RESEARCH PAPER

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A fusion protein with the receptor-binding domain of vascular endothelial growth factor-A (VEGF-A) is an antagonist of angiogenesis in cancer treatment

Simultaneous blocking of VEGF receptor-1 and 2

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Vascular endothelial growth factor (VEGF) is an angiogenic factor that signals through VEGFR-1 and VEGFR-2, which are expressed preferentially in proliferating endothelial cells. Thus, simultaneous blockage of both VEGF receptors may provide a more efficient therapeutic response in cancer treatment. We created a recombinant fusion protein (RBDV-IgG1 Fc), which is composed of the receptor binding domain of human VEGF-A (residues 8-109) and the Fc region of human IgG1 immunoglobulin. The recombinant protein can bind to both mouse VEGFR-1 and VEGFR-2 to decrease VEGF-induced proliferation and tube formation of endothelial cells in vitro. In this study, the RBDV-IgG1 Fc fusion protein reduced the effects of proliferation, migration and tube formation induced by VEGF in murine endothelial cells in vitro. In vivo tumor therapy with RBDV-IgG1 Fc resulted in tumor inhibition by reducing angiogenesis. Pathological evidence also shows that RBDV-IgG1 Fc can seriously damage vessels, causing the death of tumor cells. These findings suggest that this chimeric protein has potential as an angiogenesis antagonist in tumor therapy.

Introduction

Tumor cells usually represent the main source of vascular endothelial growth factor (VEGF), although several studies have shown that tumor-associated stroma is also a site of VEGF production.[1,2] In situ hybridization studies have demonstrated that VEGF mRNA is expressed in many tumors, including lung, breast, gastrointestinal tract, renal and ovarian carcinomas.[3] The VEGF-related gene family of angiogenic and lymphangiogenic growth factors comprises five secreted glycol-proteins referred to as VEGF-A, VEGF-B, VEGF-C, VEGF-D and placenta growth factors (PlGF).[4] VEGF-A is a major angiogenic factor of the VEGF family. VEGF-B selectively binds to VEGFR1 and has a role in the regulation of extracellular matrix degradation, cell adhesion and migration.[5] Both VEGF-C and VEGF-D bind to VEGFR-2 and VEGFR-3 and regulate lymph-angiogenesis and VEGF-C may also be involved in wound healing.[6,7] In addition, alternative exon splicing of human VEGF-A gene shows that it is comprised eight exons, denoted as: VEGF-A121, VEGF-A145, VEGF-A165, VEGF-A165b, VEGF-A189 and VEGF-A206.[8] VEGFs initiate signals through two receptor tyrosine kinases, VEGFR-1 and VEGFR-2,[9] which are involved in the regulation of extracellular matrix degradation, cell adhesion and migration.[5]

In 1971, Folkman proposed that inhibition of angiogenesis was a strategy to treat cancer and VEGF is now generally considered central in the process of angiogenesis.[10] Several different strategies have been designed to target VEGF/VEGFR signal transduction, including small molecules such as TNP-470 inhibiting VEGFR signal transduction,[11] humanized anti-VEGF

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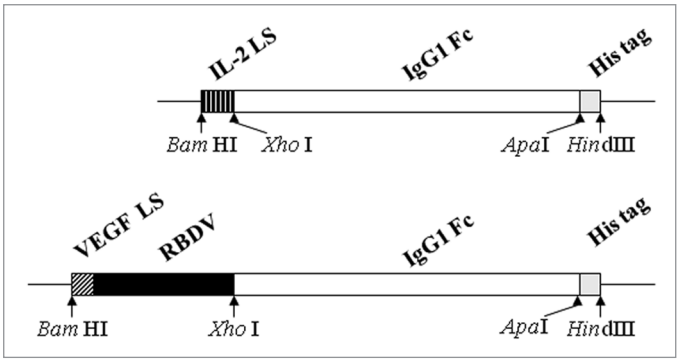

Figure 1. Scheme of the chimeric gene construction. The DNA fragment encoding the receptor binding domain of human vascular endothelial growth factor (RBDV) was fused to the 5'-end of DNA fragment which encoding the Fc portion of human IgG1. LS refers to the leader sequence; IgG1 Fc, the constant region (Hinge, CH2 and CH3 domains) of the Fc domain of human immunoglobulin G_1. As a control, DNA fragment encoding IL-2 leader sequence was fused with the Fc portion of human IgG1 and subcloned.

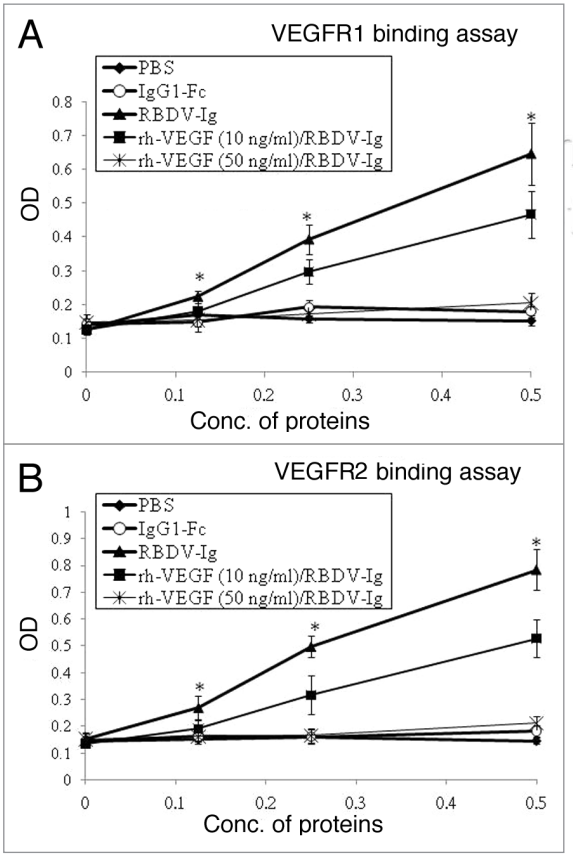

Figure 2. The binding activities of purified recombinant chimeric proteins to mouse recombinant VEGF receptor 1 and 2. A two-fold serial dilution of biotinylated RBDV-IgG1 Fc or IgG1 Fc proteins from 0.5 to 0.125 μg were incubated with (A) mouse VEGFR1 or (B) VEGFR2 competing with or without 10 or 50 ng/ml VEGF. The binding activities are represented by OD_{450} values. The results shown are mean ± SD and data were obtained from three independent experiments. *p < 0.05 (n = 6).

monoclonal antibody e.g., bevacizumab,[12] anti-VEGFR-1 antibody, anti-VEGFR-2 antibody and a VEGFR chimeric protein.[13] In addition, some peptides selected with phage display have also been used as antagonists of VEGF to its receptor.[14-16] In addition, a cyclic peptide corresponding to amino acids 79–93 of the VEGF sequence was reported to inhibit angiogenesis.[17]

Its biological properties make VEGF an important therapeutic target, and it has been shown that the anti-VEGF signal pathway can inhibit tumor growth in vivo. At the molecular level, VEGF activity is mediated by its interaction with its two receptors. Information in the literature has allowed identification of the receptor-binding domain of VEGF (RBDV), in which amino acids are lined between 8 and 109 of full VEGF-A.[18] According to this information, RBDV-IgG1 Fc chimeric proteins were constructed by fusing the RBDV with the constant region of human IgG1. In this study, we further confirmed that RBDV-IgG1 Fc could also bind to both murine VEGF receptors to reduce the angiogenestic activities of VEGF in vitro. In addition, parallel unpublished data also indicate similar binding antitumor activity towards the human receptors to block angiogenesis in vitro. In a mouse model, in vivo treatments with the RBDV-IgG1 Fc chimeric proteins significantly resulted in tumor inhibition. In addition, the pathological evidence indicated these proteins indeed targeted tumors and destroyed the vessels in B16/F10 tumors. Together these results suggest that RBDV-IgG1 Fc is a good candidate for development as an anti-angiogenestic drug for tumor suppression.

Results

Determining the binding activities of RBDV-Ig to murine VEGF-Rs. Bioinformation on the comparison of the protein sequences between human VEGF and murine VEGF led to a hypothesis that the receptor binding domain of human VEGF (RBDV) could also bind to murine VEGFR. The information showed that the protein sequence of RBDV is highly similar to 1–109 amino acids of mature murine VEGF164 (92.7% similarity).

Thus, RBDV-IgG1 Fc was biotinylated to directly determine its binding activities to mouse VEGF receptors. The results showed that RBDV-IgG1 Fc could bind to immobilized VEGFR-1 (**Fig. 2A**) and VEGFR-2 (**Fig. 2B**) in a dose dependent manner. In contrast, neither VEGFR-1 nor VEGFR-2 could interact with human IgG1 Fc. Furthermore, additions of VEGF-A could compete with RBDV-IgG1 Fc to bind to VEGFR-1 and VEGFR-2. According to the literature, MS1 cells have high expression of VEGFR-2 and SVEC4-10 cells express VEGFR-1 and VEGFR-2. Thus, RBDV-Ig Fc was also examined to determine whether it could engage with murine VEGF receptors expressed on the cell surface of these two cells. Unlike to IgG1-Fc, RBDV-IgG1 Fc could bind to both MS1 and SVEC4-10 cells (**Fig. 3**).

The suppressive potency of RBDV-IgG1 Fc in murine SVEC4-10 endothelial cells in vitro. As previously described, RBDV-IgG1 Fc can suppress VEGF-induced proliferation of human endothelial cells. Therefore, the effect of RBDV-IgG1 Fc on the proliferation of murine endothelial cells was examined. As

expected, **Figure 4A** indicates that RBDV-IgG1 Fc could inhibit proliferation of SVEC4-10 cells with or without the addition of VEGF. However, control chimeric protein IgG1 Fc had no effect on the proliferation of SVEC4-10 cells. B16/F10 cells were also tested to determine whether RBDV-IgG1 Fc affected the proliferation of tumor cells. In contrast to endothelial cells, no significant difference between the experimental treatments was identified (**Fig. 4B**). Furthermore, we examined the other effects of RBDV-IgG1 Fc in vitro and the activities of migration and tube formation affected by the chimeric proteins were allowed to represent their functions in angiogenesis. RBDV-IgG1 Fc (10 μg/ml) induced a significant inhibition of VEGF-stimulated endothelial cell migration from the upper chamber to the lower one through the membrane (**Fig. 5C**) whereas IgG1 Fc had no significant effect on the migration ability of SVEC4-10 cells (**Fig. 5B**). The numbers of migrating branches in each group were calculated and treatment with RBDV-IgG1 Fc resulted in more than an 80% decrease in cell migration (**Fig. 5D**). This result indicates that RBDV-IgG1 Fc could suppress angiogenic factor-stimulated cell locomotion in response to VEGF-attractive surroundings. Similarly, RBDV-IgG1 Fc also had a stronger inhibitory effect on VEGF-induced tube formation and capillary connection than VEGF alone or IgG1 Fc with VEGF stimulation (**Fig. 6A–C**). In the presence of RBDV-IgG1 Fc, SVEC4-10 cells could not extend their morphology to form a tube-like structure and accumulated in aggregates. The numbers of capillary network connections in each group were calculated and treatment with RBDV-IgG1 Fc resulted in more than a 60% decrease in tube formation (**Fig. 6D**). Together these results indicated RBDV-IgG1 Fc also has the ability to suppress the activity of VEGF in angiogenesis in a mouse model, similar to that in humans.

In vivo tumor suppression with RBDV-Ig Fc treatment. When the average tumor volume of B16/F10 melanoma in the mice was up to 50 mm^3, chimeric proteins were injected in situ into the tumors to determine their activities in tumor suppression. **Figure 7** shows that the mean tumor sizes in mice treated with RBDV IgG1 Fc were significantly ($p < 0.05$) smaller than those in untreated or IgG1 Fc treated mice (**Fig. 7**). Furthermore, the formation of vessels in the dorsal skin was examined after the mice were sacrificed. Many new vessels developed in the mice treated with PBS and IgG1 Fc (**Fig. 8A and B**). In contrast, treatment with RBDV-IgG1 Fc reduced the formation of new vessels in vivo (**Fig. 8C**).

The mice with B16/F10 tumors were also treated with an intravenous injection of RBDV-IgG1 Fc twice. As expected, the tumor growth was significantly suppressed from the day four after treatment with RBDV-IgG1 Fc compared with that in the untreated and IgG1 Fc treated mice (**Fig. 9**).

H&E histological staining revealed that RBDV-IgG1 Fc treatment caused damage in the tumor region and the blood vessels and tumor cells around the blood vessels were disrupted (**Fig. 10E**). No significant damage was observed after PBS or IgG1 Fc treatment (**Fig. 10A and C**). Moreover, immunochemical staining further showed that RBDV-IgG1 Fc accumulated in the tumor area, and was bound to the endothelial cells of the blood vessels and the cells around the blood vessels (**Fig. 10F**). However, IgG1 Fc administration did not cause fusion protein accumulation in the tumor area and the results were the same as with PBS treatment (**Fig. 10B and D**). These results indicate that systemic administration of RBDV-IgG1 Fc can result in preferential targeting to tumor mass and active chimeric proteins disrupt the endothelial cells and tumor cells around vessels. The numbers of vessels in the tumors with different treatments were calculated respectively and the results accord with the previous finding that the administration of RBDV-IgG1 Fc could decrease the numbers of vessels in tumor.

Discussion

In cancer therapy, inhibition of the VEGF/VEGF receptor signal pathway has been shown to suppress angiogenesis in many models, including genetic models of cancer, which has led to clinical development of VEGF inhibitors. Consequently, a novel chimeric protein was created by fusing the receptor binding domain of human VEGFA165 to the Fc portion of human IgG1 as a fusion protein, which is designed as an antagonist that blocks the physiological interaction between VEGF ligands and its receptors, VEGFR-1 and VEGFR-2. In parallel unpublished data, it has been shown that RBDV-IgG1 Fc could bind to human VEGFRs, VEGFR-1 and VEGFR-2 and inhibit angiogenesis through VEGF and interaction with its receptors in HUVE cells in vitro.

To examine the tumor inhibition ability of RBDV-IgG1 Fc in vivo by antagonizing angiogenesis in a mouse model, this chimeric protein was investigated to determine whether it could "cross-react" on mouse VEGFR. Since the sequence alignments of RBDV from humans and mice share a 92.7% similarity, it is possible that RBDV-IgG1 Fc can work in a mouse model. As expected, **Figure 2** shows that RBDV-IgG1 Fc fusion proteins can bind to immobilized mouse VEGF receptors VEGFR-1 and VEGFR-2. In addition, MS1 cells have high expression of VEGFR-2 and SVEC cells also express VEGFR-1 and VEGFR-2.[19,20] Both cells were significantly probed with RBDV-IgG1 Fc (**Fig. 3**). We therefore demonstrated that this receptor binding domain containing a fusion protein could also bind to mouse cells which express VEGF receptors on the cell surface.

Further, we needed to confirm whether RBDV-IgG1 Fc could not only abolish the mitogenic activity of VEGF but also inhibit signal transduction cascades in mouse endothelial cells. Clearly, we discovered that RBDV-IgG1 Fc consistently and significantly inhibited the proliferation of SVEC4-10 mouse endothelial cells with and without the addition of VEGF (**Fig. 4A**). The heparin binding region (amino acids 110–165) of VEGF has been strongly identified and heparin, through simultaneous binding to VEGF and its receptors, increases in signal amplitude and duration.[17] Most importantly, the loss of the heparin-binding domain, the amino acids 111–165, results in a reduction in the mitogenic activity of VEGF.[21] Also, RBDV-Ig Fc's mitogenetic activity conformed to these previous experiments. Without mitogenetic activity, however, the chimeric protein still had receptor binding activity and logically, it could be proposed that the mechanism of endothelial cell growth inhibition mediated by the chimeric proteins may be due to the direct blocking of VEGF/VEGFR

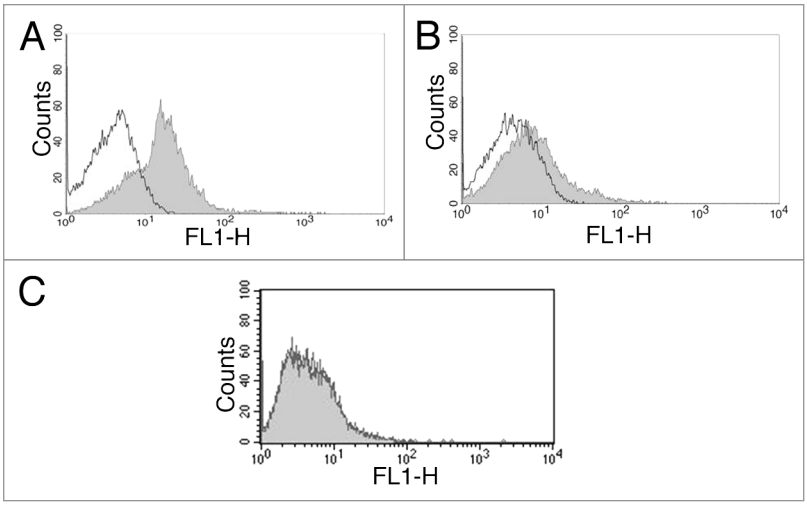

Figure 3. The binding activities of purified recombinant chimeric proteins to mouse endothelial cells. (A) MS1, (B) SVEC4-10 (C) Balb/3T3 cells were respectively stained with RBDV-IgG1 Fc (filled curve) or IgG Fc (bold line) chimeric proteins, followed by FITC-conjugated goat anti-human IgG antibody. Immunofluorescence of the stained cells was measued by flow cytometer.

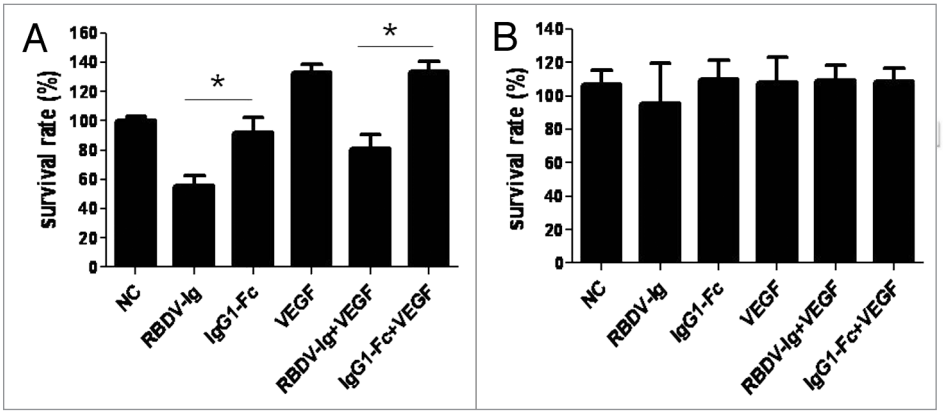

Figure 4. The effects of RBDV-IgG1 Fc on cell proliferation. (A) SVEC4-10 cells and (B) B16/F10 cells were incubated with chimeric proteins and the proliferation profile was determined. NC: treatment with PBS as a control (100%). Cells were treated with 100 μl 5 μg/ml of RBDV-Ig (lane 2) or IgG1-Fc (lane 3) without or with VEGF (lanes 5 and 6). Lane 4 cells were treated with VEGF without chimeric protein treatment. The data shown here are the mean ± SD of proliferation inhibition percentages obtained from three independent experiments (n = 9). *p < 0.05.

interaction, resulting in inhibition of VEGF-induced VEGFR activation. There are many studies indicating that VEGFR-2 is the major mediator of the mitogenesis, survival and permeability enhancing effects of VEGF-A in endothelial cells.[3] Therefore, this growth inhibition may be due to the blocking of VEGFR-2 signaling.

It was proposed that the disturbance of VEGFR-1 activity results in vascular malformation,[22] although the role of VEGFR-1 in angiogenesis is still debated. As expected, VEGF-driven, capillary-like tube formation and morphological changes in SVEC4-10 cells were inhibited by RBDV-Ig Fc (**Fig. 6**). Moreover, the migration ability of SVEC4-10 cells, which was induced by VEGF, was dramatically suppressed by RBDV-IgG1 Fc (**Fig. 5**). According to the above results, we confirmed that RBDV-IgG1 Fc could inhibit signal transduction through VEGF and VEGFR-1 in vitro. Therefore, RBDV-IgG1 Fc could completely abolish the process of angiogenesis, because the chimeric protein can block the signaling of both VEGFRs.

Several strategies have previously been reported to function as VEGF binding antagonists, including anti-VEGF antibody,[23,24] anti-VEGFR-2 receptor antibody,[25] RNA-based aptamers,[26] and various peptides.[14-16] RBDV-IgG1 Fc has some theoretical advantages over the above molecules because it can target both VEGF main receptors, VEGFR-1 and VEGFR-2. Furthermore, it has been reported that the Ig Fc portion can help maintain the tertiary structure.[27] Therefore, coupling of the RBDV sequence to the human IgG Fc region could sufficiently prolong the half-life of the RBDV peptide in antagonizing VEGF activity. In addition, glycosylation of the IgG1 molecule has been shown to significantly affect the resulting ability of the antibody to participate in antibody dependent cellular cytotoxicity (ADCC) and the complement system.[28] Fusion of IgG1 Fc would allow the chimeric protein the ability to directly kill the target cell by immune responses as described above.

There are several advantages for targeting VEGFR receptors on endothelial cells in cancer therapeutics. First, VEGFR-2 is expressed exclusively on proliferating endothelial cells at tumor sites;[29] therefore, antagonists against the receptor may offer high selective cytotoxicity for tumor inhibition. Moreover, tumor cells have heterogeneity for antigen expressions. In contrast, endothelial cells in tumor vessels are normal cells with a normal genetic expression. Thus they are homogeneous and a better target for "specific targeting therapy" than heterogeneous tumor cells. In some cases, VEGFR-1 is expressed by tumor cells, potentially extending the role of this receptor in cancer growth. Wu and collaborates indicated that VEGF-A autocrine growth activity is acquired by certain human breast tumor cell lines defined by expression of VEGFR-1.[30] Therefore, certain tumor cells can also be directly targeted by RBDV-IgG1 Fc.

With in situ injection of RBDV-IgG1 Fc, B16/F10 tumor growth was suppressed and tumors were significantly smaller than in the control groups treated with PBS and IgG1 Fc. However, the proliferation of B16/F10 was not inhibited by RBDV-IgG1 Fc in vitro (**Fig. 4B**). Furthermore, RBDV-IgG1 Fc could inhibit B16/F10 melanoma growth in vivo, which may be through suppression of the angiogenesis and proliferation

of endothelial cells. In agreement with our results, another study also showed that blockade of angiogenesis which was activated by VEGFR-1 and VEGFR-2 signaling was necessary to efficiently inhibit B16/F10 melanoma growth and metastasis.[31] Tumor sections were obtained and stained with H&E and tumor-associated blood vessels were disrupted (**Fig. 10E**) with RBDV-IgG1 Fc treatment. This may be due to targeting by RBDV-IgG1 Fc against the endothelial cells of tumor-associated vessels with the preferential expression of VEGFR-1 and VEGFR-2. Furthermore, damage in tumor associated vessels may result in the effortless penetration and accumulation of chimeric proteins, causing more serious hypoxia in the tumor mass (**Fig. 10F**). The tumor cells may die because of RBDV-IgG1 Fc-induced hypoxia (**Fig. 10E**). Moreover, a previous study showed that B16F10 cells have low surface expression of VEGFR-1 and VEGFR-2. Thus, tumor cells may also be directly targeted by RBDV-IgG1 Fc. An IgG-like fusion protein was proposed to be a better choice over smaller molecule substances or fragments by providing the Fc domain which not only confers a long pharmacokinetic half-life[27] but also supports secondary immune functions, such as ADCC and complement-dependent cytotoxicity (CDC). Tumor cell killing by ADCC is triggered by the interaction between the Fc region of an antibody bound to a tumor cell and the Fcγ receptors on immune effector cells, such as neutrophils, macrophages and natural killer cells.[32,33] CDC is initiated by complement component C1q binding to the Fc region of IgG1, because the Fc regions of IgG1 are strong complement activators.[34,35] According to our results, the damage in tumor cells occurs in the cytoplasm (**Fig. 10**), and there are not a lot of immune cells in the tumor region. We propose that the Ig Fc region of chimeric protein damages tumor cells and endothelial cells by CDC.

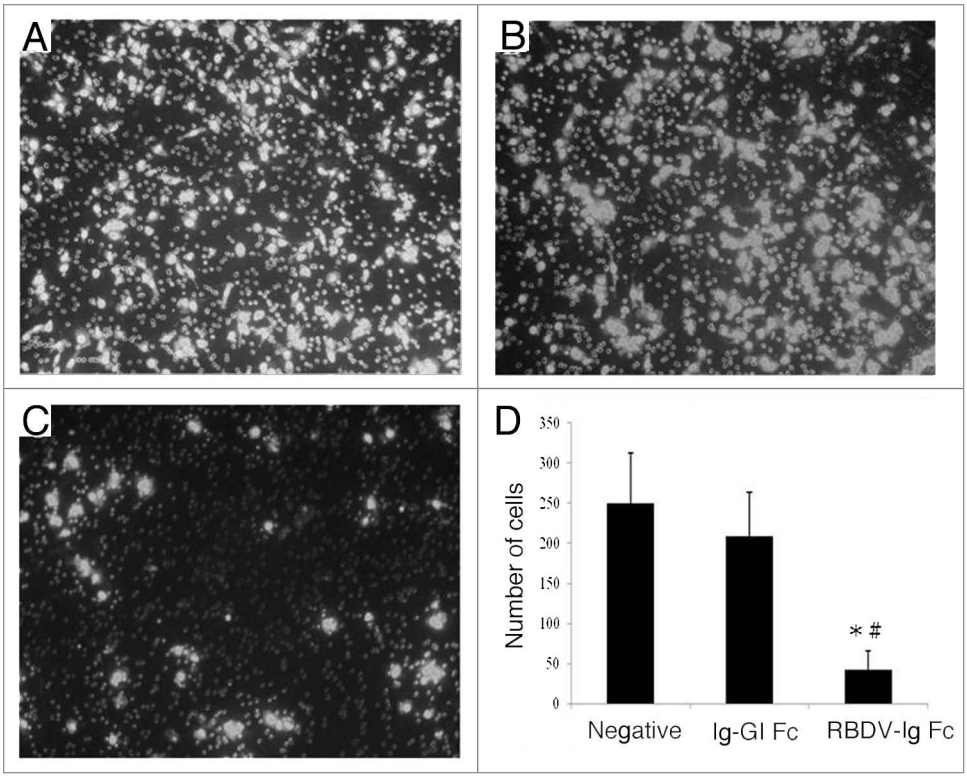

Figure 5. Effects of RBDV-IgG1 Fc on in vitro cell migration. (A) Migration abilities of SVEC4-10 cells are enhanced in the presence of VEGF (10 ng/ml) as seen under a microscope (x100 magnification). SVEC4-10 cells were also treated with (B) IgG1 Fc or (C) RBDV-IgG1 Fc to monitor their effects on the migration abilities of SVEC4-10 cells. (D) Three random fields were counted per well and the total number of cells were calculated. The data represent the mean ± SD of the number of migrating cells obtained from two independent experiments (n = 4). *$p < 0.05$ compared with the VEGF group. #$p < 0.05$ compared with the IgG1 Fc group.

In summary, an effective strategy was demonstrated in the engineering of the receptor binding domain of ligands against specific targeted cells which were fused with an appropriate Fc portion of IgG for their therapeutic capacity. Also, this chimeric protein, RBDV-IgG1 Fc, was effective in anti-angiogenesis

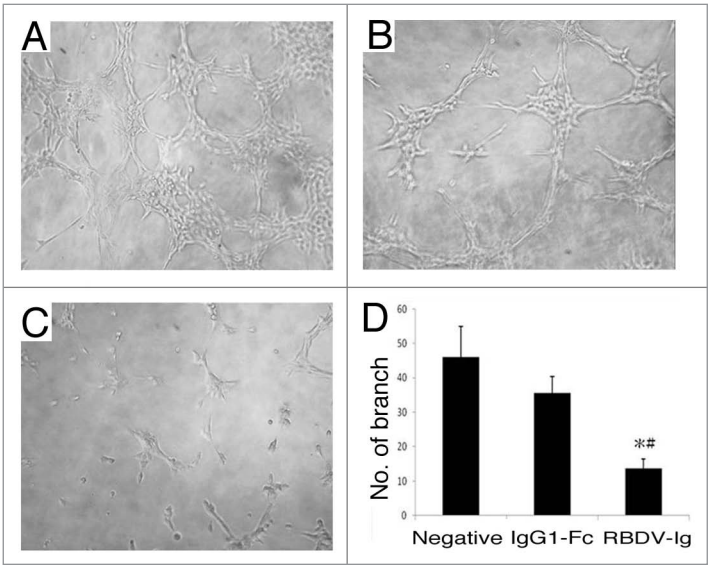

Figure 6. Effects of RBDV-IgG1 Fc on in vitro tube formation. (A) Under normal conditions, SVEC4-10 cells form a network of tubes as seen under a microscope (x100 magnification). SVEC4-10 cells were also treated with (B) IgG1 Fc or (C) RBDV-IgG1 Fc to monitor their effects on tube formation. (D) Three random fields were counted per well and the total number of branches of the tube-like structures per field were calculated. The data represent the mean ± SD of the number of branches of tube-like structures obtained from three independent experiments (n = 6). *$p < 0.05$ compared with the VEGF group. #$p < 0.05$ compared with the VEGF + IgG1 Fc group.

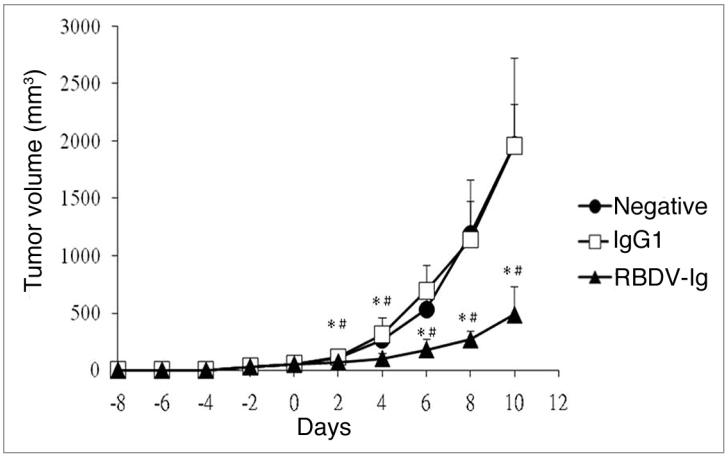

Figure 7. In vivo suppression of tumor growth with in situ RBDV-Ig Fc treatment. When the average tumor volume was up to 50 mm^3, mice were injected with 150 μg recombinant proteins in situ. Tumor volumes were measured every 2 days after injections. The data represent the mean ± SD of tumor volumes obtained from two independent experiments (n = 6). For the RBDV-Ig group, $p < 0.05$ compared with the negative group, $^{\#}p < 0.05$ compared with the IgG1 Fc group.

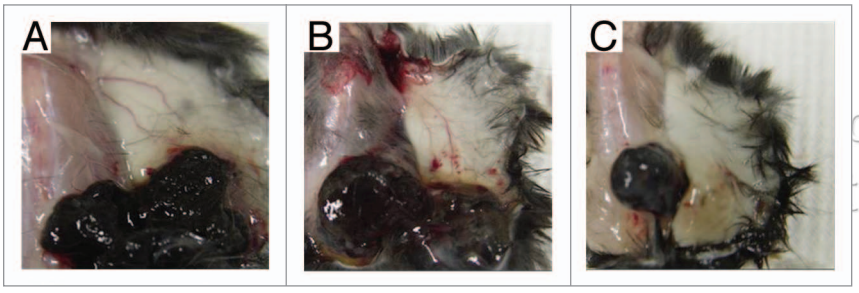

Figure 8. Subcutaneous vascularization of mouse dorsum. Mice were sacrificed and the dorsal skin was cut from the mice after in situ injection of recombinant proteins. The vessels were obvious in the (A) negative and (B) Ig-G1 Fc groups, but not in (C) the RBDV-Ig group.

in vitro and suppressing B16/F10 tumor growth in a C57/BL6 mouse model. It is believed that RBDV-IgG1 Fc can be used in the treatment of the growth of human cancer cells, which are dependent on angiogenesis. The concept of using a receptor binding domain of a growth factor conjugated with the Fc portion of IgG might be a useful strategy for cancer therapy.

Materials and Methods

Construction of pAAV-MCS/IgG1 and pAAV-MCS/RBDV-IgG1. The angiogenestic fusion protein (RBDV-IgG1 Fc) and vehicle protein (IgG1 Fc) were designed and shown in **Figure 1**. and the transgene encoding RBDV IgG1 Fc was constructed (data not shown). Briefly, a human vascular endothelial growth factor (VEGF) cDNA fragment from the leader sequence to the receptor binding domain of human VEGF-A (RBDV, amino acids 1–109) was amplified using forward primer: 5'-AGG ATC CAT GAA CTT TCT GCT GTC TTG G-3' and reverse primer: 5'-ACT CGA GTT AGA TCC GCA TAA TCT GCA TGG T-5'. The PCR fragments of RBDV were ligated prior to the fragment encoding the Fc portion of IgG1 and the chimeric transgene was further subcloned into the pAAV/MCS vector (Stratagene, La Jolla, CA, USA), with the poly his6-tag at the C terminals. For the control group, a DNA fragment encoding IL-2 leader sequence was fused with the Fc portion of human IgG1 and the transgene was subcloned into the pAAV/MCS vector (Stratagene) as above.

Preparation of recombinant proteins. The two vectors, pAAV-MCS/IgG1 and pAAV-MCS/RBDV-IgG1, were respectively transfected into 293T cells, using the calcium-phosphate method as described in the instruction manual of the pAAV helper-free system (Stratagene). After incubation for 48 h, the supernatants of the transfectants were collected and purified by Protein G-Agarose (Upstate Inc., Lake Placid, NY, USA) according to the instruction manual. Then, the eluted fractions were further purified by a nickel-charged HisTrap HP affinity column (Amersham Biosciences, Piscataway, NJ, USA) as described in the instruction manual. Finally, the buffer was exchanged to PBS by a Sephadex G-25 prepacked column (Amersham Biosciences, Uppsala, Sweden) and the recombinant proteins were concentrated by the Microcon Centrifugal Filter Unit (Millipore, Bedford, MA, USA). The recombinant proteins were produced successfully (**Sup. Fig. 1 and 2**).

Cell lines and cell cultures. All cells were obtained from BCRC (Food Industry and Development Institute, Hsinchu, Taiwan). Human epithelial kidney (HEK) 293T cells, mouse melanoma cells (B16/F10), mouse vascular endothelial cells (SVEC4-10) and mouse embryonic fibroblast cells (Balb/3T3) were maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Gaithersburg, MD, USA) containing 10% heat-inactivated FBS (fetal bovine serum qualified; Invitrogen) and 1% penicillin-streptomycin amphotericin B (PSA; Biological industries, NY, USA). Mouse endothelial cells (MS1) were cultured in DMEM supplemented with 5% heat-inactivated FBS and 1% PSA. All cells were incubated in a tissue culture incubator with 5% CO_2 at 37°C.

Receptor binding assay in vitro. To prepare biotinylated fusion proteins, 500 μl of protein was reacted with 2 μl of No-Weight™ Sulfo-SBED [Sulfosuccinimidyl (2-6-[biotinamido]-2-[p-azidobenzamido]-hexanoamido)-ethyl-1,3-dithiopropionate; Pierce, NY, USA] at room temperature for 30 min and later non-reacted Sulfo-SBED was removed using a sephadex G-25 column.

The extracellular domain of mouse VEGFR-1 (0.5 μg/well; R&D systems, Minneapolis, MN, USA) or the extracellular domain of mouse VEGFR-2 (0.5 μg/well; R&D systems) were coated onto a 96-well microtiter plate (Nunc, Denmark), blocked and washed. Then, the biotin-labeled proteins were added into the coated plates for 1 h. After washing, the plates were then incubated with HRP-conjugate streptavidin (R&D systems). Finally, the reactions were developed by TMB substrate (KPL, Gaithersburg, MD, USA) for 10 min, the colorimetric

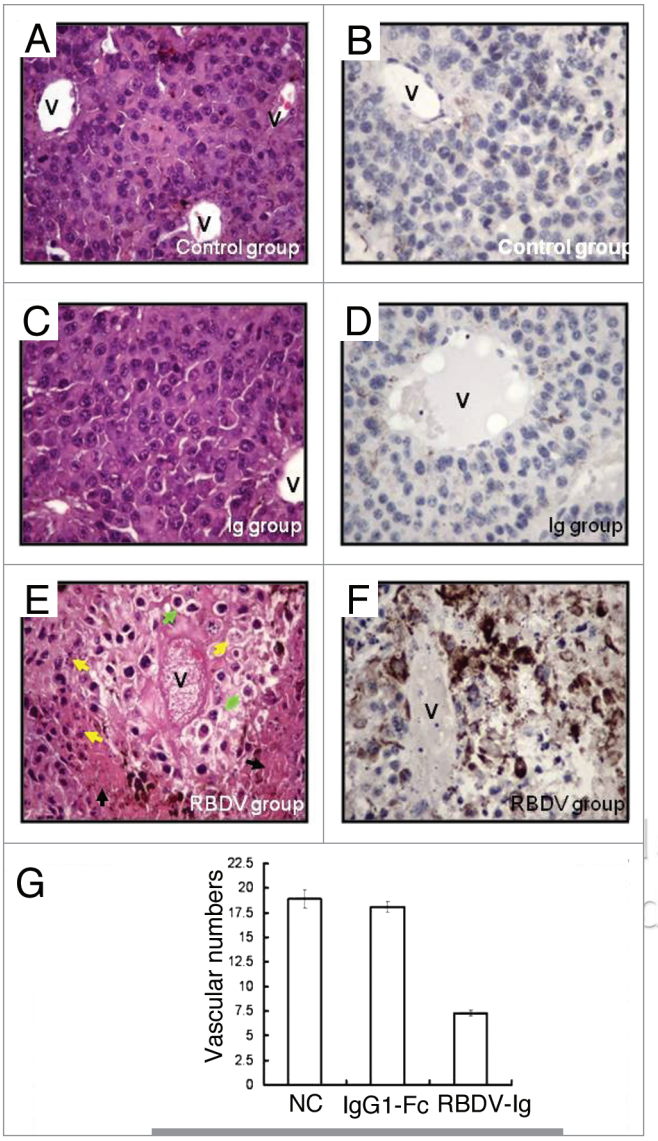

Figure 10. Effects of RBDV-IgG Fc on histological damage in melanoma tumor growth in C57 mice. H&E and immunohistochemical staining analysis were done in mice melanoma tumor tissues. Representative photographs of sections in the control group (A and B), Ig group (C and D) and RBDV-treated group (E and F) are shown. After treatment, mice were sacrificed and the tumors were subjected to histology examination (A, C and E). Nucleic degradation (yellow arrow), a cavitous cytosol appearance (green arrow) and tumor cell death (black arrow) are demonstrated around the vascular area of the tumors of RBDV- treated mice. Immunohistochemical staining for RBDV binding activity with anti-mouse Ig-HRP (B, D and F) was also done. The RBDV binding-positive cells are stained brown and those with hematoxylin are stained blue as a counter staining (x400). The mean values of vessel numbers in tumor with different treatments were respectively calculated and showed at (G).

reactions were stopped with 1 N HCl and the absorbance was measured by an ELISA reader (Sunrise™, Tecan Group Ltd., Mannedorf, Switzerland) at 450 nm.

Cell surface binding assay. MS1, SVEC4-10 and Balb/3TT3 cells were washed with PBS, detached by Versine (0.2% EDTA in PBS, pH = 7.4), washed and resuspended in

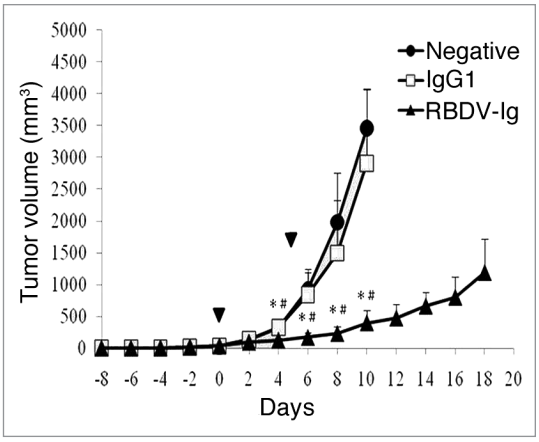

Figure 9. In vivo tumor therapy with intravenous. injections of RBDV-Ig Fc. Female C57/BL6 mice (6–8 weeks of age) was inoculated subcutaneously with 1 x 10^6 cells in 200 μl PBS. When the average tumor volume was up to 30 mm^3, mice were intravenously injected with 150 μg protein and reinjected again after five days. Tumor volumes were measured every 2 days and mice were sacrificed when tumors grew to 2,500 mm^3. The data represent the mean ± SD of the tumor volumes obtained from two independent experiments (n = 9). For the RBDV-Ig group, $p < 0.05$ compared with the negative group, $^{\#}p < 0.05$ compared with the IgG1 Fc group. ▼ = day of protein injection.

flow cytometry buffer (1% bovine serum albumin in PBS, pH 7.4). One μg of purified recombinant proteins were incubated with the cell suspension for 1 hour at 4°C, followed by 1 hour of incubation with FITC-conjugated goat anti-human IgG antibody (Acris Antibodies GmbH, Hiddenhausen, Germany). The cells were washed three times with ice-cold flow cytometer buffer after incubation. Cell pellets were suspended in 1 ml flow cytomer buffer and analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA).

SVEC 4–10 and B16/F10 cell proliferation assay. Five thousand SVEC 4–10 or B16/F10 cells were seeded into the flat-bottomed well of a 96-well plate (Corning Inc., Corning, NY, USA) in DMEM growth medium, allowed to settled for 16 hours, incubated with recombinant proteins for 2 hours, and then challenged for 70 hours with human VEGF165 (Upstate Inc., Lake Placid, NY, USA) at a final concentration of 10 ng/ml. The proliferative response was measured by adding 1.9 mg/ml of MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carbo-xymethoxyphenyl)-2-(4-sulfophenyl)-2H- tetrazolium) (Promega, Madison, WI, USA) to each well followed by a further 2 hour incubation and spectrophotometric analysis at 492 nm. The survival percentage was calculated as follows: mean OD values of the recombinant protein-treated cells/mean OD values of cells without treatment X 100%.

Tube formation assay. Fifty microliters of growth factor reduced matrigel (BD Biosciences, San Jose, CA, USA) was added to each well of a 96-well plate (Corning Inc.) and polymerized for 2 hours at 37°C. SVEC 4–10 cells (3 x 10^4) were incubated with or without recombinant proteins (10 μg/ml) for 1 hour in growth medium containing 16 ng/ml of VEGF (Upstate Inc.). Cells were incubated for a further 16 hours at 37°C and photographed under a microscope (x40 magnification). The total number of network formations was counted.

Cell migration assay. SVEC4-10 cells were pre-cultured with 10 μg/ml protein for 1 h (37°C, 5% CO_2), then plated (30,000 cells/well) in the upper well of a transwell plate (8-μm pore; Costar, Corning, NY, USA). The SVEC4-10 cells were allowed to migrate toward the lower well with DMEM growth medium with 10 ng/ml VEGF for 6 hrs incubation (37°C, 5% CO_2). The cells on the top of the filter were removed with a cotton swab and migrated cells on the underside were fixed with methanol, stained with propidium iodide (PI, 50 μg/ml) and counted with a fluorescence microscope.

In situ tumor therapy. Female C57/BL6 mice (6–8 weeks old) were inoculated subcutaneously with 1 x 10^6 B16/F10 cells. When the average tumor volumes in each group were up to 50 mm^3, mice were injected with 150 μg proteins in situ. Tumor volumes were then measured every 2 days.

Tumor therapy with intravenous injections. When the average tumor volumes were up to 30 mm^3, the mice were injected intravenously with 150 μg/kg proteins. The tumor volumes were then measured every 2 days. Mice were sacrificed when the tumors grew up to 2,500 mm^3. Tumors and organs were obtained, fixed by paraformaldehyde and examined by microscopic hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining.

H&E staining. For the histological H&E staining of the B16/F10 melanoma tumor tissues (s.c., melanoma tumors with or without RBDV treatment), the tumors were harvested and fixed with 10% neutral formalin. After dehydration and embedding in paraffin wax, tissue sections (4 μm/section) from paraffin-embedded blocks were collected on clean glass slides and dehydrated in an oven for 30 min at 60°C. Prior to staining, tissue slides were deparaffinized and rehydrated, then stained with Mayer's hemotoxylin and Eosin Y solution for 3 min. After air-drying, tissue slides were mounted with mounting media and visualized under a Nikon light microscope camera system.

IHC staining. Paraffin-embedded sections (7 μm/section) were obtained from the tumors and processed for immunohistochemical staining. Briefly, the slides were treated with 3% hydrogen peroxide in 1x PBS for 10 min to block endogenous peroxidase activity after the dewaxing and rehydrating processes. Next, the sections were washed three times with PBS-T (1x PBS containing 0.05% Tween-20) for 5 min each time and non-specific reactions were blocked by 10% FBS in PBS for 10 min at room temperature. The sections were incubated with biotin-conjugated anti-rat IgG antisera (1/1,000 dilution) for 1 hour at room temperature, and the immune complexes were visualized using the horseradish peroxidase-conjugated streptavidin. LSAB2 system (DAKO, Carpinteria, CA, USA), and incubated with 0.5 mg/ml diaminobenzidine and 0.03% (v/v) H_2O_2 in PBS for 10 min. Finally, sections were counterstained with hematoxylin, mounted and observed under a light microscope at a magnification of x400 and photographed.

Statistical analysis. Statistical analyses were done using SPSS statistics software (SPSS Inc., Chicago, IL, USA). The t-test was used when comparing two independent samples and ANOVA was used when comparing multiple samples. A value of $p < 0.05$ was considered significant.

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Note

Supplemental materials can be found at:
www.landesbioscience.com/supplement/TsengCBT10-9-sup.pdf

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