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Inhibition of ovarian cancer by RGD-P125A-endostatin-Fc fusion proteins

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Abstract

Previous studies have shown that a single point mutation in endostatin at position 125 (P125A) can improve the biological activity of endostatin. Addition of an integrin-targeting moiety, R-G-D, resulted in better localization to tumor vasculature and improved the anti-angiogenic activity of endostatin. Since endostatin has relatively shorter serum half-life, frequent dosing was required for inhibiting tumor growth. In the present study, we have genetically fused RGD-P125A-endostatin to Fc of IgG4 isotype and evaluated its anti-angiogenic and anti-tumor effects in athymic mice. Two genetic constructs were made, RGD-P125A-endostatin-Fc (RE-Fc) and P125A-endostatin-RGD-Fc (ER-Fc). Both constructs were cloned and expressed in mammalian cells. Purified fusion proteins inhibited endothelial cell migration and proliferation better than yeast derived P125A-endostatin. Both RE-Fc and ER-Fc inhibited ovarian cancer growth and were found to be as effective as Bevacizumab treatment. Fusion protein showed marked increased half-life. Combination treatment with Bevacizumab and ER-Fc showed additive inhibition of ovarian cancer growth. These studies demonstrate that genetic fusion with human IgG4-Fc increases the half-life of P125A-endostatin and can be used along with Bevacizumab to improve anti-angiogenic and anti-tumor activity.

Keywords

Tumor angiogenesis; mutant endostatin; Fc-fusion protein; ovarian cancer; Bevacizumab

Introduction

About 225, 000 new cases of ovarian cancer are diagnosed in the year 2008 around the world, accounting for around 3.7 % of all cancers diagnosed in women¹. Adjuvant chemotherapy with *cis*- or carboplatin and paclitaxel is commonly used to treat ovarian cancer patients leading to complete clinical remission in about 50% of the treated population. Yet 90% of suboptimally debulked patients and 70% of optimally debulked patients relapse within 18 to 24 months². Subsequent repeated treatments with chemotherapy show progressively reduced responses, finally resulting in broad resistance to chemotherapy³. Based on the clinical trial experience of the Gynecologic Oncology Group (GOG), a small fraction of recurrent ovarian cancer patients retain platinum sensitivity if

they remain free of clinical symptoms for more than six months after the initial treatment. However, the vast majority of the patients fall into the categories of resistant ovarian cancer, persistent disease or refractory to treatment⁴. Thus there is a need for second line chemotherapy for these patients. Currently, recurrent ovarian cancer is treated with drugs such as Topotecan, liposomal doxorubicin (Doxil), oral etoposide, Ifosfamide and Gemcitabine. Clinical response to these drugs ranges from only 10 to 25% and the majority of ovarian cancer patients are left with no choice after initial therapy. Therefore, new therapeutic methods targeting novel pathways such as angiogenesis are warranted.

Angiogenesis is a hallmark of ovarian cancer growth and peritoneal metastasis⁵⁻⁷. In fact, anti-VEGF (Bevacizumab) antibody is clinically used to inhibit tumor angiogenesis. Recent clinical findings reveal emergence of resistance to Bevacizumab treatment which necessitates development of alternate drugs to inhibit angiogenesis^{8,9}. Nearly 30 endogenous angiogenesis inhibitors are currently being investigated at various stages of clinical development^{10,11}. Several endogenous angiogenesis inhibitors such as endostatin have been evaluated in phase I/II clinical trials, either alone or in combination with chemotherapy. Endostatin is a C-terminal fragment of the NC-1 domain of collagen XVIII¹². Endostatin inhibits angiogenesis by modulating integrin-mediated signaling ($\alpha_v\beta_3/\alpha_5\beta_1$) and interfering with canonical *wnt*-signaling pathways^{13,14}. While endostatin was very effective in inhibiting tumor growth in several preclinical models, response in clinical trials were less impressive. Some of the potential reasons for the observed discrepancy include a) bioavailability, b) shorter half-life, c) protein folding differences in transgene expression using yeast vectors and d) reduced biological activity. To mitigate some of these problems we have genetically modified endostatin to improve its biological activity¹⁵. One of the approaches to increase half-life of proteins in systemic circulation is either conjugating it to Fc fragment of IgG or genetically engineering a fusion protein with Fc^{16,17}. Towards this goal, we have prepared genetically fused mutant endostatins containing an integrin targeting sequence engineered at the amino terminus or spliced between the angiogenesis inhibitor and Fc fragment. Fusion proteins were then evaluated for anti-angiogenic and anti-tumor activities in xenotransplant model systems. Endostatin-Fc fusion proteins inhibited tumor growth as effectively as Bevacizumab and showed additive effects when both the treatment modalities were combined.

Material and methods

Cell lines and media

HEK-293 cells were grown in DMEM medium supplemented with 10% FBS. HEK-293 cells expressing fusion protein were cultured in protein expression medium (Gibco) supplemented with L-glutamine. Primary human umbilical vein endothelial cells (HUVECs), kindly provided by Dr. Vercelotti (University of Minnesota), were grown in complete endothelial growth medium (EGM; Lonza). WM35, a melanoma cell line and A2780, a human ovarian cancer cell line, were grown in RPMI 1640 medium supplemented with 10% FBS.

Genetic fusion constructs of RGD-P125A-Endo-Fc (RE-Fc) and P125A-Endo-RGD-Fc (ER-Fc)

The following primers were used to PCR amplification and cloning.

RE Up 1: 5'-AAA GAA TTC ACA CAG CCA CCG CGA C -3'

RE Down 1: 5'-GGG AGA TCT ATC TCC TCT CTT GGA GGC AG -3'

ER Up 2: 5'-GGG GAA TTC AAG AGG AGA TCA CAG CCA-3'

ER Down 2: 5'-GGG AGA TCT CTT GGA GGC AGT CAT GAA-3'

Previously described Pichia vectors, pPICZα containing P125A-endostatin cDNA insert were used as templates for PCR amplification¹⁵. PCR products were cloned into pFUSE-hIgG4-Fc2 (IL2-secretory sequence) vector (InVivogen, San Diego, CA, USA) and sequence was verified. HEK293 cells were then transfected with Lipofectamine (LTX and Plus reagent, InVitrogen) and stable transfectants were selected for Zeocin resistance. Limiting dilution plating was used to clone and screen for fusion protein secretion. Selected clones were then expanded and the fusion protein was purified from the culture supernatants by HiTrap-Protein G column (GE Healthcare) according to manufacture's suggested protocol.

Cell attachment assay

Previously published method was used for cell attachment assays¹⁵.

Endothelial cell proliferation assay

Essentially, the methods described by O'Reilly *et al.*¹² were used. DNA synthesis in control and treated cells was determined by BrdU (Roche) incorporation after 48hr incubation.

Endothelial cell migration assay

HUVEC (1×10^4 /well) were seeded into gelatin-coated, 24-well plates. After serum starvation for 16 hours in M199 medium with 0.5% FBS, pipette tip was used to create scratch wounds. Cells were first pretreated with different concentrations of fusion proteins and 15min later 10 ng/ml basic fibroblast growth factor (bFGF) was added to cells in triplicate. FGF-induced HUVEC migration was determined 16hr later and digital images were acquired by an inverted phase microscope (Zeiss).

Matrigel plug assay

Matrigel plug assay was used to determine inhibition of tumour cell-induced angiogenesis *in vivo*. A2780 cells were resuspended in matrigel and injected subcutaneously at right flanks of athymic nude mice. Mice were then treated i.p. with RE-Fc, ER-Fc, P125A-endostatin or anti-VEGF antibody (Bevacizumab) at a dose of 100 µg per mouse on day 1. Control mice were treated with an equal volume of sterile PBS. Treatment was started immediately after the matrigel implantation. At day 8, matrigel plugs was removed, snap frozen and used to prepare cryostat sections. Frozen sections (10 µm) of matrigel plugs were fixed in cold acetone, blocked and stained with anti-mouse CD31 monoclonal antibody conjugated to phycoerythrin (MEC 13.3, BD PharMingen, San Diego, CA, USA) Morphometric analysis was used to determine vessel density¹⁸.

Tumor growth inhibition

A2780 cells were harvested by trypsinization, suspended in fresh medium, and injected (2×10^6 cells/mouse) s.c. into the right flank of female, athymic nu/nu mice. After 1 week, to allow tumor establishment, mice were randomly divided into six groups and injected i.p. once a week with 0.1, 1, 10, or 30 µg/mouse of RE-Fc fusion protein. Control group of mice were treated with sterile PBS under similar conditions. Tumor growth was monitored by periodic caliper measurements. Tumor volume was calculated by the following formula: tumor volume (mm^3) = length $\times$ width² / 2. A minimum of eight mice were used in each group and the experiment was repeated at least twice. Same procedure was used to investigate the tumor growth inhibition by Bevacizumab at 1, 10, 30, 100 µg/mouse/week dosing. One week after the last treatment, blood samples were collected from mice treated with RE-Fc fusion protein and plasma samples were used in ELISA to determine residual

human endostatin fusion protein levels in the blood. For orthotopic model, 2×10^6 A2780 cells were injected i.p. and the mice were treated with fusion protein at 30 $\mu\text{g}/\text{mouse}/\text{dose}$. Inhibition of tumor growth was evaluated by differences in the wet weight of the intraperitoneal tumors at the time of sacrifice. Bevacizumab was administered at 30 μg dose following a similar schedule.

In a parallel study, effect of combination therapy was evaluated. Tumors were established by injecting A2780 cells s.c. One week later, mice were randomized into groups and injected i.p. with either Bevacizumab alone (30 $\mu\text{g}/\text{mouse}/\text{dose}$) or in combination with fusion proteins (30 $\mu\text{g}/\text{mouse}/\text{dose}$). Control group was treated with sterile PBS under similar conditions. Tumor growth was monitored weekly by caliper measurements. Ten mice were used in each group and the experiment was repeated twice.

Plasma half-life

RE-Fc fusion protein (30 $\mu\text{g}/\text{mouse}$) was injected i.p. to simulate treatment dosing. Animals were bled at 1 hour, 1, 2, 4, 8, 16, and 28 days following the fusion protein injection and the serum samples were stored at -20°C until further analysis. Human Endostatin ELISA Kit (RayBiotech, Inc) was used to determine the levels of endostatin in circulation.

Statistical methods

All quantitative data are presented as mean $\pm$ standard errors. Student's t-test (1 tail, 1 type) was used to calculate statistical significance in vessel density. P values <0.05 was considered statistically significant. Number of animals used in experimental groups was based on power analysis using a significance criterion of 0.05.

Results

Expression and purification of RE-Fc and ER-Fc fusion proteins

Human mammalian cell line HEK293 was used to express recombinant human RE-Fc and ER-Fc fusion proteins in soluble form. A schematic diagram of the fusion constructs is shown in Fig. 1A and B. Protein-G, which binds to the Fc region of IgG from a variety of mammalian species, was used to purify the fusion proteins. Both RE-Fc and ER-Fc fusion proteins showed similar elution profile from the HiTrap Protein G HP column (Fig. 1C and D). SDS-PAGE analysis of the eluted proteins showed an expected molecular weight of 46kDa. Then we investigated whether the RGD moiety is accessible in either of the genetic constructs to interact with cell surface integrins. Tumor vasculatures are known to express high levels of $\alpha_v\beta_3$ / $\alpha_v\beta_5$ and $\alpha_5\beta_1$ integrins. We have shown earlier that RGD modification can increase homing of endostatin to tumor vasculature. In the fusion proteins, RGD moiety is either located at the NH_2 -terminus (RE-Fc) or spliced between endostatin and Fc (ER-Fc). To investigate accessibility of RGD moiety to integrin-binding, human melanoma cell line WM35, expressing high levels of $\alpha_v\beta_3$ integrin but no detectable level of $\alpha_5\beta_1$ was used. WM35 cells allowed us to discriminate between the binding of endostatin to $\alpha_5\beta_1$ integrin and RGD-mediated interaction with $\alpha_v\beta_3$ and $\alpha_v\beta_5$. Representative photomicrographs of cell attachment to protein-coated wells are shown in Fig 2A. WM35 cells did not attach to P125A endostatin-coated wells. WM35 cells attached to RE-Fc or ER-Fc-coated wells. Both RE-Fc and ER-Fc coated wells showed a similar attachment profile indicating that the RGD moiety in both RE-Fc and ER-Fc constructs has equal access to integrin binding and are not structurally constrained. To prove that the WM35 cell attachment was specific and mediated through integrin binding to RGD sequence in the Fc-fusion proteins, free RGD peptide was used as a competitive inhibitor. In the presence of excess, free RGD peptide, WM35 cell attachment to fusion proteins coated wells was significantly blocked. These studies suggest

the RGD moiety in the fusion proteins is readily available to bind cell surface integrins in a specific manner.

Inhibition of endothelial cell migration

Angiogenic growth factors secreted into the tumor microenvironment can induce the endothelial cells proliferation, migration and tube formation. Inhibition of endothelial cell migration is one of the hall marks of anti-angiogenic effects. Therefore, we investigated the relative capacity of fusion proteins to inhibit endothelial cell migration in a scratch wound assay (Fig. 2B). Cultures were stimulated with 10 ng/ml bFGF to induce cell migration across the wound width. Control cultures showed complete narrowing of the wound width by 16 hours. Under similar conditions, treatment with 1.0 μ M of P125A-endostatin showed significant inhibition of endothelial cell migration. Lower concentrations (250 nM) did not inhibit cell migration. Fusion proteins were more effective when compared to P125A-endostatin in inhibiting cell migration. Even at 50 nM levels, fusion proteins prevented wound closure suggesting that the fusion protein was at least 20-fold better than the native protein to inhibit endothelial cell migration.

Inhibition of endothelial cell proliferation

To investigate whether the biologic activity of endostatin was not affected by the addition of Fc fusion, HUVEC proliferation was determined by analyzing BrdU incorporation. Data in Fig. 2C show the relative ability of RE-Fc, ER-Fc and P125A-endostatin to inhibit bFGF induced proliferation. Compared to P125A-endostatin, which showed 32% inhibition at 1 μ M (IC₅₀ about 2.0 μ M), both fusion proteins were much more effective in reducing bFGF-induced BrdU incorporation in a dose dependent manner. ER-Fc fusion protein showed an IC₅₀ value of 80 nM. RE-Fc fusion protein showed an IC₅₀ value of 60 nM. Interestingly, RE-Fc treatment not only completely inhibited bFGF-induced cell proliferation, but also inhibited the basal proliferation at higher concentration. These results suggest that fusion proteins had significantly improved antiproliferative activity against endothelial cells when compared to P125A-endostatin.

Anti-angiogenic activity of fusion proteins *in vivo*

Matrigel plug assay was used to determine the effect of RE-Fc and ER-Fc to inhibit angiogenesis *in vivo*. Matrigel plugs containing tumor cells were subcutaneously injected into female, athymic mice. Groups of mice were then systemically treated with either ER-Fc or RE-Fc. As a positive control, one group of mice was treated with humanized monoclonal antibody to VEGF, Bevacizumab. After one week, matrigel plugs were removed and analyzed for vessel density using antibody to CD-31. Representative images from the control and treated animals are shown in Fig. 3. Compared to PBS treated control group, vessel densities are markedly reduced in animals treated with Bevacizumab. Under similar conditions, both ER-Fc and RE-Fc treatment resulted in significant reduction in vessel density when compared to the control group. To further investigate the effect of fusion proteins to inhibit angiogenesis, morphometric analyses were carried out. About 20 – 30 overlapping areas from multiple sections from individual matrigel plugs were binarized and the number of vessel ends, nodes and average length per unit area were calculated using a previously published method¹⁸. Results from morphometric analysis are shown in Fig. 3G-I. RE-Fc treatment reduced the number of vessel ends by 77%, nodes by 94% and length by 91% when compared to control matrigel plugs. Bevacizumab treatment resulted in 62%, 61% and 59% reduction in vessel ends, nodes and length respectively. These data suggest that fusion proteins efficiently inhibit tumor cell-induced angiogenesis *in vivo*.

Inhibition of ovarian tumor growth by RE-Fc fusion protein and Bevacizumab

Before initiating *in vivo* efficacy studies, we determined the biological half-life of RE-Fc fusion protein. Following a single dose of RE-Fc fusion protein i.p., serum levels were monitored over a periods of time. Data in Fig. 4E show the mean serum levels of RE-Fc protein from five animals. The β -phase of clearance rate indicated that the fusion protein has a biological half-life of four days in the serum. To evaluate the antitumor activity of RE-Fc fusion protein, human ovarian cancer cell line A2780 were injected s.c. to athymic nude mice. After 1 week, different doses of RE-Fc were given i.p., once a week, for 3 weeks. Changes in the tumor volume were measured by caliper. Data in Fig. 4A show the relative growth of ovarian tumors in control and treated animals. Control group showed a progressive increase in tumor volume reaching a maximum of 50-fold during the period of observation (28 days). RE-Fc treatment inhibited tumor growth and at doses between 0.1 and 10 μ g, tumor volume increased by 25-fold from the initial size in the RE-Fc treatment group. When the RE-Fc dose was increased to 30 μ g tumor growth was further inhibited. These mice showed an 11-fold increase in tumor volume. In comparison, Bevacizumab treatment resulted in a dose-dependent inhibition of tumor growth. In this experiment, control group showed a 60-fold increase in tumor volume over a period of 28 days. Treatment with Bevacizumab at 1 μ g/dose did not show any appreciable change in tumor growth. However, at higher doses there was a progressive decrease in tumor volume (Fig. 4B). For example, treatment with 30 μ g Bevacizumab decreased the tumor growth (35-fold change in initial tumor volume) and at 100 μ g dose the treated animals showed 20-fold increase in initial tumor volume. Mean size of the tumors harvested at the end of the experiment is summarized in Fig. 4C and 4D. Mean tumor volume from the control animals at the time of sacrifice was considered as 100% to compare tumor burden in the treatment groups. RE-Fc fusion protein treated animals showed a shallow bell-shaped dose response. At 0.1 μ g there was 55% decrease in tumor volume. Up to 10 μ g dose there was no statistically significant change in tumor growth inhibition. RE-Fc treatment resulted in 77% inhibition in tumor volume at 30 μ g dose (Fig. 4C). Bevacizumab inhibited tumor growth in a dose-dependent manner and at 30 μ g dose showed a 57% decrease in tumor volume at the time of sacrifice. In order to verify that the endostatin-Fc fusion protein can be effective against peritoneal growth of ovarian cancer, A2780 cells were injected i.p. After one week, groups of five mice were treated once a week with either RE-Fc or Bevacizumab at a dose of 30 μ g/mouse/dose for three weeks. Data in Fig. 4F show the extent of cumulative tumor burden in the control and treated mice. Control group of animals showed a mean cumulative tumor burden of 9.31 gm/mouse. Treatment with RE-Fc reduced the mean tumor burden to 4.56 gm/mouse while Bevacizumab treatment showed mean tumor burden of 4.74 gm/mouse. These studies showed that endostatin-Fc fusion protein is effective in inhibiting intraperitoneal growth (orthotopic) of ovarian cancer.

Next we compared the effect of ER-Fc and RE-Fc in combination with Bevacizumab on ovarian cancer growth. In this experiment tumor bearing animals were treated with either the fusion protein alone or in combination with Bevacizumab (30 μ g/dose). Combination therapy with Bevacizumab resulted in substantial (additive) improvements in anti-tumor effect. Relative changes in tumor growth during monotherapy and combination therapy are shown in Fig. 5A. Control tumors grew 37-fold increase when compared to initial tumor volume during the observation period reaching a maximum size of 2.22 cm³ at the time of sacrifice. RE-Fc at 30 μ g dose showed reduced tumor growth (only 21-fold increase when compared to initial tumor volume). Bevacizumab treatment alone at 30 μ g resulted in a similar inhibition of tumor growth (20-fold change in tumor volume). ER-Fc treated animals showed a 30-fold change in tumor volume. Combination with Bevacizumab showed lowest tumor growth (RE-Fc + Bevacizumab 17-fold change and ER-Fc + Bevacizumab, 11-fold change in tumor volume). Summary of mean tumor volume from various treatment groups

at the time of sacrifice is shown in Fig. 5B. Combination treatment with RE-Fc and Bevacizumab inhibited tumor growth by 78% when compared to control animals and ER-Fc plus Bevacizumab showed 92% inhibition in tumor volume. Survival data is summarized in Fig. 5C. Monotherapy with either of the fusion proteins or Bevacizumab showed 20% long-term survivors. Animals treated with ER-Fc and Bevacizumab improved survival rate to 60% which coincided with reduced mean tumor burden in these animals. As a measure of gross toxicity we determined the body weight changes in the treated animals. When compared to control animals, fusion protein-treated group showed 7 % loss in body weight at the end of the experiment. However, the change in body weight was not statistically significant ($p=0.1$). Bevacizumab treatment alone resulted in 14 % loss in body weight which was statistically significant ($p=0.0006$). Combination group showed lesser body weight loss when compared to antibody treatment alone (12% decrease) which was however significant when compared to the control group. Bevacizumab treatment alone or in combination is likely to have significant body weight loss when compared to the fusion protein treatment. These studies demonstrate that P125A-endostatin-hIgG-Fc fusion proteins are effective in inhibiting ovarian cancer and that the fusion proteins along with Bevacizumab additively inhibit ovarian cancer growth.

Discussion

Angiogenesis is a critical step in tumor development and metastasis. A number of strategies to inhibit angiogenesis have been evaluated to complement existing methods of cancer treatment. One of the clinically validated methods is to neutralize VEGF with Bevacizumab, a humanized monoclonal antibody. On the other hand, a number of anti-angiogenic molecules such as endostatin have been shown to be very active in preclinical models but found to be less effective in clinical trials. Several factors such as shorter half-life, bioavailability and potency could have contributed to the less than impressive effect of endostatin in clinical trials. To overcome some of these limitations, we genetically engineered a mutant endostatin (P125A-endostatin) with an integrin-targeting RGD moiety to improve bioavailability and potency. These constructs have shown better biological activity *in vitro* and *in vivo*. The half-life of endostatin is about 2 hours in mice and therefore to maintain sustained levels of endostatin frequent administration or continuous infusion is necessary. This limitation can be however overcome by slow release formulations or by linking endostatin to immunoglobulin heavy chain Fc fragment to increase the biological half-life. This strategy has been successfully applied to a variety of therapeutically useful proteins, such as extracellular receptor regions (e.g. TNF receptor 2), growth factors (e.g. EPO, EGF) ¹⁷. Lee et al ¹⁹ indeed showed that endostatin half-life can be increased to more than a week by genetically fusing it to the carboxyl terminus of human Fc-heavy chain. This construct contains Fc fragment at the NH₂-terminus followed by human endostatin. In the present study, we have reserved the orientation of endostatin in the fusion proteins so that it is located at the amino terminus instead of carboxyl terminus. P125A-mutant endostatin was placed at the amino terminus followed by human Fc to mimic the heavy chain of human IgG. In addition, we incorporated the integrin targeting moiety, RGD, either at the amino terminus or at the junction between endostatin and Fc-molecule. These constructs were expressed as soluble proteins in mammalian cells (HEK 293) and evaluated their effect against ovarian cancer. Furthermore, we chose the Fc fragment from human IgG4 isotype which is least likely to elicit ADCC and therefore, may not generate immunological reaction towards endothelial cells (normal and tumor). RGD moiety in the fusion proteins was found to be accessible to cell surface integrins irrespective of its location either at the amino terminus or at the splice junction. Cell attachment assays using WM35 cells clearly demonstrated RGD-mediated binding was reversible and selective. WM35 cells do not express $\alpha_5\beta_1$ integrin and therefore, the interaction between P125A-endostatin and $\alpha_5\beta_1$ was negated in these studies.

The Fc-fusion proteins showed enhanced biologic activity compared to P125A-Endostatin. Fusion proteins effectively inhibited endothelial cell proliferation and bFGF-induced migration. Similar improvements in biological activity were also reported earlier by Lee et al.¹⁹. Increased potency of fusion proteins could be due to changes improved by protein folding and stability. P125A-endostatin was expressed in yeast and the fusion proteins were expressed in mammalian cells. It is likely that over production of a human protein in a non-mammalian host cell could constraint the availability of chaperones and disulfide isomerizes thereby limiting properly folded recombinant proteins. This notion is further validated in gene therapy methods wherein in situ expression of P125A-endostatin was found to be very effective in inhibiting tumor growth even though the serum levels of endostatin were only 28 ng/ml^{20, 21}. Endostatin expressed in yeast (*Pichia pastoris*) showed comparable efficacy when injected at a dose of 400 µg/mouse daily. Such a dosing schedule results in >40.0 µg/ml of endostatin in the serum. Further structural studies are needed to accurately identify the folding dependent differences in the biological activity of endostatin. Interestingly, the recombinant protein used in clinical trials was made in the yeast.

Earlier studies have reported that anti-tumor responses ranging from 47% to 91% by endostatin treatment over a dose range of 10 mg/kg to 100 mg/kg/day²². In the present studies we used about 466-fold lesser dose of the fusion proteins. The enhanced biological activity can be attributed to longer serum half-life of the fusion protein. The concentrations of endostatin in circulation ranged from 40 ng/ml (0.01 mg/dose) to 1500 ng/ml (30 µg/dose) 7 days after treatment with different doses of RE-Fc fusion protein. This is consistent with published data that the half-life of fusion protein is at least a week and a half and possibly longer^{19, 23, 24}. Increased half-life and integrin-targeting motif can also enhance anti-angiogenic and anti-tumor activity. Both RE-Fc and Bevacizumab treatment were effective against intraperitoneal growth of ovarian cancer as well.

Anti-angiogenic therapy can be combined with chemotherapy to enhance tumor growth inhibition^{20, 25}. In deed, anti-angiogenic therapy using Bevacizumab (Avastin) was found to be clinically beneficial only in combination with chemotherapy. Despite their initial promise in patients with a variety of cancers, resistant to Bevacizumab has been observed in patients and in preclinical models^{9, 26}. Evidently, tumors became resistant to Bevacizumab therapy by compensatory production of other angiogenic factors such as PDGF-C and chemokine, SDF-1^{27, 28}. Furthermore, the tumor microenvironment was found to be altered following anti-VEGF therapy. Preclinical models have demonstrated that CD11⁺, Gr-1⁺ macrophages and neutrophils to infiltrate into the tumors of anti-VEGF treated animals²⁹. Infiltrating cells are suggested to remodel the tumor milieu and provide alternate, VEGF-independent angiogenic stimulation³⁰. Therefore, strategies to inhibit infiltrating cells can be therapeutically useful in preventing resistance to Bevacizumab. Antibodies to CD11 for example is able to inhibit the infiltration of macrophage and prolong the effectiveness of anti-VEGF treatment³¹. In the present studies we evaluated the potential use of RE-Fc fusion protein to co-operatively inhibit tumor growth along with Bevacizumab. Our results show that 30 µg of Bevacizumab per mouse (about 1.5 mg/kg) once a week, which is 6~7 times less than dosing used in patients (10 mg/kg), significantly enhanced the fusion protein-induced inhibition of tumor growth. It is interesting to note that ER-Fc construct had better efficacy when compared to RE-Fc in the combination treatment with Bevacizumab. This difference cannot be attributed to accessibility of RGD moiety to target tumor vasculature since RGD-dependent cell attachment assays showed that both ER-Fc and RE-Fc were equally accessible to integrin. One possibility is that amino terminal processing in vivo could have removed RGD from a fraction of RE-Fc fusion protein thereby rendering them less effective in targeting to tumor vasculature. Whereas, RGD moiety is spliced between endostatin and Fc in the ER-Fc fusion protein, it could have been protected from amino terminal processing in circulation. Under this circumstance ER-Fc could home onto tumor

vasculature better when compared to RE-Fc fusion protein resulting in better synergy between the former and Bevacizumab. Amino terminus sequencing of fusion proteins in circulation will answer this question. Preliminary studies suggest that Bevacizumab treatment is likely to induce significant body weight loss when compared to endostatin-Fc fusion protein. Conclusive evidence for toxicity however should come from detailed toxicological analyses.

The mechanism underlying the enhanced activity of combining endostatin fusion proteins with Bevacizumab is not fully understood. Recent studies have shown that in addition to antiangiogenic effects, endostatin could reduce tumor cell adhesion³². Endostatin also can suppress the production of IL-1 α , which is known to upregulate cytokines and activate anti-apoptotic genes³³. Endostatin is known to inhibit basic fibroblast growth factor and ephrin-A1 expression suggesting that combination treatment would target independent signaling pathways to enhance tumor growth inhibition³⁴. Our studies demonstrate that, Fc-fusion prolongs the serum half-life of endostatin and improves anti-tumor activity. Fc-fusion protein in combination with anti-VEGF antibody showed additive inhibition of tumor growth.

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Fig. 1. Purification and characterization of recombinant fusion proteins. A and B show the construct of RE-Fc and ER-Fc, respectively. Endostatin-Fc fusion proteins were purified using a HiTrap protein G affinity column. Elution profiles of RE-Fc and ER-Fc are shown in C and D. E. Fusion proteins were analyzed by SDS- polyacrylamide gel electrophoresis (Lane 1, molecular weight markers; lane 2, RE-Fc; lane 3, ER-Fc).

Fig. 2.

A, Effect of endostatin-Fc fusion proteins on WM35 cell attachment. Control and RGD peptide pretreated WM35 cells were added into the triplicate wells coated with indicated proteins. Photographs show representative images of cells attached to wells (Inverted microscope, Olympus).

B. Endothelial cell migration assay. HUVE cells were seeded onto gelatin-coated 24-well plates, and a scratch wound was made by scraping cell monolayer as indicated. Cells treated with fusion proteins or P125A-Endo plus bFGF were incubated for 16 hours to allow migration into the wounded area. Three different areas were randomly chosen from each wounded area, and experiments were repeated at least twice. Photographs show

representative images of cell migration. In comparison with P125A-Endo, fusion proteins markedly reduced bFGF-induced endothelial cells migration. Lines indicate margins of wounds. Values represent changes in the relative area (% of initial width) of wounds.

C. Endothelial cell proliferation assay. BrdU incorporation was used to test the antiproliferative activity of purified fusion proteins. RE-Fc (●), ER-Fc (○), P125A-Endo (Δ). Each data point represents a mean of 3 independent experiments. bFGF-induced HUVE cell proliferation was considered as 100% response to calculate relative inhibition elicited by fusion proteins or P125A-Endo. BrdU incorporation in the absence of bFGF was considered as basal level of proliferation (0%). In comparison with P125A-Endo, fusion proteins markedly inhibited bFGF-induced cell proliferation.

Fig. 3.

Anti-angiogenic activity of fusion proteins. A2780 ovarian cancer cells containing matrigel plugs were s.c. implanted into athymic mice. Groups of mice were treated with either PBS or Bevacizumab or fusion proteins. Bevacizumab treated mice were used as positive control. Cryostat sections of matrigel stained with anti-mouse CD31 antibody conjugated to phycoerythrin to show blood vessels (red) and with DAPI (blue) to show nuclei are shown. Sections were analysed by fluorescence microscopy (x100 magnification). A and B, control, C, RE-Fc, D, ER-Fc, E, P125A-Endo, F, Bevacizumab. Each group includes at least five mice and vessel density and branching points were determined by morphometric analysis. G, vessel length, H, vessel ends, I, vessel nodes. Asterisks (*) denote statistically significant values.

Fig. 4.

Inhibition of ovarian tumor growth by RE-Fc fusion protein and Bevacizumab. Female, athymic nude mice transplanted with human ovarian carcinoma cell line A2780 were randomized into 5 groups and treated with different doses of RE-Fc fusion protein (A, C) or Bevacizumab (B, D). 0.1 μg (◇), 1 μg (○), 10 μg (■), 30 μg (□), 100 μg (▲). Treatment was given once a week for 3 weeks. Mice treated with sterile PBS were used as a control (●). Fold changes from initial tumor volume are shown. Initial tumor volume from each mouse at the beginning of the treatment was considered as 1 to calculate fold change in tumor volume. Data are from two independent experiments using eight animals per group. At the end of experiment, tumors were resected out and measured. Mean volume of control tumors were considered as 100% to calculate relative inhibition of tumor growth in the treated animals. Data in E, show serum half-life of RE-Fc fusion protein. Mice were injected with 30 μg of fusion protein i.p. Human endostatin level in the serum was determined by ELISA. Each data point represents mean value from five different samples. Inhibition of intraperitoneal tumor growth (orthotopic model) is shown in panel F. Asterisks indicate statistically significant values.

Fig. 5. Combination effect of fusion proteins and Bevacizumab on ovarian tumor growth (A) and survival (C). Female athymic nude mice were transplanted with A2780 cells and randomized into 6 groups and treated with 30 μ g/mouse/week of RE-Fc (Δ), ER-Fc ($\square$), Bevacizumab ($\diamond$), RE-Fc plus Bevacizumab ($\blacktriangle$), or ER-Fc plus Bevacizumab ($\blacksquare$). Mice treated with sterile PBS were used as control ($\bullet$). Combination treatment with RE-Fc or ER-Fc and Bevacizumab significantly inhibited tumor growth (fold change in tumor volume is shown in Fig. 5A). Panel B shows mean tumor volume from various treatment groups at the time of sacrifice. Panel C shows data on survival of control and treated animals. Values represent mean of two independent experiments using 10 animals per group. Asterisks indicate statistically significant values.