Linking Antibody Fc Domain to Endostatin Significantly Improves Endostatin Half-life and Efficacy

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Abstract

Purpose: The half-life of the antiangiogenic molecule endostatin that has been used in clinical trial is short (~2 h). In addition, ~ 50% of the clinical grade endostatin molecules lack four amino acids at their NH2 termini. Lack of these amino acids gives rise to a molecule that is devoid of zinc, resulting in no antitumor activity. Our goal was to develop a new version of endostatin that does not show such deficiency.

Experimental Design: A recombinant human endostatin conjugated to the Fc domain of IgG was constructed and expressed in mammalian cell culture. The presence of Fc has been shown by previous investigators to play a major role in increasing the half-life of the molecule. Fc-endostatin was tested in tumor-bearing mice, and its half-life was compared with the clinical grade endostatin.

Results: The antitumor dose of Fc-endostatin was found to be ~100 times less than the clinical grade endostatin. The half-life of Fc-endostatin in the circulation was found to be weeks rather than hours, as observed for endostatin alone. In addition, a U-shaped curve was observed for antitumor activity of endostatin as a function of endostatin concentration delivered to the animals.

Conclusion: Fc-endostatin is a superior molecule to the original clinical endostatin. Due to its long half-life, the amount of protein required is substantially reduced compared with the clinically tested endostatin. Furthermore, in view of the U-shaped curve of efficacy observed for endostatin, we estimate that the requirement for Fc-endostatin is ~700-fold less than endostatin alone. The half-life of endostatin is similar to that of vascular endothelial growth factor—Trap and Avastin, two other antiangiogenic reagents. We conclude that a new clinical trial of endostatin, incorporating Fc, may benefit cancer patients.

Endostatin, a proteolytic fragment from collagen 18, has been shown to be a potent antiangiogenic protein (1, 2). The antitumor activity of endostatin is well-established. At the time of this writing, there are >950 publications on endostatin. The antitumor properties of endostatin have been confirmed in the vast majority of these publications using a large variety of tumor models (2). The protein does not cause toxicity in patients.

The mechanism of endostatin action is still not clear. Integrin α5β1 has been implicated by binding endostatin and inhibiting angiogenesis (3). Its generation from collagen 18 is regulated by cell pathways initiated by the master tumor suppressor p53 (4, 5). P53 up-regulates transcription of α (II) collagen prolyl-4-hydroxylase, releasing tumstatin and endostatin from collagen 4 and 18, respectively.

Clinical trials of human endostatin in phase I and II used a recombinant molecule that was expressed in yeast. This formulation of endostatin carried two major handicaps. The half-life of the protein in circulation was very short. The decay of endostatin represented a biexponential model that resulted in a half-life (t1/2) of 42.3 min and a β t1/2 of 12.9 h (6). The second problem, which has not been appreciated, is the fact that ~50% of the injected recombinant human endostatin used in the original clinical trials lacked four amino acids at the NH2 terminus of the molecule (7). Deletion of these four amino acids gave rise to a molecule which did not bind zinc and consequently showed a relatively decreased antitumor activity (8).

To overcome these two deficiencies, we have constructed a molecule of endostatin that is fused to the Fc region of an IgG molecule (9, 10). The presence of Fc increases the half-life to longer than a week, analogous to the two angiogenesis inhibitors Avastin and vascular endothelial growth factor (VEGF)-Trap. Both of these reagents contain an Fc domain of the injected recombinant human endostatin used in the original clinical trials lacked four amino acids at the NH2 terminus of the molecule (7). Deletion of these four amino acids gave rise to a molecule which did not bind zinc and consequently showed a relatively decreased antitumor activity (8).

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Another aspect of endostatin, its biphasic, U-shaped anti-tumor activity (14, 15), is also revealed for recombinant human Fc-endostatin (hFc-endostatin) here. We show here that Fc-endostatin, when its biphasic, U-shaped antitumor activity is taken into account, can achieve optimum antitumor efficacy when administered at doses that are at least 100-fold lower than endostatin lacking Fc.

We anticipate that by using Fc-endostatin, it should be possible to reach endostatin concentrations in tens of µg/ml in the circulation of patients, analogous to Avastin and VEGF-Trap (11, 16). Finally, we report the antitumor activities for two endostatin mutants. One of the mutants has alanine substitutions of two histidines responsible for its zinc binding (H1A and H3A). The second mutant lacks heparin binding due to replacement of two critical arginines by alanines (R27A and R139A). Both of these mutants decrease antitumor activity of the native endostatin. The zinc-deficient mutant is more effective than the heparin-deficient endostatin. The improved formulation of endostatin reported here may provide for more effective clinical trials.

Materials and Methods

Expression and purification. Construction, expression, and purification of hFc-endostatin and mouse Fc-endostatin (mFc-endostatin) have been described previously (9, 10). The recombinant constructs were prepared by placing the Fc regions at the NH2 terminus of endostatin. Stable cell lines of these constructs were produced in NS/0 murine myeloma cells. The proteins were expressed and secreted into the medium. Protein A was used for purification of the recombinant proteins (at least 90% purity; refs. 9, 10). We obtained ~50 mg/liter of Fc-endostatin by using fermentors of 10- to 18-liter capacity.

hFc-endostatin (H1A and H3A) is a mutant in which the histidines 1 and 3 of endostatin are substituted by alanines, resulting in a molecule that lacks zinc binding. The expression plasmid for hFc-endostatin (H1A and H3A) was constructed by using a synthetic HindIII-SexA1 oligonucleotide duplex containing the two codon substitutions to replace the corresponding restriction fragment in pdCs-Fc(D4K)-endostatin (9). The sense strand has the sequence 5’-AGCTTGCTAGCGCCAGCCAGCTCCGGTCTC-3’, and the nonsense strand has the sequence 5’-CCAGGTGGAGCACCGGCTGGAAGTCGCGTGCAGC-3’, where the H1A and H3A codons and anticodons are in bold. The resultant construct was used to transfect NS/0 murine myeloma cells. hFc-endostatin (H1A and H3A) was purified from conditioned medium of stable clones as previously described (9). Construction, expression, and purification of endostatin deficient in heparin binding have been previously described (17). We have previously reported the inhibition of endothelial cell migration in vitro and the reduction of tumor vessel density in vivo by Fc-endostatin (8, 14).

Animal studies. All animal procedures were carried out in compliance with Children’s Hospital Boston guidelines. Protocols were approved by the Institutional Animal Care and Use Committee. Male (24-27 g) immunocompetent C57Bl/6J (Jackson Laboratory) and immunocompromised severe combined immunodeficiency (SCID) mice (Massachusetts General Hospital) were used. Mice were ages 7 to 9 weeks. Mice were acclimated, caged in groups of five in a barrier care facility, and fed animal chow and water ad libitum. Animals were euthanized by CO2 inhalation.

ELISA determination of Fc-endostatin. Serum samples were obtained by retroorbital puncture with nonheparinized capillary tubes under...
anesthesia. Samples were placed at 4°C overnight, and serum was collected after centrifugation at 10,000 rpm for 10 min. Concentrations were determined by competition ELISA (Cytimmune Sciences) following the manufacturer’s protocol.

**Tumor models.** Human melanoma A2058 and ASPC-1 (a human pancreatic tumor cell line) were cultured in DMEM and RPMI 1640 with l-glutamine, respectively and supplemented with 10% FCS and antibiotics. SCID mice were shaved and the dorsal skin was cleaned with ethanol before cell injection. A suspension of $5 \times 10^6$ tumor cells in 0.1 mL of PBS was injected s.c. into the dorsa of mice at the proximal midline. Mice were weighed and tumors were monitored twice a week in 2 diameters with a digital calipers. Tumor volumes were determined using $a^2 \times b \times 0.52$ (where $a$ is the shortest and $b$ is the longest diameter). Tumors were allowed to grow to $~100 \text{ mm}^3$ and mice were randomized. Treatment was by bolus s.c. injections. Concentrations of Fc-endostatin were corrected for the Fc contribution. Consequently, all indicated concentrations refer to concentrations of endostatin in Fc-endostatin. After experiments were completed, tumors were excised and fixed in either 4% paraformaldehyde or were snap frozen. Ten mice were treated with each dose of endostatin.

Unless otherwise specified, antitumor studies were done with injection doses delivered every 6 days. The dose amounts were converted into mg/kg/d to compare with our previous published data for endostatin alone (no Fc).

**Immunohistology.** Melanomas (A2058) were removed and fixed with 4% paraformaldehyde for 2 h and then incubated in 30% sucrose in PBS overnight. Tumors were embedded in ornithine carbamyl transferase medium (Tissue-Tek). Sections were treated with protease K (20 µg/mL) for 20 min before staining. Fc-endostatin was detected by FITC-labeled polyclonal antibody against human Fc fragment (Sigma). Blood vessels were visualized with monoclonal antibody against CD-31 (BD PharMingen). The primary antibody was detected by biotin-labeled goat anti-rat antibody (Vector) followed by Alexa 594-labeled streptavidin (Molecular Probes). The sections were imaged by confocal microscopy (model DM IRE2; Leica).

**Terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling assay.** Apoptosis was examined by use of the terminal deoxynucleotidyltransferase–mediated dUTP nick end labeling (TUNEL) assay (18). To detect apoptotic cells in vivo, frozen tumor sections were treated with proteinase K (20 µg/mL) for 20 min, and the slides were stained by manufacturer’s protocol (Promega). Hoechst 33258 (Molecular Probes) was used as a counter stain. Quantitative results were expressed as the number of apoptotic cells per total cells at magnification $\times 200$.

**Statistical method.** Data are expressed as mean ± SD. Statistical significance was assessed by using the Student’s $t$ test.

**Results**

**Pharmacokinetics of Fc-endostatin versus endostatin alone is significantly different.** We used mouse endostatin, human endostatin, and their Fc counterparts to investigate their pharmacokinetics. Immunocompetent mice received a single
injections s.c. and they were bled at indicated time intervals. The concentration of endostatin in serum was detected by ELISA. The data in Fig. 1A show that in contrast to human endostatin alone (blue), which has a half-life of 1 to 2 h, the half-life of hFc-endostatin (red) is at least a week and a half and possibly longer; a phenomenon known for Fc containing proteins and antibodies (19, 20). The maximum endostatin concentration is 329 ng/mL and decreases to 187 ng/mL by day 7. Similar data were obtained for mouse endostatin and mFc-endostatin (Fig. 1B). In the case of murine endostatin, the highest endostatin concentration was 595 ng/mL, which decreased to 385 ng/mL by day 12. Mouse endostatin concentrations have been corrected for the baseline value of 20 to 60 ng/mL.

**Antitumor activity of hFc-endostatin in a melanoma cancer model requires much less protein and shows a U-shaped curve.** Human melanoma cells (A2058) were injected into SCIDmice, and the animals were treated with hFc-endostatin at doses delivered once every 6 days. We show here that smaller doses administered at longer intervals resulted in optimum antitumor activity. The tumor volumes as a function of endostatin dosage are presented in Fig. 2A. The optimum antitumor effect is observed at 0.67 mg/kg/d [100 μg of endostatin per mouse every 6 days; treated versus control (T/C) = 0.52; P = 0.0006]. In Fig. 2B, the data are presented as a histogram at the completion of the experiment (8). Anti-VEGF monoclonal Avastin and clinical grade endostatin were used for comparison in Fig. 2B. The T/C for Avastin is 0.35 at the dose of 0.67 mg/kg/d. In Fig. 2C, the same data are shown by a U-shaped curve. The antitumor efficacy drops below and above the optimum concentration; consistent with a biphasic curve of efficacy.

For comparison of endostatin levels in the circulation corresponding to different doses of recombinant hFc-endostatin, we carried out an ELISA analysis (Fig. 2D). The optimum concentration (1.192 ng/mL) is a function of endostatin concentration of the dose and the frequency.

**Treatment of mice bearing pancreatic tumor cell line ASPC-1 with hFc-endostatin yields similar results to those of melanoma-bearing mice.** For a second tumor model, we used a human pancreatic cancer cell line ASPC-1. The cells were injected into the dorsa of SCID mice. After tumors reached a mean volume of ~100 mm³, the mice were treated with hFc-endostatin (Fig. 3). The optimum antitumor activity was achieved with a dose of 0.67 mg/kg/d (100 μg of endostatin per mouse every 6 days; Fig. 3A). In Fig. 3B, the tumor sizes are plotted as a function of endostatin doses (8). The U-shaped curve for these data are presented in Fig. 3C.

We conclude that the U-shaped curve is a property of endostatin in a number of tumor models, based on our data presented here, our previous data, and those generated by another group of investigators in our laboratory (14, 15).

**Histidine and arginine mutations in hFc-endostatin affect antitumor activity of endostatin.** We previously reported that the antitumor activity of endostatin is mimicked by a 25-amino-acid peptide corresponding to the NH₂ terminus of the molecule (8). Substitution of histidines 1 and 3 by alanines in this peptide eliminated its antitumor activity. This result showed that zinc binding of endostatin is required for its activity. The zinc binding requirement has been controversial and has been reviewed by us (8). Boehm et. al. (21) reported the first evidence for the criticality of zinc binding for the antitumor activity of endostatin. For antitumor activity, the authors used an *Escherichia coli* suspension of endostatin that was poorly soluble. The data showed that zinc binding was necessary for antiangiogenic and antitumor activity but did not include comparison to a soluble form of endostatin.

In the construct reported here, we have substituted alanines for the two crucial histidine residues essential for zinc binding (H1A and H3A). The antitumor activity of this mutant in the ASPC-1 tumor model is shown in Fig. 3 (A and B). Consistent with our previous data, we conclude that zinc binding of endostatin is critical for its antitumor activity. Substitution for the two histidine residues reduces this activity from 50% to 20%. For comparison of endostatin levels in the circulation corresponding to different doses of recombinant hFc-endostatin, we carried out an ELISA analysis (Fig. 2D). The optimum concentration (1.192 ng/mL) is a function of endostatin concentration of the dose and the frequency.

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The U-shaped curve of ASPC-1 is not as symmetrical as that of melanoma (compare Figs. 2C and 3C). The antitumor activity of the mutant endostatin resembles the nonoptimum doses of the wild-type molecule. Based on these data, in addition to the zinc binding contribution to the antitumor activity, other constituents of the endostatin molecule may play a smaller role in such an activity.

The heparin binding domain of endostatin is clustered on the protein surface (which cannot be mimicked by a linear peptide). We prepared a mutant of endostatin lacking heparin binding (R27A and R139A) in the context of hFc-endostatin as described previously (17). Mutation of these two arginines eliminates the heparin binding property of endostatin (22). Antitumor activity of this mutant, using ASPC-1 tumor cells, is shown in Fig. 3A and B. We observed 38% tumor inhibition for mice treated with endostatin (deficient for heparin binding), in contrast to 50% tumor inhibition for mice treated with wild-type endostatin. Both zinc-binding and heparin-binding mutant Fc-endostatin will need to be tested in a number of tumor models to reach a more definite conclusion.

Clinical grade yeast–expressed endostatin shows a doublet on PAGE with ~50% of endostatin having reduced antitumor activity. It has been reported that the endostatin protein used in clinical trials showed a doublet on PAGE (7). Based on their animal studies, the authors concluded that deletion of four amino acids from the NH2 terminus (HSHR), which included two zinc-binding histidines in endostatin, did not affect its antitumor activity. In this study, it was found that histidine-deleted endostatin bound two atoms of zinc, whereas wild-type endostatin bound 10 atoms of zinc. However, the zinc binding data are inconsistent with the crystal structure of human endostatin and our zinc measurements of native endostatin where one atom of zinc is bound to the endostatin molecule (9). We conclude that the reported high zinc-binding endostatin was probably the result of nonspecific interactions between zinc and endostatin. This artifact occurs under certain conditions during the reconstitution of endostatin with zinc. For example, during reconstitution of zinc with endostatin, the protein should be dialyzed against a large volume of buffer containing low zinc concentration to allow a gradual addition of the metal ion to the protein.

We previously reported that endostatin antitumor activity requires zinc binding (8, 21). In Fig. 4, clinical grade endostatin and hFc-endostatin are subjected to PAGE. The faster migrating protein in endostatin (lane 2) lacks four amino acids HSHR (data not shown). Based on PAGE, we speculate that the antiangiogenic and antitumor activity of recombinant endostatin used in clinical trials may have been reduced by at least 30%. This is because ~50% of the final endostatin product lacked zinc. In contrast, hFc-endostatin seems to show a single protein band under the same conditions (lane 3).

hFc-endostatin accumulates in tumor vessels. To verify the location of exogenous hFc-endostatin, histologic sections of melanoma were incubated with FITC-labeled anti-human Fc fragment antibody (green) and anti-mouse CD31 antibody (red). The FITC-labeled antibody reacted with the histologic sections of tumors from mice treated with endostatin but not with the histologic sections of tumors treated only with PBS (Fig. 5A). We conclude that the injected hFc-endostatin is selectively localized in tumor vessels.

TUNEL-positive apoptotic cells are increased after hFc-endostatin treatment. To confirm the role of hFc-endostatin as an apoptosis inducer, we did TUNEL analysis on histologic sections of melanoma (Fig. 5B). TUNEL-positive apoptotic cells were increased in hFc-endostatin–treated sections. The mean percentage of apoptotic tumor cells was 34.7% ± 6.82% in the histologic sections of the hFc-endostatin–treated (0.67 mg/kg/d) mice but was only 8.77% ± 0.87% in PBS-treated tumor sections (Fig. 5C).

Discussion

A major problem observed with clinical grade recombinant human endostatin produced in yeast has been its short half-life. Forty-two patients with advanced neuroendocrine tumors who had failed conventional therapy were treated with daily self-administered subcutaneous injections of recombinant human endostatin (from yeast) in a phase II study at a dose of 90 mg/m2 day (~ 2.5 mg/kg/d; ref. 23). There was minimal or no toxicity, and the disease remained stable in 80% of patients. Four patients had stable disease for >3.5 years of uninterrupted therapy. However, there was no partial response to therapy (i.e., 50% tumor regression), as defined by WHO criteria.
The median steady-state trough serum level after dose escalation was 331 ng/mL. The fact that only half of the endostatin bound zinc (deletion of four amino acids at the NH$_2$ terminus) suggests that the actual median protein concentration of active endostatin was $\sim 200$ ng/mL. This estimate is based on the assumption that the ELISA detects both intact and deleted endostatin equally well. The level of endogenous endostatin was reported to be 61 ng/mL. We conclude that the measured median steady-state trough in serum was only 3.3-fold higher than the baseline value.

In contrast, when Avastin (anti-VEGF monoclonal antibody) was administered to cancer patients at a dose of 0.1 to 10 mg/kg, the maximum concentration of circulating antibody was determined to be linear with a range of 2.8 to 284 $\mu$g/mL and with a half-life of 3 weeks (11, 24). The dose of antibody administered to patients with colorectal cancer was 5 mg/kg. This translates to 140 $\mu$g/mL for maximum circulating antibody. The long half-life of Avastin is mainly due to the presence of the Fc domain. Of interest is that Lucentis, a Fab fragment derived from Avastin, has a half-life of only a few hours (13).

**Fig. 5.** Immunohistochemistry and TUNEL assay. A, immunohistochemistry results for one of the mice treated at 0.67 mg/kg/d and a control mouse. B and C, TUNEL assay (scale, 20 $\mu$m).
Although the modes of actions of endostatin and Avastin are different, and the requirements for the two drugs are not equivalent, we believe that the poor pharmacokinetics of recombinant endostatin from yeast may be a critical basis for its lack of robust response in patients. Therefore, we have formulated Fc-endostatin, in the event that this construct will increase the serum concentration of endostatin in patients by at least 100-fold compared with endostatin lacking the Fc domain. From our experimental results in tumor-bearing mice, we predict that the half-life of Fc-endostatin in humans may be ~2 to 3 weeks, in contrast to hours for endostatin lacking the Fc-fragment. In other words, Fc-endostatin would be expected to have a similar half-life in the circulation as Avastin or VEGF-Trap.

For VEGF-Trap, treatment of patients with 0.8 mg/kg of the protein resulted in a maximum concentration of ~14 μg/mL with a half-life of 25 days (16). Based on the observed linear pharmacodynamics for Avastin in patients (11), at a dose of 0.8 mg/kg of the antibody, the maximum circulating antibody is calculated to be 22 μg/mL with a half-life of ~3 weeks. We conclude that the concentration of endostatin in the serum of cancer patients may have to reach 10 to 100 μg/mL with a 2- to 3-week half-life to achieve antitumor efficacy for endostatin. hFc-endostatin is potentially capable of achieving such a level.

We also report here that the antitumor activity of endostatin is biphasic and reveals a U-shaped curve for efficacy. Other proteins that regulate angiogenesis have also been reported to show similar biphasic curves of antitumor efficacy. These include IFN-α (25), rosiglitazone (26), and thrombospondin (27). In our study of hFc-endostatin in mice, we determined that maximum antitumor activity was achieved by administration of ~0.7 mg/kg/d for the two tumor models. We compared Fc-endostatin from this study with clinical endostatin in the tumor models ASPC-1 and BxPC-3 (15). Maximum antitumor activity was achieved with Fc-endostatin at 0.67 mg/kg/d. In contrast, maximum antitumor activity for endostatin lacking the Fc-fragment was achieved at ~100 mg/kg/d for BxPC-3 and 500 mg/kg/d for ASPC-1. Thus, the optimum antitumor dose for Fc-endostatin is 150- to 700-fold lower than the optimum antitumor dose for endostatin that lacks the Fc-fusion domain. However, when the clinical grade recombinant lacking the Fc-domain (from yeast) was used in tumor-bearing animals, the authors reported 90% inhibition of tumor growth. We observed ~55% inhibition of tumor growth in mice given recombinant Fc-endostatin and 65% inhibition of tumor growth in mice treated with Avastin.

In conclusion, the new data presented here: (a) provide a molecular explanation for why the limited clinical trials of endostatin did not achieve the full potential expected for this broad-spectrum, endogenous antiangiogenic protein (2); (b) show that the antitumor potency of human endostatin can be increased at least by 100 fold by fusing an Fc domain to the NH₂ terminus, thus increasing the half-life of the protein, stabilizing the terminal histidines, and conserving the zinc affinity of endostatin; (c) reveal a biphasic U-shaped curve of antitumor activity for Fc-endostatin, as we previously reported for endostatin lacking the Fc-domain; and (d) make a compelling case for initiation of clinical trials of recombinant hFc-endostatin.

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