A recombinant, fully human, bispecific antibody neutralizes the biological activities mediated by both vascular endothelial growth factor receptors 2 and 3

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Abstract

Vascular endothelial growth factors (VEGF) and their receptors (VEGFR) have been implicated to play important roles in tumor-associated angiogenesis and lymphangiogenesis, and hence in tumor growth and metastasis. We previously produced a number of fully human antibodies directed against VEGF receptor 2 (VEGFR2) and VEGF receptor 3 (VEGFR3) and showed that these antibodies are capable of inhibiting growth factor (VEGF and VEGF-C)-induced receptor activation, migration, and proliferation of human endothelial cells. In this report, we constructed and produced a bispecific antibody, a diabody, using the variable domain genes of two neutralizing antibodies, IMC-1121 to VEGFR2 and hF4-3C5 to VEGFR3. The diabody binds to both VEGFR2 and VEGFR3 in a dose-dependent manner, and blocks interaction between VEGF/VEGFR2, VEGF-C/VEGFR2, and VEGF-C/VEGFR3. In cell-based assays, the diabody neutralized both VEGF and VEGF-C-stimulated activation of VEGFR2, VEGFR3, and p44/p42 mitogen-activated protein kinase in endothelial cells. Furthermore, the diabody was able to inhibit both VEGF and VEGF-C-induced migration of endothelial cells. Taken together, our results suggest that a dual blockade of both VEGFR2 and VEGFR3 simultaneously may represent a more potent approach to effective cancer therapy. [Mol Cancer Ther 2005;4(3):427–34]

Introduction

Vascular endothelial growth factor (VEGF) is a strong inducer of vascular permeability, stimulator of endothelial cell migration and proliferation, and is an important survival factor for newly formed blood vessels (1–3). Numerous studies have shown that overexpression of VEGF and its receptors (VEGFR) play an important role in tumor-associated angiogenesis, and hence in both tumor growth and metastasis (4–6). VEGF (i.e., VEGF-A) binds to and mediates its activity mainly through two tyrosine kinase receptors, VEGF receptor 1 (VEGFR1), or *fms*-like tyrosine receptor 1 (Flt-1), and VEGF receptor 2 (VEGFR2), or kinase insert domain–containing receptor (KDR, and Flk-1 in mice; refs. 1–6). Among these two receptors, KDR seems to be the major transducer of VEGF signals in endothelial cells, thus constituting an excellent target for antiangiogenic intervention. This is supported by numerous studies demonstrating tumor growth inhibition in animal models by antibodies to the receptor (7, 8), receptor ribozyme (9), soluble receptor (10), and small molecular receptor kinase inhibitors (11, 12).

VEGFR3 (Flt-4), a receptor for VEGF-C and D, was believed to be critical for the development of the embryonic vascular system but to be postnatally restricted to the endothelial cells of lymphatic vessels and specialized fenestrated capillaries (13, 14). Transgenic expression of Flt-4-specific mutant of VEGF-C in mouse skin resulted in increased growth of dermal lymphatic but not vascular endothelium (15). Similarly, missense mutations that inactivate Flt-4 primarily disrupt lymphatic but not blood vessels (16). However, recent reports suggest that Flt-4 may also be expressed and functioning in adult vascular endothelial cells, at least under pathologic conditions. For example, overexpression of VEGF-C and D has been associated with abnormal growth and enlargement of both vascular and lymphatic vessels of certain human tumor specimens (17–20). In addition, enhanced expression of Flt-4 has been observed in neoplastic colonic mucosa (21), and the level of Flt-4 expression was correlated with prognosis in patients of breast cancer and cutaneous melanoma (22, 23). Finally, a rat antagonist antibody to mouse Flt-4, AFL-4, has been shown to suppress growth of tumor xenografts via disruption of the microvasculature (24), and to reduce induction of lymphangiogenesis induced by fibroblast growth factor 2 in a mouse corneal model (25). Taken together, these observations indicate that Flt-4, like KDR, may also represent a good therapeutic target for inhibiting tumor growth and metastasis (26–28).

We previously produced a panel of neutralizing antibodies to KDR from single chain Fv (scFv) and Fab fragments originally isolated from antibody phage display libraries (7, 29, 30). One of the fully human antibodies, IMC-1121, binds to KDR with high affinity and efficiently blocks the KDR/VEGF interaction. IMC-1121 also inhibited VEGF-stimulated KDR activation and endothelial cell

migration and proliferation (29, 30). Using the same antibody phage display libraries, we recently identified a high-affinity neutralizing antibody to human Flt-4, hF4-3C5. The antibody binds specifically to human Flt-4 and blocks Flt-4/VEGF and Flt-4/VEGF-C interaction. Furthermore, hF4-3C5 showed a strong inhibitory effect on bovine aortic endothelial (bovine aortic endothelial) cell invasion and sprouting induced by VEGF-C (31). More importantly, a combination of hF4-3C5 and an anti-KDR antibody completely abolished the activity of VEGF-C, indicating that both KDR and Flt-4 are involved in the angiogenic response of bovine aortic endothelial to VEGF-C (31).

In this report, we produced a bispecific antibody, a diabody, using the variable domain genes of IMC-1121 (specific to KDR) and hF4-3C5 (specific to Flt-4) as the ''building blocks,'' and investigate whether simultaneous blockade of both KDR and Flt-4 with the diabody would provide additional benefits compare with the use of a single antibody that only neutralizes an individual receptor. The diabody binds to both KDR and Flt-4 in a dose-dependent manner and blocks interaction between VEGF/KDR, VEGF-C/KDR, and VEGF-C/Flt-4. In cell-based assays, the diabody neutralized both VEGF and VEGF-C-stimulated endothelial cell migration and activation of the receptors and p44/p42 mitogen-activated protein kinases (MAPK) in endothelial cells. These results suggest that a simultaneous dual blockade of both KDR and Flt-4 may represent a more potent approach to effective cancer therapy.

Materials and Methods

Cell Lines and Proteins

Primary-cultured human umbilical vein endothelial cells (HUVEC) were maintained in EBM-2 medium (Clonetics, Walkersville, MD) at 37°C, 5% CO_2. The bovine aortic endothelial cells were maintained in DMEM (Invitrogen, Carlsbad, CA) containing 10% FCS and grown at 37°C with 5% CO_2. The soluble fusion proteins, KDR-alkaline phosphatase (AP) and Flt-4-AP were expressed in stably transfected NIH 3T3 cells and purified from cell culture supernatant by affinity chromatography using immobilized monoclonal antibody to AP as previously described (32). VEGF protein was expressed in baculovirus and purified following previously described procedures (32). VEGF-C was purchased from R&D Systems (Minneapolis, MN).

Construction of the Anti-KDR × Anti-Flt-4 Diabody

PCR fragments encoding the variable light and heavy chain genes (VL and VH, respectively) of IMC-1121 and hF4-3C5 were first used to assemble the scFv of the antibodies, scFv 1121 and scFv 3C5, respectively, using an overlapping PCR following a previously described protocol (33). In the scFv, the COOH terminal of the VL was linked to the NH2 terminal of the cognate VH via a 15−amino acid linker (glycine-glycine-glycine-glycine-serine)3 or (GGGGS)3 (Fig. 1A). The scFv-encoding gene

was then cloned into vector pCANTAB 5E (Amersham Pharmacia Biotech, Piscataway, NJ) for the expression of the soluble scFv protein. To construct the diabody, V domains of scFv 1121 and scFv 3C5 were used for PCR-directed assembly to create the expression construct (Fig. 1A). Briefly, two cross-over scFv polypeptides were first created by fusing the VL domain of IMC-1121 at its COOH-terminus to the NH2-terminus of the VH domain of hF4-3C5 via a five−amino acid linker (GGGGS), and the VL domain of hF4-3C5 at its COOH-terminus to the NH2-terminus of the VH domain of IMC-1121 via the same five−amino acid linker. Following a PCR step to introduce appropriate restriction sites as well as the leader peptide sequence for bacteria secretion, the two cross-over scFv were subcloned into vector pCANTAB 5E for *E. coli* expression. All sequences encoding the cross-over scFv fragments were verified by DNA sequencing.

Expression and Purification of the Diabody

The diabody was secreted from *E. coli* strain HB2151 containing the expression plasmid grown at 30°C in a shaker flask following the procedure previously described (33). A periplasmic extract of the cells was prepared by resuspending the cell pellet in 25 mmol/L Tris (pH 7.5) containing 20% (w/v) sucrose, 200 mmol/L NaCl, 1 mmol/L EDTA and 0.1 mmol/L phenylmethyl-sulfonyl fluoride, followed by incubation at 4°C with gentle shaking for 1 hour. After centrifugation at 15,000 rpm for 15 minutes, the soluble diabody was purified from the supernatant by anti-E tag affinity chromatography using
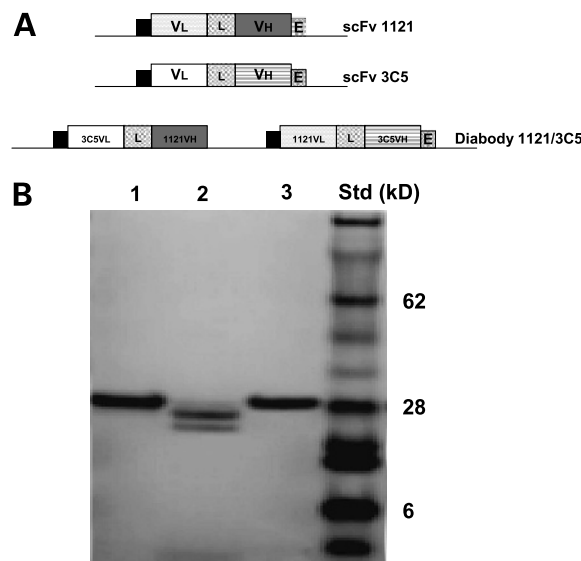

Figure 1. Construction and expression of the anti-KDR (1121) × anti-Flt-4 (3C5) bispecific diabody. **A,** schematic representation of the expression constructs for scFv 1121, scFv 3C5, and the diabody (drawings are not to scale). **B,** expression and purification of the scFv and the diabody. The antibodies were expressed in *E. coli*, purified by affinity chromatography, and analyzed by SDS-PAGE. *Lane 1,* scFv 1121; *lane 2,* the diabody; *lane 3,* scFv 3C5; *right,* positions of molecular weight markers in kilodaltons (kDa).

the RPAS purification module (Amersham Pharmacia Biotech). To examine the purity of the preparation, the purified diabodies were electrophoresed in an 18% poly-acrylamide gel (Novex, San Diego, CA) and visualized by staining with colloidal blue stain kit (Novex).

Dual Antigen Binding of the Diabody to KDR and Flt-4

Various amounts of diabody or scFv were added to KDR or Flt-4 coated 96-well plates and incubated at room temperature for 1 hour, after which the plates were washed thrice with PBST. The plates were then incubated at room temperature for 1 hour with 100 μL of an anti-E tag antibody-horseradish peroxidase conjugate (Amersham Pharmacia Biotech). The plates were washed, peroxidase substrate added, and the absorbance at 450 nm read following procedures described previously (33).

VEGF/KDR, VEGF-C/KDR, and VEGF-C/Flt-4 Blocking Assays

The assay was carried out following a previously described protocol (33). Briefly, various amounts of the diabody or scFv were mixed with a fixed amount of KDR-AP (100 ng) or Flt-4-AP (100 ng) and incubated at room temperature for 1 hour. The mixture were then transferred to 96-well microtiter plates precoated with VEGF (200 ng/well) or VEGF-C (200 ng/well) and incubated at room temperature for an additional 2 hours, after which the plates were washed five times with PBS. The substrate for AP was added and the absorbance at 405 nm was read to quantify the plate-bound KDR-AP or Flt-4-AP molecules. The IC_{50}, i.e., the antibody concentration required for 50% inhibition of KDR or Flt-4 binding to VEGF or VEGF-C, was then calculated.

Binding Kinetics Analysis of the Diabody

The binding kinetics of the diabody and its parent scFv to KDR and Flt-4 were measured using BIAcore biosensor (Biacore, Neuchatel, Switzerland). KDR-AP or Flt-4-AP fusion protein was immobilized onto a sensor chip at approximately 850 reference units and soluble antibodies were injected at concentrations ranging from 1.5 to 100 nmol/L. Sensorgrams were obtained at each concentration and were evaluated using the program, BIA Evaluation 2.0, to determine the rate constants k_{on} and k_{off}. The affinity constant, K_d, was calculated from the ratio of rate constants k_{off}/k_{on}.

Bovine Aortic Endothelial Migration Assay

The chemotaxis assay was done using 48-well chemo-taxis chambers with 8 μm polycarbonate membranes (Neuro Probe, Gaithersburg, MD). VEGF or VEGF-C was added to the bottom wells at 5 ng/mL and 1.5×10^4 bovine aortic endothelial cells were added to the top wells with and without the testing antibodies at 20 nmol/L. After 4 hours at 37°C, the cells on the top portion of the filter were removed by scraping and transmigrated cells were stained with Hoechst 33342. Membranes were mounted on glass slides and the nuclei were imaged using epifluorescence microscopy with a 20× objective. Images were analyzed by automated object counting using the AlphaEase software package (Alpha Innotech Corporation, San Leandro, CA).

Inhibition of VEGF- and VEGF-C-Stimulated Phosphorylation of KDR and Flt-4 and MAPK

HUVEC cells were plated into 75 mm dishes and grown to 70% to 80% confluence, after which the cells were washed twice in PBS and cultured overnight in serum-free medium. The cells were first incubated with various antibodies at 200 nmol/L at 37°C for 30 minutes, followed by stimulation with 100 ng/mL of VEGF, VEGF-C, or both at 37°C for 20 minutes. The cells were lysed and KDR and Flt-4 were immunoprecipitated from the cell lysate by using polyclonal rabbit antibodies to KDR or Flt-4 (ImClone Systems) followed by ProA/G-sepharose beads (Santa Cruz Biotech, Santa Cruz, CA). The precipitated receptor proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Phospho-KDR and phospho-Flt-4 were detected on the blot using an anti-phosphotyrosine antibody-horseradish peroxidase conjugate (Santa Cruz Biotech). Total Flt-4 protein was determined with an anti-Flt-4 antibody (SC-321 antibody, Santa Cruz Biotech). For phosphorylation of p44/p42 MAPK, whole cell lysate was resolved by SDS-PAGE and the phospho-p44/p42 were detected with an anti-phospho-p44/p42 antibody (Cell Signaling), followed by an anti-mouse antibody-horseradish peroxidase conjugate. Total p44/p42 proteins were assayed with an anti-p44/p42 antibody (Cell Signaling, Beverly, MA). All signals were visualized with the enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Results

Construction and Expression of the scFv and the Diabody

The VH and VL domains from two previously described human antibodies, IMC-1121 and hF4-3C5, directed against KDR and Flt-4, respectively, were used as the building blocks to construct the scFv and the bispecific diabody (Fig. 1A). The most commonly used 15-amino-acid linker (Gly-Gly-Gly-Gly-Ser)3, was used for the construction of scFv, whereas a shorter, five–amino acid linker (Gly-Gly-Gly-Gly-Ser) was used as the linker for the construction of the diabody. In constructing the diabody, the COOH-terminus of the IMC-1121 VL domain was first connected to the NH2-terminus of the hF4-3C5 VH domain using a five–amino acid linker (GGGGS), to restrict intrachain pairing of VL and VH. A second cross-over scFv was then constructed similarly by connecting the COOH-terminus of the hF4-3C5 VL domain to the NH2-terminus of the IMC-1121 VH domain using the same linker. The two cross-over scFv were then PCR-amplified and subcloned into expression vector pCANTAB 5E to create the expression vector for the diabody (Fig. 1A). A polypeptide tag, the E tag, was fused immediately to the COOH-terminus of the second cross-over scFv for purification and detection purposes. Both the scFv and the diabody were produced in *E. coli* strain HB2151 containing the expression vector grown in shake flasks. The proteins were released from the periplasmic space of *E. coli* by osmotic shock, and purified with anti-E tag affinity chromatography.

The purified scFv and the diabody were analyzed by SDS-PAGE. The two scFv gave rise to a single protein band of expected mobility, whereas the two component cross-over scFv in the diabody were resolved under the electrophoretic conditions and gave rise to two major bands (Fig. 1B, *lane 2*); the lower band represents the first cross-over scFv, and the upper band correlates with the second cross-over scFv with E-tag.

Dual Specificity of the Diabody

The dual antigen binding activity of the diabody was determined on immobilized KDR and Flt-4. As expected, the diabody bound to both KDR and Flt-4, whereas scFv 1121 only reacted with KDR and scFv 3C5 only with Flt-4 (Fig. 2). The binding efficiency of the diabody to KDR and Flt-4 was, however, about 5-fold and 15-fold lower, respectively, than that of the parent scFv to their respective targets, as judged by the EC_{50} values, i.e., the antibody concentrations that yielded 50% of maximum binding.

The binding kinetics of the diabody to KDR and Flt-4 were determined by surface plasmon resonance using a BIAcore instrument (Table 1). The diabody binds to KDR with a K_d of 0.61 nmol/L, which is comparable to that of 0.41 nmol/L for the scFv 1121. The binding affinity of the diabody to Flt-4 was moderately reduced compared with that of the scFv 3C5 (5.0 versus 0.71 nmol/L), due to both a slower on-rate and a faster off-rate of the diabody (Table 1).

Figure 3A and B show that the diabody blocks both VEGF and VEGF-C from binding to KDR, in a dose-dependent manner, with an IC_{50}, i.e., the antibody concentration that yielded 50% blockade of ligand/receptor interaction, of approximately 2 to 3 nmol/L. As a comparison, scFv 1121 showed an IC_{50} value of about 0.8 to 1 nmol/L. The diabody also blocks Flt-4 from binding to VEGF-C with an IC_{50} of ~12 nmol/L, which is about 7-fold higher than that of the parent scFv 3C5 (Fig. 3C). As expected, scFv 1121 had no effects on Flt-4/VEGF-C, whereas scFv 3C5 had no effects on KDR/VEGF and KDR/VEGF-C interaction.

Inhibition of VEGF- and VEGF-C-Stimulated Receptor Phosphorylation and Activation of MAPK

The effect of the diabody on VEGF- and VEGF-C-stimulated activation of both KDR and Flt-4 was examined using HUVEC. VEGF stimulation only resulted in phosphorylation of KDR (Fig. 4A, *lane 2*), whereas VEGF-C, as well as a combination of VEGF and VEGF-C, led to phosphorylation of both KDR and Flt-4 (*lanes 3* and *4*). When the HUVEC were incubated with both VEGF and VEGF-C, scFv 3C5 or scFv 1121 alone only blocked the phosphorylation of its respective receptor but had no effect on the phosphorylation of the other receptor (*lanes 5* and *6*). Combination of scFv 1121 and scFv 3C5, either as a simple mixture or in the diabody format, completely inhibited activation of both KDR and Flt-4 by VEGF and VEGF-C (*lanes 7* and *8*). Treatment by the diabody alone, in the absence of any ligand, did not affect the activation status of KDR and Flt-4 in the HUVEC (*lane 9*).

Activation of p44/p42 MAPK, one major component of the downstream signaling pathways of VEGF and VEGF-C, was also examined. Both VEGF and VEGF-C, alone or in combination, stimulate significant phosphorylation of p44/p42 MAPK (Fig. 4B, *lanes 2-4*). ScFv 3C5 or scFv 1121 alone only partially (~50%) blocked the combined effect of VEGF and VEGF-C on p44/p42 phosphorylation (*lanes 5* and *6*), whereas the diabody, as well as the mixture of both scFv, completely abolished the activation of the kinases (*lanes 7* and *8*). Similar to the observation on the receptors, the diabody alone showed no effect on the phosphorylation status of p44/p42 MAPK (*lane 9*).

Inhibition of VEGF- and VEGF-C-Induced Migration of Endothelial Cells

The scFv and the diabody were tested for its activity in inhibiting VEGF and VEGF-C-induced endothelial cell migration. Both VEGF and VEGF-C induced significant migration of bovine aortic endothelial cells (Fig. 5). scFv 1121 inhibited ~50% of the VEGF-induced (Fig. 5A), but did not affect VEGF-C-induced (Fig. 5B), bovine aortic
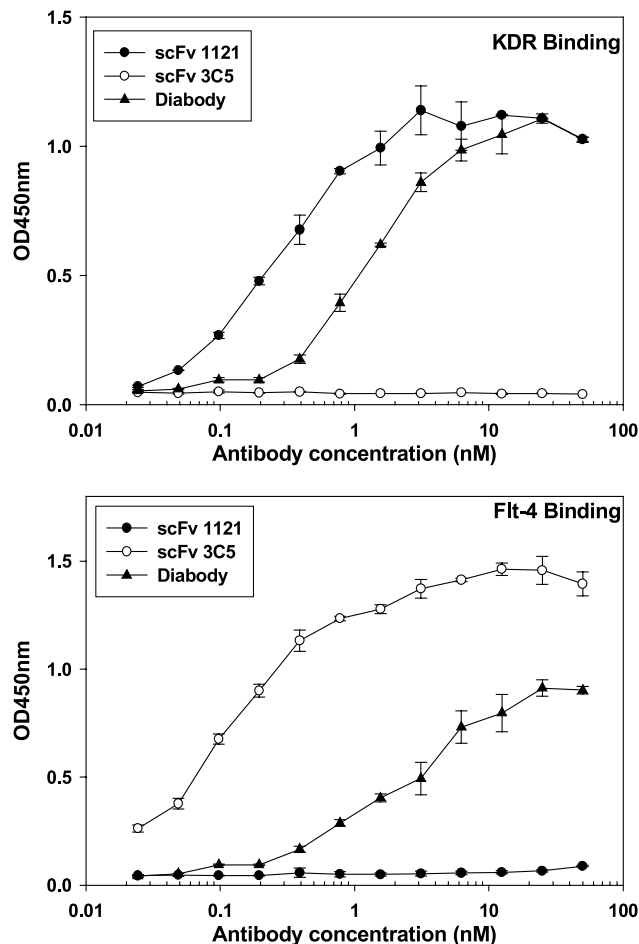

Figure 2. Antigen-binding efficiency of the scFv and the diabody to their respective targets, KDR and Flt-4. Various amounts of scFv or diabody were added to 96-well plates coated with KDR or Flt-4 (1.0 μg/mL) and incubated at room temperature for 1 h, after which the plates were incubated with a mouse anti-E tag antibody-horseradish peroxidase conjugate for an additional 1 h. The plates were washed, peroxidase substrate was added, and $OD_{450\ nm}$ was read. *Points,* mean; *bars,* ±SD.

Table 1. Binding affinity of the bispecific diabody to KDR and Flt-4

Antibody	KDR binding			Flt-4 binding		
	k_{on} ($\times 10^5$ mol/L^{-1} s^{-1})	k_{off} ($\times 10^{-4}$ s^{-1})	K_d (nmol/L)	k_{on} ($\times 10^5$ mol/L^{-1} s^{-1})	k_{off} ($\times 10^{-4}$ s^{-1})	K_d (nmol/L)
scFv 1121	3.4 ± 0.19	1.4 ± 0.01	0.41 ± 0.03	NB	NB	NB
scFv 3C5	NB	NB	NB	15.6 ± 6.5	9.6 ± 0.1	0.71 ± 0.36
Diabody	1.5 ± 0.4	0.88 ± 0.04	0.61 ± 0.19	5.3 ± 1.8	25.1 ± 6.1	5.0 ± 1.4

NOTE: All numbers are determined by BIAcore analysis and represent the mean ± SE of four to six separate determinations. K_d values are calculated as the ratios of k_{off}/k_{on}.
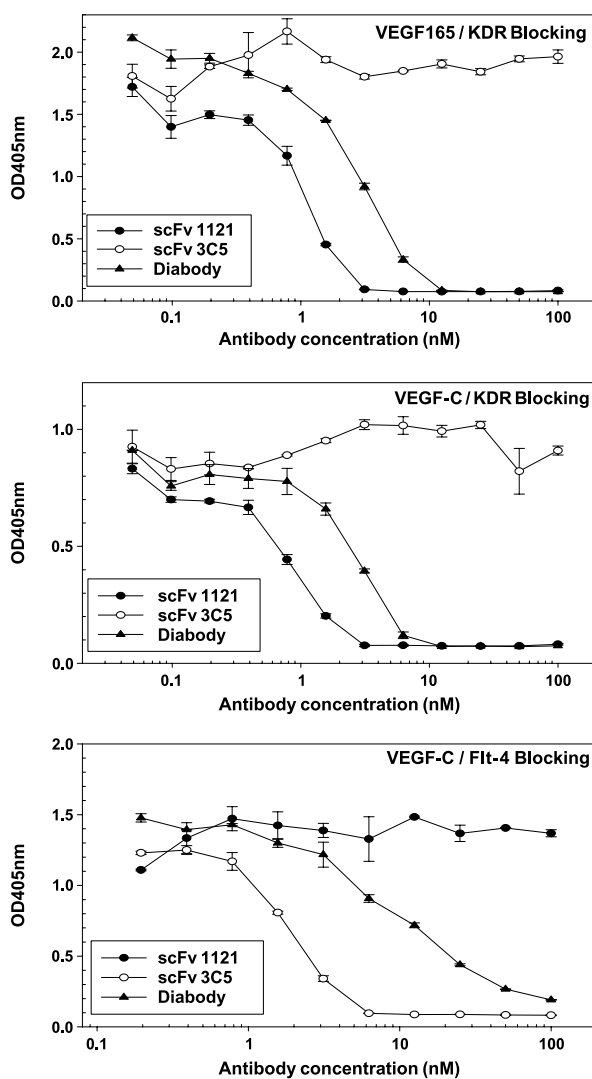Abbreviation: NB, no binding.

Figure 3. Inhibition of binding of VEGF and VEGF-C to KDR and Flt-4 by the scFv and the diabody. Various amounts of antibodies were incubated with a fixed amount of KDR-AP or Flt-4-AP fusion in solution at room temperature for 1 h, after which the mixtures were transferred to 96-well plates coated with VEGF or VEGF-C. The amount of KDR-AP or Flt-4-AP that bound to the immobilized VEGF or VEGF-C was quantified by incubation of the plates with AP substrate and reading at A$_{405\ nm}$. *Points,* mean; *bars,* ±SD.

endothelial migration. On the other hand, scFv 3C5 was a stronger inhibitor of VEGF-C-induced cell migration (Fig. 5B, ~65% inhibition) but had only marginal effect on VEGF activity (Fig. 5A). Combination of scFv 1121 and scFv 3C5, either as a simple mixture or in the diabody format, blocked both VEGF- and VEGF-C-induced cell migration at a potency that is comparable to each individual parent scFv to their respective ligand/receptor. A control scFv, scFv p3S5, an antibody that binds KDR but does not interfere with KDR/VEGF interaction, did not show any inhibition in cell migration in this assay.

Discussion

The importance of VEGFs and its receptors in tumor angiogenesis suggests that blockade of these pathways by antibody therapy would be an effective therapeutic strategy for inhibiting tumor growth and metastasis (1–6). Among the three VEGF receptors, KDR seems to be the major transducer of VEGF signals in endothelial cells which result in cell proliferation, migration, differentiation, tube formation, increase of vascular permeability, and maintenance of vascular integrity (3–6). Inhibition of KDR-mediated signal transduction, therefore, constitutes an excellent approach for antiangiogenic intervention. On the other hand, Flt-4 is considered as the primary receptor mediating the proliferation and survival of lymphatic endothelial cells. A number of recent reports suggested that Flt-4 also played a role in adult angiogenesis, especially under pathologic conditions (21–23). Taken together, these findings indicate that Flt-4 may represent a good target of antimetastatic therapy (26–28). To this end, we postulate that therapeutic approaches simultaneously targeting both KDR and Flt-4 would lead to enhanced antitumor activity by blocking both receptor-mediated tumor growth events including angiogenesis and metastasis.

In this study, we constructed a neutralizing bispecific antibody directed against both KDR and Flt-4, and investigated whether an antibody-based dual targeting approach would simultaneously neutralize both KDR and Flt-4-mediated biological activities in endothelial cells *in vitro*. The bispecific antibody, a diabody, was constructed from the variable domains of an anti-KDR antibody, IMC-1121 and an anti-Flt-4 antibody, hF4-3C5, and produced recombinantly from bacteria expression.
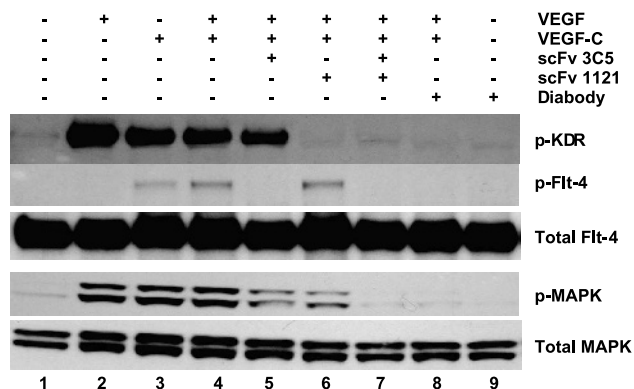
Figure 4. Inhibition of VEGF- and VEGF-C-stimulated activation of KDR, Flt-4, and p44/p42 MAPK in HUVEC by the diabody. Serum-starved subconfluent HUVEC were first incubated with various antibodies (200 nmol/L) at room temperature for 30 min, followed by stimulation with VEGF (100 ng/mL), VEGF-C (100 ng/mL), or a combination of both ligands at room temperature for 15 min. The level of receptor and MAPK phosphorylation was assayed by Western blotting analysis. *Lane 1,* control; *lane 2,* VEGF; *lane 3,* VEGF-C; *lanes 4-8,* VEGF plus VEGF-C in the presence of PBS (*lane 4*), scFv 3C5 (*lane 5*), scFv 1121 (*lane 6*), the diabody (*lane 7*), or the mixture of scFv 3C5 and scFv 1121 (*lane 8*); *lane 9,* diabody alone in the absence of any ligand.

As expected, the diabody specifically bound both KDR and Flt-4 (Fig. 2) and effectively blocked the receptors from binding to their respective ligands, VEGF, and VEGF-C (Fig. 3). In HUVEC cells stimulated with VEGF and VEGF-C, the diabody completely inhibited activation of both KDR and Flt-4, as effectively as the combination of the two parent antibodies. On the contrary, the parent antibodies, scFv 3C5 or scFv 1121 alone only blocked the phosphorylation of its respective receptor but had no effect on the phosphorylation of the other receptor. The diabody also totally abolished VEGF and VEGF-C-induced phosphorylation of a downstream signaling molecule, MAPK, whereas each individual antibody only showed a modest inhibitory effect (Fig. 4). Taken together, these observations confirmed that the diabody retained the biological activities of both its parent antibodies, and is functionally a dual specific KDR and Flt-4-neutralizing molecule.

In our previous work, we showed that collagen gel invasion and sprouting of bovine aortic endothelial induced by VEGF could be completely inhibited by an antagonistic anti-KDR antibody, IMC-1C11, whereas those induced by VEGF-C was inhibited by ~67% by the same antibody (34). On the other hand, hF4-3C5 blocked ~65% of VEGF-C-induced bovine aortic endothelial sprouting but had no effect on VEGF activity (31). Combination of both IMC-1C11 and hF4-3C5 completely abolished bovine aortic endothelial sprouting induced by VEGF-C (31). Taken together, these observations suggest that, whereas VEGF mediates its angiogenic activity on bovine aortic endothelial through KDR only, VEGF-C exerts it effect via both KDR and Flt-4. In this study, we used a different assay, the chemotactic (cell migration) assay, to examine the role of both KDR and Flt-4 in the response of bovine aortic endothelial towards VEGF and VEGF-C. In contrast to the

observations with the cell sprouting assay (31, 34), the anti-KDR antibody, scFv 1121, only partially (~50%) inhibited VEGF-induced, but had not effect on VEGF-C-induced cell migration, and the reverse held true for the anti-Flt-4 antibody scFv 3C5. The bispecific diabody inhibited bovine aortic endothelial migration induced by both VEGF and VEGF-C, as potently as the combination of scFv 1121 and scFv 3C5. It is interesting to note that simultaneous blockade of both KDR and Flt-4, either by the diabody or the mixture of both scFv, did not show additive effects in blocking VEGF and VEGF-C-induced chemotactic response of bovine aortic endothelial. Similar results were also obtained when IMC-1C11 and hF4-3C5 were used in the same cell migration assay (31). These results indicate that the other VEGF receptor, Flt-1, may also play a significant role in VEGF-induced bovine aortic endothelial cell migration (since bovine aortic endothelial also expresses Flt-1). For example, we have shown that both anti-KDR and
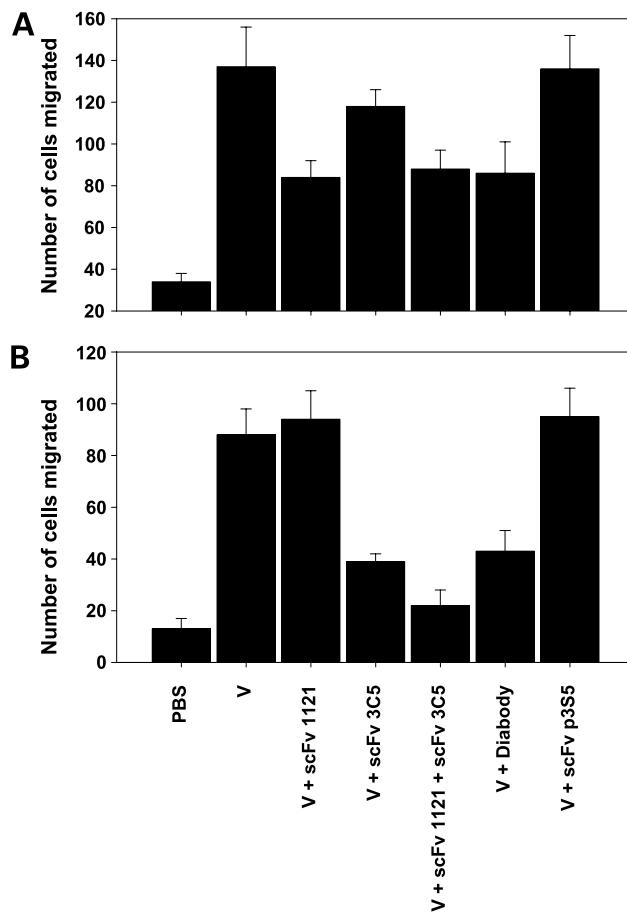

Figure 5. Inhibition of (**A**) VEGF- and (**B**) VEGF-C-induced migration of bovine aortic endothelial cells by the diabody. Bovine aortic endothelial cells in upper wells were induced to transmigrate in the presence or absence of the antibodies (20 nmol/L), through 8 μm polycarbonate filters towards the ligand, VEGF, or VEGF-C (5 ng/mL), added in the bottom wells. The transmigrated cells were stained with Hoechst stain, imaged under epifluorescence using 20× lens and counted using automatic image analysis. *Columns,* mean; *bars,* ±SD; *V,* VEGF (**A**) or VEGF-C (**B**).

anti-Flt-1 antibodies were able to inhibit VEGF-induced migration of leukemia cells that are KDR[+]/Flt-1^{+}, whereas only the anti-Flt-1 antibody was effective in leukemia cells that express Flt-1 but not KDR (30, 33). Similarly, it has been shown that peripheral blood monocytes (Flt-1^{+}/KDR[−]) migrated towards both VEGF and placenta growth factor (a Flt-1-specific ligand), and the response to VEGF could be blocked by an anti-Flt-1 neutralizing antibody (35, 36). The apparent discrepancy between the antibody effects observed in this report in cell migration assay and those reported when using the cell sprouting assay (31, 34) might be explained by the fact that cell sprouting involves a complex multistep mechanism including not only cell migration but also adhesion and assembly (tube formation), and is thus more prone to anti-VEGF and anti-VEGFR inhibition.

There are a number of advantages associated with the development of bispecific antibodies that simultaneously target two tumor-associated targets. Apart from VEGF and VEGF-C, several other growth factors related to VEGF have now been identified: VEGF-B, VEGF-C, VEGF-D, and VEGF-E. Whereas VEGF and VEGF-E are specific for KDR, VEGF-C and VEGF-D can bind to both KDR and Flt-4 (1–6). In theory, antibodies to an individual growth factor such as VEGF would only specifically neutralize the angiogenic activity of a single ligand. In contrast, antagonistic antibodies to a VEGF receptor will not only block the angiogenic activity of VEGF, but also that of other growth factors exerting their angiogenic effects via the receptor. For example, an anti-KDR antibody will potentially block the angiogenic activity of VEGF, VEGF-C, VEGF-D, and VEGF-E, whereas an antibody to Flt-4 will inhibit the activity of VEGF-C and VEGF-D. A combination of both anti-KDR and anti-Flt-4 antibodies would thus neutralize the angiogenic and lymphangiogenic activities of all four growth factors, VEGF, VEGF-C, VEGF-D, and VEGF-E. To support this notion, there is accumulating evidence from both *in vitro* and in animal studies suggest that combination of antitumor antibodies directed against different tumor-associated targets may yield enhanced therapeutic activity without adding severe unwanted toxicity (33, 37–40). Clinical application of combinational antibody therapy is, however, greatly hindered by a number of factors, including limited availability of antibody products, high cost of each product, and Food and Drug Administration–associated regulatory issues (e.g., every antibody in combination may require separate approval by the agency). To this end, the development of bispecific or multispecific antibodies that target two or more tumor-associated antigens simultaneously may offer a novel and promising solution. Here, we produced a bispecific diabody that blocks both KDR and Flt-4, and showed that this diabody is an effective inhibitor of VEGF and VEGF-C. Our results suggest that dual KDR/Flt-4 blockade with the bispecific diabody may represent a more efficient approach in tumor treatment by inhibiting both tumor angiogenesis and lymphangiogenesis. Taken together, these results lend strong support for further evaluation of the bispecific diabody as an antitumor agent.

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