localization and biodistribution, we analyzed the tumor/blood ratio of injected radiolabeled protein over time. Ninety-six hours after an i.v. injection into mice bearing CT26-HER2 tumors, anti-HER2 IgG3

Thus, the endostatin moiety of αHER2-Endo is significantly more stable in the circulation than endostatin (P = 0.0014, comparing 5-minute time point with 1-hour time point).

### Table 1. Pharmacokinetic variables for $^{125}$I-labeled proteins in mice with or without tumors

<table>
<thead>
<tr>
<th>Mice</th>
<th>Variable*</th>
<th>Anti-dansyl IgG3</th>
<th>Anti-HER2 IgG3</th>
<th>αHER2-Endo</th>
<th>Endostatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>No tumor</td>
<td>$T_{1/2}$ (h): distribution</td>
<td>0.70 ± 0.44</td>
<td>0.48 ± 0.06</td>
<td>0.028 ± 0.004</td>
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<tr>
<td></td>
<td>$T_{1/2}$ (h): elimination</td>
<td>39.9 ± 18.3</td>
<td>40.2 ± 2.9</td>
<td>3.75 ± 2.18</td>
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<tr>
<td></td>
<td>AUC$_{0.06}$ (%ID h/mL)</td>
<td>658 ± 113</td>
<td>389 ± 87</td>
<td>3.3 ± 0.4</td>
<td></td>
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<tr>
<td></td>
<td>AUC$_{0.06}$ (%ID h/mL)</td>
<td>1,474 ± 593</td>
<td>667 ± 135</td>
<td>18.2 ± 8.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean residence time (h)</td>
<td>54.8 ± 24.2</td>
<td>56.5 ± 3.8</td>
<td>5.25 ± 2.75</td>
<td></td>
</tr>
<tr>
<td>CT26</td>
<td>$T_{1/2}$ (h): distribution</td>
<td>2.62 ± 1.07</td>
<td>1.61 ± 0.21</td>
<td>10.33 ± 8.47</td>
<td>0.028 ± 0.004</td>
</tr>
<tr>
<td></td>
<td>$T_{1/2}$ (h): elimination</td>
<td>43.7 ± 2.2</td>
<td>62.2 ± 3.8</td>
<td>43.3 ± 22.7</td>
<td>1.10 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>AUC$_{0.06}$ (%ID h/mL)</td>
<td>847 ± 165</td>
<td>1,157 ± 60</td>
<td>358 ± 76</td>
<td>2.8 ± 0.2</td>
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<tr>
<td></td>
<td>AUC$_{0.06}$ (%ID h/mL)</td>
<td>1,063 ± 247</td>
<td>1,717 ± 88</td>
<td>470 ± 109</td>
<td>5.4 ± 0.6</td>
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<tr>
<td></td>
<td>Mean residence time (h)</td>
<td>56.0 ± 6.2</td>
<td>85.3 ± 4.6</td>
<td>66.0 ± 16.2</td>
<td>1.44 ± 0.14</td>
</tr>
<tr>
<td>CT26-HER2/neu</td>
<td>$T_{1/2}$ (h): distribution</td>
<td>15.73 ± 8.52</td>
<td>4.93 ± 1.17</td>
<td>3.37 ± 0.95</td>
<td>0.014 ± 0.007</td>
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<td></td>
<td>$T_{1/2}$ (h): elimination</td>
<td>46.5 ± 22.2</td>
<td>63.0 ± 6.7</td>
<td>44.0 ± 4.0</td>
<td>0.63 ± 0.29</td>
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<td>AUC$_{0.06}$ (%ID h/mL)</td>
<td>1,083 ± 123</td>
<td>887 ± 24</td>
<td>185 ± 17</td>
<td>3.0 ± 0.7</td>
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<td>AUC$_{0.06}$ (%ID h/mL)</td>
<td>1,453 ± 242</td>
<td>1,243 ± 50</td>
<td>218 ± 20</td>
<td>3.9 ± 0.3</td>
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<td></td>
<td>Mean residence time (h)</td>
<td>67.7 ± 12.0</td>
<td>76.3 ± 10.1</td>
<td>46.7 ± 10.6</td>
<td>0.83 ± 0.35</td>
</tr>
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</table>

*For the pharmacokinetic variables, the superscript 1 represents the distribution phase and the superscript 2 represents the elimination phase. AUC$_{0-96}$ and AUC$_{0-1/2}$ are the first 96 hours and steady-state AUC, respectively. To calculate pharmacokinetic variables, the plasma radioactivity results were fit to a biexponential model (endostatin, anti-HER2 IgG3, and αHER2-Endo) with a derivative-free nonlinear regression analysis. Data are mean ± SE (n = 3 BALB/c mice).

Measurements of endostatin were made 1 hour after i.v. injection in the mice.

Measurements of $^{125}$I-labeled proteins in mice without tumors were made 48 hours after i.v. injection in the mice.
was found mainly in the tumor and blood (5.67% and 2.10% ID/g, respectively; data not shown). The radio-localization indices at 96 hours after injection (the %ID/g in tumor divided by the %ID/g in blood) of αHER2-Endo and anti-HER2 IgG3 were similar (Fig. 3A). αHER2-Endo showed a tumor/blood ratio of 3.76 for CT26-HER2 and 0.50 for CT26, whereas anti-HER2 IgG3 showed tumor/blood ratios of 2.83 and 0.47 for CT26-HER2 and CT26, respectively (Fig. 3A). Therefore, both anti-HER2 antibody and anti-HER2 antibody-endostatin fusion protein preferentially localized to HER2-expressing tumors.

To directly measure localization of antibody-endostatin fusion proteins to the antigenic target, mice simultaneously implanted with CT26 and CT26-HER2 tumors on opposite flanks were injected i.v. with either 125I-labeled αHER2-Endo or 125I-labeled anti-HER2 IgG3 (Fig. 3). The biodistribution and biolocalization of the labeled proteins was examined at different times (6, 24, and 96 hours) after injection of labeled proteins (Fig. 3B). αHER2-Endo and anti-HER2 IgG3 preferentially localized to CT26-HER2 tumors (Fig. 3C and D). Specific tumor radio-localization indices (the %ID/g in CT26-HER2 tumor divided by the %ID/g in CT26) of αHER2-Endo (5.34, 7.42, and 3.55 at 6, 24, and 96 hours, respectively) were consistently equal to or greater than those of anti-HER2 IgG3 (1.12, 2.12, and 2.60, respectively; Fig. 3C and D). This indicates that the relative localization of targeted antibody-endostatin fusions to tumor is largely due to binding to the HER2 target antigen, and targeting functions of the antibody are well preserved. Whether the endostatin domain also contributes to the localization into tumor we observed is not known, because endostatin did not preferentially localize to HER2-expressing tumor in our hands (Fig. 3A).

**Antitumor Activity of αHER2-Endo In Vivo**

Preliminary experiments revealed that the CT26-HER2 tumors implanted in BALB/c mice grew at the same rate as the parental CT26 tumors. HER2 expression was maintained in CT26-HER2 implanted into BALB/c mice for up to 1 month following implantation as determined by immunohistochemistry (21). In initial experiments using the CT26-HER2 model, αHER2-Endo administration inhibited tumor growth more efficiently than either anti-HER2 IgG3 or endostatin alone (data not shown). To determine whether targeting to HER2 antigen was important, mice were simultaneously implanted with CT26 and CT26-HER2 on opposite flanks. Administration of αHER2-Endo showed preferential inhibition of CT26-HER2 compared with contralaterally implanted CT26 parental tumor (Fig. 4). αHER2-Endo inhibited more effectively than anti-HER2 IgG3 antibody, endostatin, or the combination of antibody and endostatin ($P = 0.041$, 0.041, and 0.022, respectively). These results suggest that targeting to HER2 antigen may have increased efficacy of the fusion protein.

We next determined whether αHER2-Endo, endostatin, anti-HER2 IgG3 antibody, or both antibody and endostatin in combination would inhibit the growth of SK-BR-3 human breast cancer xenografts in SCID mice. SK-BR-3

![Figure 4. Antitumor activity of αHER2-Endo fusion protein. A–C, syngeneic mouse model. BALB/c mice (C; n = 8 per group) were implanted s.c. contralaterally with CT26 (A) and CT26-HER2 (B; 1 × 10^6 cells per mouse) followed on day 7 by equimolar injections every other day (arrows; seven times) of PBS (○), αHER2-Endo (●), anti-HER2 IgG3 (■), endostatin (▲), or the combination of anti-HER2 IgG3 and endostatin (▲) as indicated in Materials and Methods. D, SCID mice model bearing human breast cancer SK-BR-3 xenografts. SCID mice (n = 8 per group) were implanted s.c. with SK-BR-3 (1 × 10^6 cells per mouse). On day 15, equimolar amounts of αHER2-Endo (○), anti-HER2 IgG3 (■), endostatin (▲), and combination of anti-HER2 IgG3 and endostatin (▲) or PBS (○) were injected every other day (arrows, 10 times). Points, mean tumor measurements; bars, SE. Statistical analysis was done using ANOVA and Student’s t test (paired, two-tailed distribution).](https://mct.aacrjournals.org/content/mct/4/6/962)
was implanted on the flank of SCID mice. Repeated administration of αHER2-Endo resulted in a significantly greater reduction of tumor volume compared with either anti-HER2 antibody alone, endostatin alone, or antibody and endostatin given in combination ($P = 0.010, 0.046,$ and $0.038$, respectively; Fig. 4D).

**Blood Vessel Formation in CT26-HER2 Tumors Treated with the αHER2-Endo Fusion Protein**

Mice were simultaneously implanted with CT26 and CT26-HER2 tumors on opposite flanks and tumors allowed to grow to a diameter of 4 to 6 mm at which time the mice were i.v. treated with αHER2-Endo fusion proteins. CT26-HER2 tumors grew more slowly in mice treated with αHER2-Endo compared with control mice, and kinetics were similar to those shown in Fig. 4 (data not shown). After five treatments, tumors were removed and cryosections were immunohistochemically stained for endothelial cells with anti–CD31 antibody or anti-HER2 antigen (F and G). CT26 tumor (A, B, and F), CT26-HER2 tumor (C–E and G), no treatment (PBS; A and C), treatment with αHER2-Endo (B and D), treatment with anti-HER2 IgG3 (E). Magnification, ×400. H and I, visualization of blood vessel formation in representative CT26-HER2 tumor sections. Tumor sections were prepared from CT26-HER2 tumors without treatment (PBS; H) or CT26-HER2 tumors treated with αHER2-Endo (I). Each cryosection was stained with rat anti-mouse CD31 and anti-rat IgG-Alexa 594 (red fluorescence). Fourteen to 21 digital images (magnified ×400) were obtained per section, and the above images are composite figures. J, quantification of blood vessel area in CT26 and CT26-HER2 tumors. The composite images were analyzed using NIH ImageJ version 1.31 by color image to form a binary image to allow measurement of blood vessel density. Blood vessel area (pixel$^2$) was then computed. Columns, mean; bars, SE. Statistical comparison was done using Student’s $t$ test (paired, two-tailed distribution).

![Figure 5. Analysis of tumor vascularity. A–G, immunohistochemical staining of blood vessels in treated or untreated CT26 and CT26-HER2 tumors. Cryosections of CT26 and CT26-HER2 tumors with or without treatment were stained with anti-CD31 antibody for endothelial cells (A–E) or anti-HER2 antigen (F and G). CT26 tumor (A, B, and F), CT26-HER2 tumor (C–E and G), no treatment (PBS; A and C), treatment with αHER2-Endo (B and D), treatment with anti-HER2 IgG3 (E). Magnification, ×400. H and I, visualization of blood vessel formation in representative CT26-HER2 tumor sections. Tumor sections were prepared from CT26-HER2 tumors without treatment (PBS; H) or CT26-HER2 tumors treated with αHER2-Endo (I). Each cryosection was stained with rat anti-mouse CD31 and anti-rat IgG-Alexa 594 (red fluorescence). Fourteen to 21 digital images (magnified ×400) were obtained per section, and the above images are composite figures. J, quantification of blood vessel area in CT26 and CT26-HER2 tumors. The composite images were analyzed using NIH ImageJ version 1.31 by color image to form a binary image to allow measurement of blood vessel density. Blood vessel area (pixel$^2$) was then computed. Columns, mean; bars, SE. Statistical comparison was done using Student’s $t$ test (paired, two-tailed distribution).](mct.aacrjournals.org/article-fig5a-g)
blood vessel density was measured by determining the area that was occupied by vessels as described in Materials and Methods. CT26-HER2 tumors treated with endostatin fusion had significantly less vascular area (16% of untreated CT26-HER2; \( P = 0.028 \)) than did the untreated CT26-HER2 tumors (Fig. 5). Vessel density was also markedly reduced in the CT26-HER2 tumors treated with αHER2-Endo relative to parental CT26 or untreated CT26-HER2 tumors (Fig. 5).

Discussion

Despite promising results in murine studies involving angiogenic inhibition by endostatin, results in preliminary clinical trials in man have been disappointing. The reason for the lack of efficacy are not known but may in part relate to the short endostatin half-life and poor stability of endostatin. The short in vivo half-life and the serum instability of endostatin necessitate frequent administration and may decrease clinical efficacy. It is possible that sustained levels of endostatin will be required to effectively inhibit tumor growth. Indeed, patients with Down syndrome showed a remarkably low incidence of solid tumors. This has been attributed by some investigators to circulating endostatin levels approximately twice those seen in normals (36). This suggests that modest but sustained increases in endostatin may be capable of significantly inhibiting tumor growth. Although it may be possible to improve therapy by using continuous or higher dosage, this increases the risk of systemic side effects on normal physiologic processes involving vascular repair or vessel outgrowth, such as the angiogenic response to ischemia and wound healing (37, 38).

To produce a more effective form of trastuzumab and improve the efficacy of endostatin, we constructed an αHER2-Endo fusion protein by joining murine endostatin to the C\( _{H}3 \) terminus of the antitumor antibody, anti-HER2 IgG3. We hypothesized that several of the logistical disadvantages of the long-term treatment with high dosages of endostatin could be overcome if the half-life and stability of endostatin could be increased and if endostatin could be specifically targeted to the tumor to achieve higher local concentrations and greater specificity. In this report, we show that the serum half-life and stability of endostatin were dramatically increased by fusion to an anti-HER2 antibody. Both the antiangiogenic properties of endostatin and the tumor targeting function of the antibody were fully maintained in the fusion protein. The fusion protein selectively targets tumors that express the target antigen HER2, increasing the local delivery of endostatin and decreasing tumor growth. In addition, reduction of tumor growth by treatment with the endostatin fusion protein correlates with a reduction in tumor blood vessel density and complexity (Fig. 5).

Endostatin fused with antibody was cleared from the peripheral compartment much more slowly than free endostatin (Table 1). The antibody moiety of the endostatin fusion protein prolonged the serum half-life of the fused endostatin. A similar effect has been observed with other antibody-fusion proteins, including antibody-avidin (23, 39). The human prolactin antagonist G129R-endostatin fusion protein also had a prolonged serum half-life compared with that of G129R or endostatin and showed augmented antitumor effects on a murine mammary adenocarcinoma 4T1 in a nude mouse tumor model (40). The longer half-life and ability to target tumors of αHER2-Endo might render it effective at lower and less frequent doses.

To achieve high concentration of endostatin in circulation, transfer of a retroviral vector encoding a secretable endostatin into hematopoietic stem cells resulted high-level and long-term secretion of endostatin in hematopoietic stem cell–transplanted mice. However, no inhibition of neangiogenesis or the growth of primary or metastatic T241 fibrosarcoma was observed (41). In addition, elevated concentrations of endostatin also did not inhibit the growth of human B-lineage acute lymphocytic leukemia in several animal models (42). The hematopoietic stem cell transplants might not work in mice because of instability of endostatin in circulation. Circulating human endostatin has been isolated from patient blood with chronic renal insufficiency, but this endostatin showed no antiproliferative effect on bovine microcapillary endothelial cells or human umbilical vascular endothelial cell following bFGF stimulation (43). Circulating endostatins may lose antiangiogenic activity over time (43). In the present study, endostatin has also been shown to be unstable in serum and was rapidly eliminated (Fig. 2B and C). In contrast, local expression of endostatin using viral vectors in the tumor vicinity did inhibit the growth of tumor in several mouse models (38, 44–47). However, it is impractical to locally inject metastases especially in man. Viral vectors may cause inflammation and elicit both an innate and an adaptive immunologic response on repeated injection. It is also difficult to titrate dose reproducibly. Thus, genetic delivery of endostatin is likely to be difficult to regulate in vivo and is unlikely to result in reliable and reproducible levels in a manner suitable for clinical application (48–50). Local delivery using an antibody fusion protein as a targeting vehicle may allow increased local concentrations and more reliably target microscopic tumor deposits. Because ~90% of αHER2-Endo in serum remained intact 96 hours following injection (Fig. 2B and C), the increased stability and/or local delivery of αHER2-Endo may render it a more effective antiangiogenic agent compared with endostatin.

Both anti-HER2 antibody and αHER2-Endo preferentially localized to HER2-bearing tumors, whereas endostatin and anti-dansyl antibody failed to localize to tumor in our studies (Fig. 3A). In vivo localization of anti-HER2 antibody and αHER2-Endo to tumor is due to binding to HER2 target antigen. Consequently, treatment with αHER2-Endo preferentially inhibited growth of CT26-HER2 compared with parental CT26 tumors (Fig. 4). αHER2-Endo inhibited
more effectively than the equimolar combination of antibody and endostatin. These data suggested that combining the targeting capability of anti-HER2 antibody with the antiangiogenic activity of endostatin improved the antitumor activity of both agents more than that which could be achieved by simultaneous administration of both agents. The targeting of antiangiogenic proteins using antibody fusion proteins may be highly selective for tumors and relatively nontoxic compared with other strategies, such as the attachment of radioligands (e.g., Zevalin or Bexxar) or chemotherapeutic agents (e.g., gemtuzumab ozogamicin; Mylotarg; anti-CD33 antibody conjugated with calicheamicin; refs. 51–53). Because the overall response rates of HER2+ breast cancers to trastuzumab remain relatively low (15–34%; refs. 54–56), this approach holds promise for increasing both response rate and durability relative to trastuzumab.

Several different mechanisms may account for the antitumor activity of trastuzumab. These include the down-regulation of HER2 in some tumors, resulting in the subsequent inhibition of its downstream phosphatidylinositol 3-kinase-Akt signaling pathway (57, 58) and the induction of G1 arrest and the cyclin-dependent kinase inhibitor p27 (59). Trastuzumab may also indirectly affect angiogenesis. VEGF expression in some primary HER2+ tumors may be decreased by trastuzumab (60–63). More recently, investigators have shown 4- to 5-fold induction of the antiangiogenic factor thrombospondin-1 in trastuzumab-treated patients (63). This suggests that further augmentation of antiangiogenic properties might further enhance trastuzumab efficacy.

Trastuzumab may also inhibit growth by inducing antibody-dependent cell-mediated cytotoxicity and/or complement-dependent cytotoxicity (64, 65). In our hands, αHER2-Endo and anti-HER2 IgG3 showed similar ability to mediate antibody-dependent cell-mediated cytotoxicity (data not shown).

αHER2-Endo also inhibited the growth of the human breast cancer SK-BR-3 xenografts in SCID mice and reduced tumor growth more than either anti-HER2 antibody alone, endostatin alone, or antibody and endostatin in combination (Fig. 4D). This inhibition seems to involve both anti-HER2 antibody and endostatin activities, because anti-HER2 IgG3 alone also had significant antitumor activity in this model. Antitumor activity of trastuzumab is largely restricted to tumors with high levels of HER2 overexpression and/or HER2 gene amplification, and the human breast tumor SK-BR-3 cell line expresses high levels of HER2 (~43 HER2 gene copies per cell; ref. 66). Unlike SK-BR-3, CT26-HER2 tumors do not require HER2 signaling for proliferation (58–61) and anti-HER2 IgG3 showed minimal activity. Therefore, the enhanced antitumor activity of αHER2-Endo in CT26-HER2 tumors is likely due to the antiangiogenic activity of the endostatin domain. In in vivo targeting experiments (Fig. 3A), αHER2-Endo localized slightly better to the CT26-HER2 than anti-HER2 IgG3. Although the difference in targeting of αHER2-Endo and anti-HER2 IgG3 on CT26-HER2 tumors was not statistically significant (P = 0.406) and the ratio of tumor/blood may not be constant over time, the possibility that the slightly better targeting of αHER2-Endo might enhance the antitumor activity of the fusion protein cannot be excluded. Although selective targeting of endostatin to tumor has been reported (67), that did not seem to be the case for CT26.

Our studies suggest that specific targeting of the antiangiogenic agent and the prolongation of endostatin half-life and stability result in targeted suppression of angiogenesis within the tumor bed. Linking endostatin to an antibody may therefore provide a unique opportunity to enhance the spectrum of trastuzumab activity and a more effective means of delivering endostatin. Application of the strategy to women with breast cancer will require careful evaluation of antibody fusion protein antigenicity and may potentially benefit from use of a human endostatin fusion domain. Thus, αHER2-Endo should be humanized to reduce the possible antigenicity of the murine endostatin domain. Targeting antiangiogenic proteins using antibody is a potentially versatile approach that could be applied to other tumor and antibody targets (such as epidermal growth factor receptor or prostate-specific membrane antigen) as well. Other antiangiogenic domains could also potentially be used (angiostatin, tumstatin, etc.). The potential use of αHER2-Endo in combination with other therapies, such as chemotherapy and/or other antiangiogenic approaches, in the setting of both minimal residual and/or metastatic disease may also further improve results and should be further investigated.

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References


Article on antibody-endostatin fusion protein

In the article on antibody-endostatin fusion protein in the May 2005 issue (1), the last name of author Dr. Hovav Nechushtan was spelled incorrectly.

Reference
Enhanced inhibition of murine tumor and human breast tumor xenografts using targeted delivery of an antibody-endostatin fusion protein

Hyun-Mi Cho, Joseph D. Rosenblatt, Young-Sook Kang, et al.

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